Thesis for Doctoral degree (Ph.D.)

ANTIMICROBIAL RESISTANCE AND MOLECULAR EPIDEMIOLOGY OF *NEISSERIA GONORRHOEAE* IN BANGLADESH

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Antimicrobial Resistance and Molecular Epidemiology of *Neisseria gonorrhoeae* in Bangladesh

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This thesis is submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) under the Faculty of Post-graduate Medical Sciences and Research, University of Dhaka. This work has been carried out at the Department of Microbiology, Sylhet MAG Osmani Medical College, Sylhet and the former RTI/STI Laboratory (later Food Microbiology Laboratory), Molecular and Serodiagnostic Laboratory, Clinical Laboratory Services, Laboratory Sciences Division, ICDDR,B, Dhaka. This is an original and innovative type of work and to the best of my knowledge, similar works has not been done anywhere in Bangladesh.

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Certification of Thesis work

The thesis entitled "Antimicrobial Resistance and Molecular Epidemiology of *Neisseria gonorrhoeae* in Bangladesh" submitted by Dr. Md. Ashraful Alam, registration number 96/ 2006-07 in fulfillment of the requirement of degree of Doctor of Philosophy (Ph.D.) under the University of Dhaka is an original and innovative work and so far has not been done elsewhere in Bangladesh. The work has been approved by the following supervisors.

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Abstract

Gonococcal infection (or gonorrhoea) is a very old venereal disease of the humans since antiquity. In spite of substantial development in the diagnosis, prevention and treatment, gonorrhoea still remains as one of the most common venereal diseases imposing a global health problem. The disease attacks individuals of almost all ages, races and sexes of the population throughout the world. Many serious disability-causing complications like sterility in both sexes caused by urethral stricture in the male and salpingitis in the female, pelvic inflammatory disease (PID) in the female, stillbirth and premature labour in the pregnant women, blindness in the newborn babies delivered from the infected mothers and arthritis-dermatitis syndrome are known to create human sufferings in addition to the burden of the treatment costs. Moreover, gonococcal infection is also known to facilitate acquisition of HIV (human immunodeficiency virus). People with high-risk sexual behaviours like sexually active individuals practicing promiscuous sexes, having multiple sex partners including CSWs and sexes without protective barriers like condoms are at especial risks of getting the infection.

Neisseria gonorrhoeae, the causative agent of the disease is highly adaptable in the human body being capable of resisting immune attacks by antigenic variability. In addition, the organism possesses some unique ability to rapidly develop resistance to the commonly used antimicrobial agents. To combat these situations, investigators realized that the knowledge of gonorrhoea epidemiology along with characterization of the circulating strains of the *N. gonorrhoeae* in a community can provide valuable information that can be useful in public health intervention for control of the disease.

In Bangladesh, few reported studies with the epidemiology of gonorrhoea and characteristics of *N. gonorrhoeae* isolates have been mostly centered among

the female commercial sex workers based in Dhaka City. The epidemiology of gonorrhoea in the male individuals, especially outside the Dhaka City is not very much known to make useful for the control program. Keeping in mind the above considerations, the present study was designed to find out sociodemographic characteristics of the male individuals in Sylhet City as well as phenotypic and genotypic characteristics of the *N. gonorrhoeae* isolates from the high-risk populations throughout the country with a view to help designing appropriate strategy for control of gonococcal infection in Bangladesh.

To address the objectives of the study, sociodemographic characteristics of 1025 male individuals were recorded by interviewing the cases with a pre-tested set of questionnaire during January, 2001 to July, 2003 in Sylhet, thse northeastern City in Bangladesh. The enrolled cases were included in two groups- one group comprised of symptomatic male individuals, attending the outpatients Department of Skin and Venereal diseases of Sylhet MAG Osmani Medical College Hospital (male-OPD, SOMCH, n=371) and the other group were the male individuals having sex with male (MSM), attending a medical centre for the MSMs in the Sylhet City (MSMs, n=654). The cases with gonococcal infection were identified by laboratory results of microscopy and culture of appropriate specimens. A total 495 *N. gonorrhoeae* isolates including 54 from Sylhet and 441 from the ICDDR,B were characterized. These organisms were isolated from different high-risk populations in Sylhet (male-OPD, SOMCH and MSMs), Dhaka (female hotel- or street-based commercial sex workers (CSWs), male-OPD, Dhaka Medical College Hospital) and Gualanda, Faridpur (brothel-based CSWs), during 2003 to 2006. The characterization of the *N. gonorrhoeae* isolates included antimicrobial susceptibility by agar dilution MIC method, serotype and serovar determination by monoclonal antibodies, epidemic PPNG and TRNG plasmids identification by PCR and genotyping by *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).

A total of 171 (16.7%) among the 1025 male cases in Sylhet were found infected with *N. gonorrhoeae* of which 143 (38.5%) infections were among the Male-OPD and remaining 28 (4.3%) were found among the MSMs. Almost all of the infections were urethral (170, 99.4%) among both the groups, while only one was rectal (0.6%) among the MSMs. The rate of gonococcal infection among the cases was found to decline following provision of directly observed therapy with the highest sensitive antibiotic (21.6% to 14.3%). The highest rate of gonococcal infection was found to occur among the 16-25 years age group in the Male-OPD cases (98/224, 43.8%) and among the older age group of above 45 years in the MSMs (2/8, 25.0%). Gonococcal infection was significantly higher among the cases having symptoms of purulent/ mucopurulent urethral discharge (92.9% found infected), cases of urethritis (74.6% were due to gonococcal infection), cases having history of sexual exposure to female CSWs (29.5% infected), and cases having heterosexual type of sex practice (40.4% infected). Illiterate individuals, drivers by profession, cases having extra-marital sex and cases not using condom during sexual act were also found more commonly infected (19.2%, 25.6%, 17.3% and 17.7% respectively).

Antimicrobial resistance phenomenon was occurring in majority of the isolates with penicillin (51.5%), tetracycline (87.9%) and ciprofloxacin (90.9%). Resistance to all three of the antibiotics together (multi-drug resistance, MDR) was found among 46.5% of the isolates. Characterizing the *N. gonorrhoeae* isolates for PPNG plasmid content, it was found that many of the isolates (169/495, 34.1%) were PPNG (penicillinase producing *N. gonorrhoeae*), demonstrating majority (169/ 255, 66.3%) plasmid-mediated penicillin resistance and all of the PPNG plasmids identified were Africa type of 3.1 kb size. Majority of the *N. gonorrhoeae* isolates (382/495, 77.2%) was found to contain TRNG (tetracycline resistant *N. gonorrhoeae*) plasmids, demonstrating 87.6% (382/436) plasmid-mediated tetracycline resistance, almost all of the plasmids being Dutch type (700bp) (377, 98.7%) and a few were American

type (1600 bp) (5, 1.3%). More than two-thirds of the characterized isolates (400, 80.8%) were found to carry plasmids of which 18 (4.5%) carried single PPNG plasmid, 230 (57.5%) carried single TRNG plasmid and remaining 152 (38.0%) isolates were found to contain both PPNG and TRNG plasmids. A total 239 of the *N. gonorrhoeae* isolates were investigated for serotype and serovars. Majority of the isolates were serotype B (142/239, 59.4%) and 41 different serovars were identified, of which "Arst" was found to include the highest (46, 19.2%) number of *N. gonorrhoeae* isolates. Many of the *N. gonorrhoeae* isolates (58, 24.3%) showed new serovars majority of which (40, 69.0%) was multidrug resistant (MDR). A few (7) of the *N. gonorrhoeae* isolates from CSWs showing MDR were by NG-MAST. Majority of the *por* alleles of the genotyped *N. gonorrhoeae* isolates were known (6/8, 75.0%) whereas majority of the *tbpB* alleles were new (8/12, 66.7%) yielding all new sequence types (STs) (7/7, 100.0%) by the NG-MAST database posting in their own website (www.ng-mast.com).

The sociodemographic characteristics, identified in this study, of the male high-risk populations were found almost similar with those of the other investigators reported throughout the world. The rate of gonococcal infection in the present study demonstrated a steady decline from the previous reports of the disease among males in Bangladesh although this was very high in comparison with the other studies in the developed as well as the developing countries. The high-level of resistance of penicillin, tetracycline and ciprofloxacin (as well as majority of the penicillin and tetracycline resistance being plasmid-mediated) indicates no hope for these antibiotics to be included in the treatment regimen in near future and these circumstances are compelling to a concern on careful usage of the currently sensitive antibiotics. Genotypes of the highly resistant isolates showing all new sequence types indicates the rapid adaptation of the gonococcal isolates by interstrain transformation and recombination among the core transmitters. Consequently, it would not be possible to predict temporal changes in susceptibility of the *N. gonorrhoeae* isolates with the previous

knowledge of antibiogram, necessitating a consistent antimicrobial susceptibility surveillance of the isolates circulating in Bangladesh for launching an effective control program of the gonorrhoea.

Therefore, it can be concluded from the findings of the present study that the gonococcal infection in Bangladesh is not under proper control and can be effectively controlled by ensuring general measures of protected sex among the sexually active high-risk populations as well as by implementing a consistent surveillance system to identify the cases of gonococcal infections as well as the antimicrobial susceptibility of *N. gonorrhoeae* isolates to monitor and update the treatment regimen periodically.

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A.D.- After Death of Christ

ADRA- Amplified Ribosomal DNA Restriction Analysis

AGSP- Australian Gonococcal Surveillance Project

AHU- Arginine, Hypoxanthine and Uracil

AIDS- Acquired Immunodeficiency Syndrome

AP- Arbitrarily Primed

A/S- Auxotype and Serological

AST- Antimicrobial Susceptibility Test

B.C.- Before Birth of Christ

BIRDEM- Bangladesh Institute of Research and Rehabilitation of Diabetic, Endocrine and Metabolic Disorders

BMRC- Bangladesh Medical Research Council

bp- Base pair

BSMM- Bangladesh Society of Medical Microbiologists

BSMMU- Bangabandhu Sheikh Mujib Medical University

BSWS- Bandhu Social Welfare Society

Cap.- Capsule

CD- Cluster of Differentiation

CDC- Center for Disease Control and Prevention

CDSC- Communicable Diseases Surveillance Centre

CFU- Colony Forming Units

CLS- Clinical Laboratory Services

CLSI- Clinical Laboratory Standards Institute

CMP- Cytidine Monophosphate

CMRNG- Chromosomally-mediated Resistant *N. gonorrhoeae*

CO_2- Carbon dioxide

CSW- Commercial Sex Worker

DGI- Disseminated Gonococcal Infection

DMCH- Dhaka Medical College Hospital

DMSO- Dimethyl Sulphoxide

DNA- Deoxyribonucleic Acid

dNTP- Dinucleotide phosphate

DST- Diagnostic Sensitivity Test

EDTA- Ethylene Diamine Tetraacetic Acid

FA- Fluorescent antibody

GASP- Gonococcal Antimicrobial Surveillance Programme

GC- Gonococci

GISP- Gonococcal Isolate Surveillance Project

GRASP- Gonococcal Resistance to Antimicrobials Surveillance Programme

GUD- Genitourinary Medicine

GUM- Genitourinary Medicine

HAM- Homosexually active Male

HPF- High Power Field

HIV- Human Immunodeficiency Virus

HSPG- Heparin Sulfate Proteoglycan

ICDDR,B- International Center for Diarrhoeal Diseases Research in Bangladesh

IgA- Immunoglobulin A

IgM- Immunoglobulin M

IM- Intramuscular

Inj.- Injection

IUD- Intrauterine Device

IV- Intravenous

kb- Kilo base

KDa- Kilo Dalton

Lbp- Lactoferrin binding protein

LCR- Ligase Chain Reaction

LF- Lactoferrin

LOS- Lipooligosaccharide

LPS- Lipopolysaccharide

LSD- Laboratory Sciences Division

MAG- Mohammad Ataul Goni

M.B.B.S.- Bachelor of Medicine and Bachelor of Surgery

M.D.- Doctor of Medicine

MDR- Multi-drug Resistance

MIC- Minimum Inhibitory Concentration

µg- Microgram

ML- Martin Lewis

ml- Millilitre

MLST- Multilocus Sequence Typing

MMWR- Mortality and Morbidity weekly report

mol. wt.- Molecular weight

M.P.H.- Master of Public Health

M.Phil.- Master of Philosophy

MSM- Male sex for Male

MTM- Modified Thayer Martin

NAAT- Nucleic acid amplification tests

NAD- Nicotine Adenine Dinucleotide

NANA- N-acetyl neuraminic acid

NASBA- Nucleic Acid Sequence Based Amplification

NCCLS- National Committee for Clinical Laboratory Standards

NGMAST- *Neisseria gonorrhoeae* Multi-antigen Sequence Typing

NIPSOM- National Institute of Preventive and Social Medicine

NYC- New York City

OMP- Outer membrane protein

OPD- Outpatients department

PAGE- Poly acrylamide gel electrophoresis

PBP- Penicillin Binding Protein

PBS- Phosphate Buffered Saline

PCR- Polymerase chain reaction

PFGE- Pulsed Field Gel Electrophoresis

PHAC- Public Health Agency of Canada

Ph.D.- Doctor of Philosophy

PHLN- Public Health Laboratory Network

PID- Pelvic Inflammatory Disease

Pil^+- Piliated

Pil^- - Non-piliated

PMN- Polymorphonuclear Neutrophil

PI- Protein I

PIA- Protein IA

PIB- Protein IB

PPNG- Penicillinase producing *Neisseria gonorrhoeae*

QRDR- Quinolone Resistance Determining Region

Rmp- Reduction modifiable protein

RNA- Ribonucleic Acid

Rpm- Revolutions per minute

RTI/ STI- Reproductive tract infection/ sexually transmitted infections

SDA- Strand Displacement Amplification

SDS- Sodium Dodecyl Sulphate

SEA- South-East Asia

SOMC- Sylhet MAG Osmani Medical College

SOMCH- Sylhet MAG Osmani Medical College Hospital

ST- Sequence type

STD- Sexually Transmitted Disease

STI- Sexually Transmitted Infections

Tbp- Trasferrin binding protein

TBE- Tris Borate EDTA

TMA- Transcription mediated Amplification

TNF- Tumour Necrosis Factor

TRNG- Tetracycline Resistant *Neisseria gonorrhoeae*

TSB- Tryptic Soya Broth

UGI- Urogenital Disease Isolate

UK- United Kingdom

US- United States

USA- United States of America

VCNT- Vancomycin, Colistin, Nystatin and Trimethoprim

VD- Venereal Diseases

WHO- World Health Organization

WPR- Western Pacific Region

Chapter 1: Introduction

Gonorrhoea is one of the oldest known human diseases (Boyd, 1955; Handsfield, 1990). Although substantial development occurred in diagnosis and treatment of gonorrhoea, the disease still remains as one of the most common venereal diseases as well as a global health problem causing a wide range of morbidity, especially among the sexually active people (De Schryver and Meheus, 1990; Fekete, 1993; Sparling, 1999; CDC, 2007a).

Although the gonorrhoea can be easily cured by medical treatment, untreated cases may possibly lead to serious health problems. The disease is known to often complicate by periurethral abscess, epididymitis, urethral strictures with or without perineal fistulae in men, urethral or tubal strictures leading to infertility and sterility in both sexes, pelvic inflammatory disease (PID) with lower abdominal pain and ectopic pregnancy in the women, and disseminated gonococcal infection (DGI) manifested by arthritis-dermatitis syndrome, endocarditis and meningitis rarely (WHO, 1978; Hook and Holmes, 1985; Knapp and Rice, 1995; Ghaly and Chien, 2000; Winn Jr. *et al.,* 2006). The pregnant women infected with *N. gonorrhoeae* are at an increased risk for pregnancy complications such as chorioamnionitis, premature rupture of membranes, preterm labour, and stillbirth (Russell, 1979; Glass *et al.*, 2005). Infected women may also pass the disease to their infants during delivery causing conjunctivitis (gonococcal ophthalmia neonatarum) leading to corneal scarring and blindness (Movitt, 1938; Sorsby, 1950; Hook and Holmes, 1985). In addition, as with other sexually transmitted infections, the gonorrhoea provides an elevated risk of acquiring infection with human immunodeficiency virus (Laga *et al.*, 1993; Weir *et al.,* 1994; Flemming and Wasserheit, 1999).

The rate of gonorrhoea is highly variable throughout the world. The incidence of the disease demonstrated a sharp decline in almost all Western countries

during 1970s and 1990s, followed by a plateau through a decade and a recent increase (De Schryver and Meheus, 1990; Fox *et al.,* 1998; Berglund *et al.,* 1999; Velicko and Unemo, 2009). After a 74% decline from 1975 through 1997 in the rate of reported gonorrhoea in the United States (US), rates of the disease remained stable up to 2004 and then increased for the following two years during 2005 and 2006 (CDC, 2007a). Gonorrhoea has been identified as the second most commonly reported communicable disease in the US, accounting for more than 300,000 cases annually (CDC, 2003; CDC, 2009). Gonococcal infection was also the second most common bacterial sexually transmitted infection (STI) in England and Wales, with over 20,000 new infections being diagnosed in genitourinary medicine (GUM) clinics in 2000 (CDSC, 2001). The infection has been also reported as the second most common bacterial STI from New Zealand as well and the number of cases of gonorrhoea in the country has increased each year since 1996, with an overall increase of 94% between 1996 and 2002 (Hefferman *et al.,* 2004). In 1999, the global number of new gonorrhoea cases among men and women (15 to 49 years of age) was estimated at 62.35 millions (WHO, 2001). Of these cases, approximately 1 million were reported in the Western Europe, 3.5 millions in Eastern Europe and Central Asia, and most of the other cases were in Sub-Saharan Africa (17 millions) and South and South-East Asia (SEA) (27 millions). Analyzing this report of the WHO, it appears that by number of new attacks of the infection, the SEA remains at top on the list, projecting the worse/ ineffective control measures in the underdeveloped / developing nations of this region. Moreover, since there are many asymptomatic carriers of the infection and inadequately effective surveillance program in majority of the developing countries reporting gonococcal infections, it is presumed that the official reports are significantly underreported. Keeping in mind the limitations of substantial under-diagnosis and underreporting of gonococcal infection, the investigators estimated approximately twice as many new infections to occur each year as has been reported (Weinstock *et al.*, 2004).

In Bangladesh, though very limited investigations were carried out, a recent surveillance study showed a continued decrease in gonorrhoea rates by 22% among the street-based commercial sex workers (CSWs) (from 42% in 1997 to 20% in 2002) (ICDDR,B, 2003a).

The gonococcal infection is now shaping up in a pandemic nature. The continued global survival of the organism is supposed inevitable because of its immunobiology, including a short incubation period of 2-7 days with high degree of transmissibility, the rapidly developing resistance of the organism to commonly-used-antimicrobial agents, the lack of natural or acquired immunity, the changing high-risk human sexual behaviour and the frequency of asymptomatic infections (Cohen and Sparling, 1992). There is a concept of gonococcal infection epidemiology that proposes the existence of core groups of effective transmitters among the commercial sex workers and their clients, who are supposed to be responsible for most, if not all, cases of the continuing endemicity of the disease (Yorke *et al.*, 1978; May, 1981; Barnes and Holmes, 1984). The clinical syndromes of gonococcal infection differ with respect to age, sex, race and sexual practiceps of the infected individuals as well as to the virulence of the infecting gonococcal strain (Barnes and Holmes, 1984).

Neisseria gonorrhoeae, the causative organism of gonorrhoea has been known since its identification by Neisser in 1879. The organism is pathogenic for humans only and is transmitted principally through sexual contact. It can grow and multiply easily in the warm, moist areas of the genital tract including cervix uteri and fallopian tubes in the women as well as in the urethra in both men and women. The organism also colonizes less efficiently to other mucous membranes of the body like oral, conjunctival and rectal mucosa, and survives poorly outside the human body. It is highly adaptable in human body and capable of resisting immune attack by the antigenic variability by which it evades host defences, thus persisting and often causing asymptomatic (and

undetected) infections (Cohen and Sparling, 1992; Knapp and Rice, 1995; Todar, 2004; Winn Jr. *et al.*, 2006).

In addition, the *N. gonorrhoeae* possesses some unique ability to develop resistance to antimicrobial agents. The only feature of gonorrhoea epidemiology feared and shared by both developing and developed countries is the increasing incidences of antibiotic resistance among the isolates of *N. gonorrhoeae*. Eventually, the treatment of gonococcal infection has become gradually more expensive with the rapid spread of strains resistant to older antimicrobials. An excellent example of the effects of selective antibiotic pressure occurring in Korea was the penicillin resistance that prompted primary therapy with spectinomycin and was ultimately followed by development of resistance to the spectinomycin as well (Boslego *et al.*, 1987). Very recently, *N. gonorrhoeae* was also found to develop widespread resistance to ciprofloxacin throughout the world. Fluoroquinolones including ciprofloxacin were found highly effective as the single oral dose therapy for uncomplicated gonococcal infections in the early nineties and soon the Centers for Disease Control and Prevention (CDC) recommended fluoroquinolones as first-line therapy for treatment of gonorrhoea (CDC, 1993). The ciprofloxacin in a 500 mg single oral dose was also incorporated into the syndromic management algorithms of vaginal and urethral discharge by the World Health Organization (WHO, 1997). Thus the fluoroquinolones, particularly ciprofloxacin were used widely for laboratory confirmed as well as suspected cases of gonorrhoea. As a result, the *N. gonorrhoeae* strains emerged rapidly with decreased susceptibility and finally high-level resistance to the ciprofloxacin in several countries, particularly in the Southeast Asia including Bangladesh (Tanaka *et al.*, 1995; Kam *et al.*, 1996; Bhuiyan *et al.*, 1999), the European countries (Fenton *et al.*, 2003; Arreaza *et al.*, 2003; Herida *et al.*, 2004) and the western Pacific areas (Tapsall *et al.*, 1995; Thompson *et al.*, 1995; Knapp *et al.*, 1997a; Fox *et al.*, 1997; Ison *et al.*, 1998; Ross, 1998).

Moreover, multidrug resistant (MDR) strains of *N. gonorrhoeae* isolates are now emerging in many countries. In Greece, the decrease in the incidence of gonorrhoea was found to accompany by changes in the epidemiology of the disease and an increase in the relative frequency of MDR *N. gonorrhoeae* isolates (Mavroidi *et al.*, 2001). Similar changes have been reported from other European countries and the US much earlier (Easmon, 1990; van Duynhoven, 1999). The pattern of antimicrobial susceptibility of *N. gonorrhoeae* isolated in Bangladesh is equally threatening the treatment modality, putting an urgency to frequent update the current treatment regimen (ICDDR,B, 2003b; ICDDR,B, 2004; Alam *et al.*, 2005; ICDDR,B, 2005; ICDDR,B, 2006).

In addition, mixed gonococcal infection, where an individual is colonized simultaneously with more than one strain of *N. gonorrhoeae* in the same anatomical site, was reported to occur frequently, particularly among highly sexually active populations (Short *et al.*, 1977; O'Rourke *et al.*, 1995; Gutjahr *et al.*, 1997; Hill, 1999). When more than one strain occurs together *in vivo*, it is hypothesized that the deoxyribonucleic acid (DNA) released from lyses of the bacteria act as source for transformation and recombination, resulting in a highly genetically variable population of the organism (Cannon and Sparling, 1984; O'Rourke *et al.*, 1995; Gutjahr *et al.*, 1997; Hill, 1999). *N. gonorrhoeae* also undergoes mutation and intragenic recombination contributing to the genetic variation (O'Rourke *et al.*, 1995). Mixed infections of gonorrhoea are likely to occur in focal points of transmission chains where individuals have multiple sexual partners and where the incidence of gonorrhoea is high, such as in the core groups of CSWs. However, it may be possible that a mixed infection is carried and transmitted simultaneously by one sexual partner (Martin and Ison, 2003), although it is difficult to establish a direct evidence of mixed infections occurring *in vivo*. Nevertheless, after isolation of the bacteria from a patient, it is possible *in vitro* to characterize the isolate to find out relatedness among themselves.

Precise characterization of the isolated strains of *N. gonorrhoeae* including antimicrobial susceptibility, serotyping and serovar determination, plasmid profile as well as genotyping can provide valuable information on gonococcal strain populations in a community, temporal changes and the emergence and spread of antibiotic-resistant strains of the organism. In this context, knowledge on epidemiology of gonococcal infection using molecular methods would be further useful for development of effective control and preventive measures. The most widely employed method for differentiation of *N. gonorrhoeae* strains was auxotyping and serological (A/S) characterization. However, A/S classification system has a number of limitations. Auxotyping is complicated, labourious and time-consuming, and serotyping requires well-characterized and specific polyclonal or monoclonal anti-gonococcal antibodies (Tam *et al.*, 1982). Furthermore, there is also scepticism that this system may not provide sufficient discrimination, in most populations, only a limited number of A/S classes account for the majority isolates. Some unrelated isolates were found to belong to the same A/S class and some isolates have been reported that were impossible to auxotype and serotype (Gill, 1991). For strains that carry plasmids, differentiation of strains belonging to the same A/S group could be achieved by identifying the plasmids. However, plasmid profiling is also of limited value when a common combination of plasmids is present (Gill, 1991). To overcome the limitations of the phenotyping methods, newer molecular methods offer greater discrimination compared to the previous phenotypic methods (Ison, 1998).

A genotypic method needs to be based on a target that accumulates genetic variation quickly, so that individuals that are sexual contacts or part of a short transmission chain should demonstrate an indistinguishable type, while individuals that are unlinked should have different types. *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) is one of the genotyping methods employing a sequence-based investigation of the diversity in two outer membrane proteins, *por* and *tbpB* allowing the precise characterization and

comparison of gonococcal isolates with all analyses performed in predesigned softwares via an internationally accessible website. The method has been shown to identify epidemiologically linked individuals and is able to distinguish between isolates/ individuals that are not linked (Martin *et al.*, 2004).

With the above considerations, the present study was conducted among high-risk populations to find out sociodemographic criteria of the selected male cases, and to characterize *N. gonorrhoeae* isolates by antimicrobial susceptibility, serotype and serovar determination, plasmid profile and genotyping. The demographic characteristics were collected using a set of questionnaire. Antimicrobial susceptibility was detected by agar dilution MIC method, serotype and serovar were determined using Phadebact monoclonal antibodies panels, plasmids for PPNG and TRNG were identified by PCR methods using appropriate primers and protocols. Finally, genotyping was done by NG-MAST submitting sequence data of two genes of *N. gonorrhoeae* isolates to the international website for this purpose.

The relationship between all these characteristics of the cases of gonorrhoea as well as of the *N. gonorrhoeae* isolates were identified to understand the disease epidemiology and transmission biology of the aetiological agents in a view to help designing a strategy for effective control of gonococcal infections in Bangladesh.

Objectives

General Objective:

To find out antimicrobial resistance and molecular epidemiology of *Neisseria gonorrhoeae* in Bangladesh

Specific Objectives:

1. To find out sociodemographic characteristics related to gonococcal infection among the male individuals;
2. To identify rates of gonococcal infection among the cases of male population;
3. To find out relationship between the sociodemographic characteristics and gonococcal infection of the cases;
4. To find out antimicrobial resistance of the *N. gonorrhoeae* isolates from high-risk populations at different areas of Bangladesh;
5. To find out penicillinase producing *N. gonorrhoeae* (PPNG) strains;
6. To identify plasmids responsible for penicillin and tetracycline resistance in *N. gonorrhoeae* isolates;
7. To determine the serotypes and serovars of the *N. gonorrhoeae* isolates;
8. To find out genotypes (sequence types) of the *N. gonorrhoeae* isolates;
9. To find out relationship of characters of the *N. gonorrhoeae* isolates with source populations and antimicrobial resistance.

Chapter 2: Review of literature

2.1: Sociodemographic characteristics of the cases of gonorrhoea

2.1.1: Gonorrhoea: Historical perspective

Gonorrhoea in men was described in the ancient Chinese, Egyptian, Roman, and Greek literatures as well as in the Old Testament of the Bible (Rosebury, 1971; Hook and Holmes, 1985; Handsfield, 1990). The contiguous nature of gonococcal infection can be found in the Biblical text within the Book of Leviticus (Lev. 15:1-15:19). Descriptions in few passages in the Old Testament and the Talmud refer to genital exudates, probably indicating gonorrhoea, making it one of the oldest recorded human diseases. Hippocrates was also found to refer to acute gonorrhoea as "strangury" obtained from the "pleasures of Venus" in the fourth and fifth centuries B.C. (Sparling, 1999). Galen in A.D. 130 confused the purulent discharge associated with gonococcal urethritis in men with semen and introduced the term gonorrhoea meaning "flow of seed" after Greek words *gonor* ("seed") and *rhoia* ("flow") (Handsfield, 1990; Sparling, 1999).

Although well-recognized as a sexually transmitted infection by the 13th century, gonorrhoea was not distinguished from syphilis until mid-16th when Paracelsus (1530), the great venereologist thought that gonorrhoea was an early symptom of syphilis. The confusion was further heightened by the English physician John Hunter, in 1767, who thought that the gonorrhoea and syphilis were caused by a single pathogen and intentionally inoculated himself with pus from a patient of gonorrhoea and supposed to wound up giving himself syphilis!

In 1897, *Neisseria gonorrhoeae,* the aetiological agent of gonorrhoea, was first observed in purulent exudates from the genital tract and conjunctiva by Albert

Neisser at the University of Breslau, Germany. However, it was not until 1882 that Leistikow and Loeffler finally cultivated the gonococcus (Handsfield, 1990; Sparling, 1999). Subsequent isolation of the organism, inoculation into human volunteers, and re-isolation of the organism by Bumm in 1885 proved the causal relationship of the organism with gonorrhoea (Winn Jr. *et al.*, 2006). The most notable history of gonococcal infection was found in the personal diary of James Boswell, the famous biographer of Samuel Johnson. Boswell described more than 19 separate infections with the gonococcus and described the consequences of asymptomatic infection in women, especially in his wife who never exhibited symptoms of gonococcal infection, but lost four of her nine pregnancies (Ober, 1970).

The treatment of gonorrhoea was introduced in 1936 with a single dose of the sulfonamides and found highly effective. However, a number of cases failed to respond to sulfonamides owing to the existence of a naturally occurring resistant strain of the gonococcus (Willcox, 1958). The first truly successful treatment for gonorrhoea came in the beginning of the twentieth century during the Second World War (1939-1945) with the use of penicillin which then became the drug of choice for next 40 years. The reduced penicillin susceptibility was initiated with chromosomally-mediated resistance and later followed by acquisition and spread of plasmids containing penicillinase genes requiring serial increase in dose of procaine penicillin from 50,000 units in 1945 to 4.8 million units by early 1970s (CDC, 1974). The progressive decline in penicillin susceptibility and few cases of penicillin allergy reinforced the introduction of tetracycline as an effective alternative (Marmell and Prigot, 1955; McLone *et al.*, 1968). In 1985, tetracycline was no longer a treatment option and by 1989, because of widespread resistance, penicillin was also no longer effective and ceftriaxone was recommended for treatment of uncomplicated gonorrhoea with ciprofloxacin as an alternative (CDC, 1985a; CDC, 1989). By 1993, because of high efficacy, safety and convenient single oral dose, fluoroquinolones (ciprofloxacin, ofloxacin, etc) were recommended

as first-line treatment regimen for uncomplicated gonococcal infection (CDC, 1993). With the increasing concerns about emerging resistance of the gonococci to antimicrobial agents, a nationwide sentinel surveillance system, gonococcal isolate surveillance project (GISP), was developed in the USA in 1986 to monitor antimicrobial susceptibility. Based on GISP data, the emergence of quinolone-resistant *N. gonorrhoeae* (QRNG) was first identified in Hawaii in 1991 (Knapp *et al.,* 1994), at the same time it was recognized in Asia (Iverson *et al.*, 2004). First report of decreased susceptibility of *N. gonorrhoeae* to ciprofloxacin in the UK was in 1990 (Gransden *et al.,* 1990), followed by another report in 1991 of ciprofloxacin-resistant isolate acquiring from Bangkok, Thailand (Corkill, *et al.,* 1991). On the basis of cumulative data on quinolone resistance throughout the world, the CDC declared that fluoroquinolones are no longer recommended for gonococcal infections and related conditions (CDC, 2007b).

2.1.2: Gonorrhoea: epidemiology

2.1.2a: Gonorrhoea: Bangladesh perspective

In Bangladesh, the first available report on gonorrhoea among the male population was in 1988, in which screening result of the attendees at skin and venereal diseases outpatient department (Skin VD OPD) of Sir Salimullah Medical College Hospital, Mitford, Dhaka showed that the gonococcal urethritis (52.7%) was the commonest among the sexually transmitted diseases (STDs) (Chowdhury and Amin, 1988). In another report, during 1988 to 1990 in Chittagong among the attendees at Skin VD OPD of Chittagong Medical College Hospital and Central Skin and Social Hygiene Centre, Agrabad, Chittagong the investigators found gonococcal infection (31%) as the highest of the STDs (Islam and Rahman, 1992). This was followed by another investigation carried out among the patients reporting to Skin OPD of Military hospital and to private chambers of practicing dermatologists during 1987 to 1992 and found 19.1% gonococcal infection (Sadeque and Rahmatullah, 1993).

The rate of gonococcal infection thus appears gradually declining among the males attending healthcare facilities in Bangladesh. (Figure 2.1) However, among the symptomatic cases with urethral discharge, the rate of gonococcal infection was reasonably found higher (46.5%) in another study conducted at the department of Microbiology, Rajshahi Medical College, Rajshahi (Ahmed *et al.*, 1999).

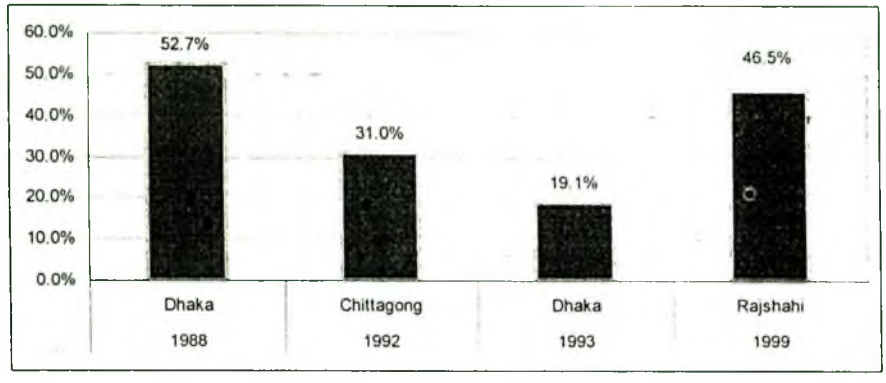


Figure 2.1: Variation in rate of gonococcal infection among the males in Bangladesh from 1988-1999

The first available report on rate of gonococcal infection among the female commercial sex workers (CSWs) in Bangladesh was 19.6% in 1994 (Sattar *et al.*, 1994). Another ICDDR,B-supported study among the CSWs in 1997 found 42% culture-positive cases of gonococcal infection (Bhuiyan *et al.*, 1999). Immediately following another study with the same population of female CSWs found 35.5% infections (Rahman *et al.*, 2000). Another similar study among CSWs supported by the ICDDR,B found 30.7% gonococcal infection (Alam *et al.*, 2002). Accordingly, the rate of gonococcal infection among the female CSWs in Bangladesh was found declining by 22% (42% to 20%) among street-based (floating) commercial sex workers in Dhaka (ICDDR,B, 2003a). (Figure 2.2)

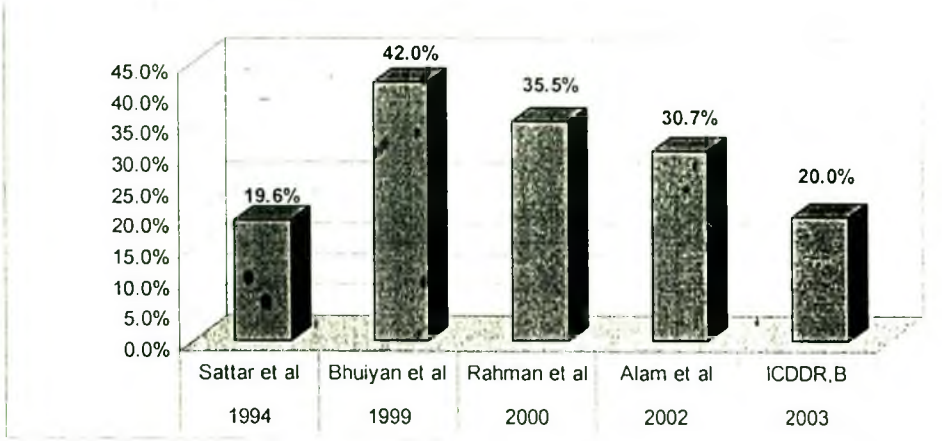


Figure 2.2: Variation in the rate of gonococcal infection among female CSWs in Bangladesh from 1994 to2003

2.1.2b: Gonorrhoea worldwide

Exact global estimates of the disease are not available but the WHO counts that 62 million new cases of gonorrhoea were occurring worldwide each year. Among the total 62 million cases of gonorrhoea in 1999 globally, most were in the South and South-East Asia (27 million), Sub-Saharan Africa (17 million), and Latin America and the Caribbean (7.5 million) (WHO, 2001).

The estimated incidences were substantially higher in developing nations than in the developed counterparts. Among the developing nations in 1995, the incidence rates of gonococcal infection per 1,000 inhabitants of 15 to 49 years age were the highest (57.7 for males and 65.5 for females) in sub-Saharan Africa, followed by South and South East Asia (30.0 for males and 31.8 for females) and the lowest in Latin America and the Caribbean (27.6 for males and 29.2 for females). Among the developed nations, the rates were the highest in North America and Australasia (10.9 for males and 12.0 for females), followed by Western Europe (5.6 for males and 6.0 for females) and the lowest in East Asia and the Pacific (4.4 for males and 3.8 for females) (Gerbase *et al.*, 1998; Tapsall, 2001). (Table 2.1, Figure 2.4)

Table 2.1: Comparison of gonococcal infections per 1000 population between developing and developed nations as estimated in 1995

Nations representations	*No. of Gonococcal infections per 1000 population among*	
	Male	*Female*
Developing nations:		
Sub-Saharan Africa	57.7	65.5
South & Southeast Asia	30.0	31.8
Latin America & the Caribbean	27.6	29.2
Developed nations:		
North America & Australasia	10.9	12.0
Western Europe	5.6	6.0
East Asia & the Pacific	4.4	3.8

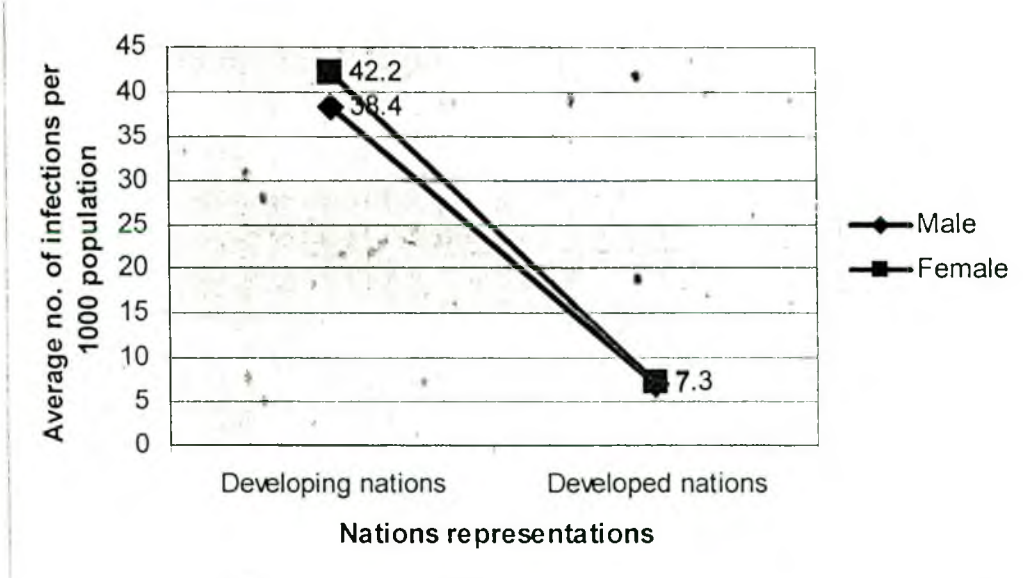


Figure 2.3: Comparison of average rate of gonococcal infections among both male and female populations in developing and developed nations

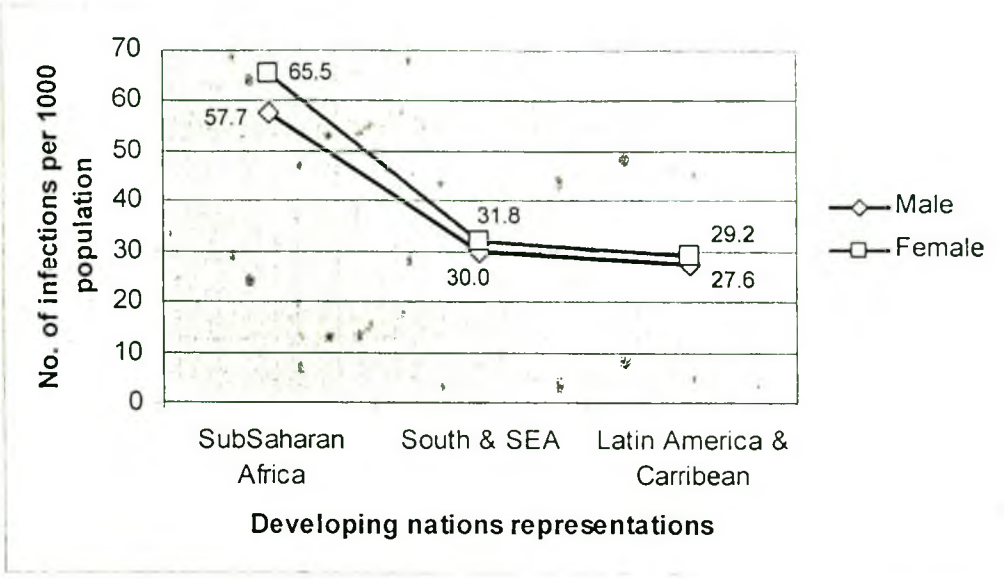


Figure 2.4: Comparison of rate of gonococcal infection among the male and females of the developing nations representative countries

In Canada, after several years of decline, the reported gonorrhoea rate was increasing considerably during the recent years. National goals for gonorrhoea control in Canada were developed in 1997 and since then, the disease surveillance showed an increase of the reported cases by 53% among men and 33% among women. The largest proportion of cases in women was among those aged 15-24 and in males among those aged 30-39 (Hansen *et al.*, 2003). A network of people with high-transmission activities was supposed to play a key role in the current prevalence levels. Case finding and partner notification were mentioned as the critical strategies for controlling gonococcal infection in the national guidelines of Canada (PHAC, 2009).

In the US, the rates of gonorrhoea decreased sharply to its lowest level in 1957, after which the incidence of the disease increased steadily during the 1960s and early 1970s, with the highest incidence of over 460 cases per 100,000 population occurring in 1975 (de Schryver and Meheus, 1990; Sparling and Handsfield, 2000). From 1975, the incidence of the disease was declined by

74.3% through 1997 following implementation of national gonorrhoea control programs in the mid-1970's (Fox *et al.*, 1998; CDC, 2000). For the next 10 years, gonorrhoea rates remained static, although during the following two years until 2006, the rates had slightly increased again in the USA. In 2006, there were 120.9 cases per 100,000 population showing an increase of 5.5% since 2005 and an increase for the second consecutive year, particularly in the western US (CDC, 2007a; CDC, 2009). Although in 2007, there were 118.9 cases per 100,000 persons with a 2.0% decrease from 2006 (CDC, 2009). Rates of gonorrhoea in the US found high among the African Americans, adolescents, young adults and men who have sex with men (MSM, homosexuals) (CDC, 2007a; Workowski *et al.*, 2008). Recent reports have also documented high rates of gonorrhoea among HIV-infected MSMs (CDC, 2007b). Gonorrhoea was identified as the second most frequently reported communicable disease in the recent years (CDC, 2006; CDC, 2008; CDC, 2009).

In the United Kingdom (UK), the gonococcal infection shows a clear increase from 1957 onwards, reaching a peak in the early seventies and then decreasing. The infected women were mostly younger than the men. The highest incidence among females was in the age group 16-19 years and among males in the age group 20-24 years (de Schryver and Meheus, 1990).

In Denmark, there was a steep increase of gonorrhoea incidence in the 1960s, followed by a sudden decline in the seventies. A plateau was observed until 1984, when even a more dramatic decline was seen. The decline was found both in men and women and throughout the country (de Schryver and Meheus, 1990). In Sweden, there was an almost continuous decline in the incidence of gonorrhoea both in men and women (de Schryver and Meheus, 1990).

In Australia, the number of cases of gonorrhoea declined from a peak of 6,599 in 1982-1983 to 1121 in 1990-1991 showing a reduction of 83% (AGSP,

1993). The epidemiology of gonococcal disease in Australia is a combination of the less developed and Western-style country patterns. Extremely high rates of disease, sometimes in excess of 1,000 cases per 100,000 populations per year are present in indigenous populations in rural Australia. In urban populations, gonorrhoea was found most frequently among homosexually active men (HAM/ MSMs), clients of often illegal CSWs and travellers entering or returning to Australia. The gonococcal populations circulating in these groups, and their susceptibility to antibiotics used to treat gonorrhoea are usually quite distinct (Donovan *et al.*, 2000).

2.1.2c: Gonorrhoea: Transmission

Although the gonococcal infection is spread almost exclusively by sexual activity, the risk of acquiring gonorrhoea is multifactorial and is also related to the frequency and sites of exposure. The gonococcal infections are transmitted more efficiently from an infected man to a woman (in 50-60% of instances of one sexual exposure) than from an infected woman to a man (in 35% of instances of one sexual exposure (Knapp and Rice, 1995; Winn Jr. *et al.*, 2006). For heterosexual males, the risk of acquiring urethral infection from an infected female was found about 20% by a single exposure and up to 80% by four exposures (Holmes *et al.*, 1970).

Anorectal infections occur in the homosexual/ bisexual men who practice unprotected receptive anal sex. Women may also acquire rectal infections by receptive anal sex, but most rectal infections in women are due to perianal contamination with infected cervicovaginal secretions. Oropharyngeal gonococcal infection is seen in homosexual and bisexual men and heterosexual women who acquire the infection by engaging in orogenital sexual contact with an infected partner (Rompalo, 1999). Transmission of rectal gonococcal infection among homosexual/ bisexual men demonstrated that urethral infection following fellatio (oral sex upon penis) from an infected partner may account for as much as 26% (Lafferty *et al.*, 1997).

Newborns may be infected by exposure during parturition and adult gonococcal ophthalmitis may also be developed due to accidental inoculation from hands during working with the organism in a laboratory (Malhotra *et al.*, 1998). The adult ocular gonococcal infections have also been reported among adults who became infected via genital secretions (Wan *et al.*, 1986).

2.1.2d: Gonorrhoea: behavioural and socio-demographic characteristics of the cases

The incidences of gonococcal infection were found to be influenced by several factors like patterns of sexual behaviour, population demographics, economic and social conditions as well as biases in the quality and quantity of the epidemiological data (Piot and Islam, 1994; Gerbase *et al.*, 1998; Tapsall, 2001).

The steady increase in the rates of gonorrhoea in the US following the Second World War (1039-1945) during 1962 and 1975 was attributable to several factors including an increasing at risk population of young adults, importation of less susceptible gonococcal strains along with the return of service-men from the Vietnam conflict, increasing use of non-barrier contraceptive methods (i.e., birth control pill and intrauterine devices), and improved screening, outreach, and contact tracing (de Schryver and Meheus, 1990; Sparling and Handsfield, 2000; Winn Jr. *et al.*, 2006). The subsequent continual decline of the disease in the US during 1980s and 1990s was found largely due to changes in sexual behaviours, particularly among gay and bi-sexual men, to more effective case finding in response to AIDS and contact tracing among women. However, data from the CDC gonococcal isolate surveillance project (GISP) has shown that the proportion of infections in gay/ bisexual men increased from 4.5% in 1992 to 13.2% in 1999 (Fox *et al.*, 2001).

While all age groups are susceptible, the highest attack rate in both men and women occurs between 15 and 29 years of age. The incidence of gonorrhoea

found high among sexually active teenagers (ages 10 through 19 years) and young adults (ages 20 through 24 years) of all races, with the highest attack rates being among 15- to 24-year old men and women (Sparling and Handsfield, 2000).

Host-related factors such as the number of sexual partners, contraceptive practices, sexual preference and population mobility contribute to the incidence of gonorrhoea. The disease is concentrated in high-density population areas with a core group of active transmitters ("core groups" or "high frequency transmitters") having high-risk sexual behaviour (Winn Jr. *et al.*, 2006; WHO, 2007) (Figure 2.5). The core transmitters include female CSWs, long distant truck drivers, sailors and migrant workers (Mabey and Mayaud, 1999). However, the mobility of individuals results in the occurrence of gonorrhoea everywhere (Rothenberg and Potterat, 1988). Both social risk factors (i.e., low socioeconomic status, urban residence, lack of education, access to healthcare, unmarried status, race/ ethnicity, male homosexuality, prostitution, histories of other STDs) as well as behavioural risk factors (i.e., unprotected sex, multiple sex partners, other high-risk partners, drug use) have been identified for targeting by outreach/ intervention and STD control programs by the developed countries (Winn Jr. *et al.*, 2006).

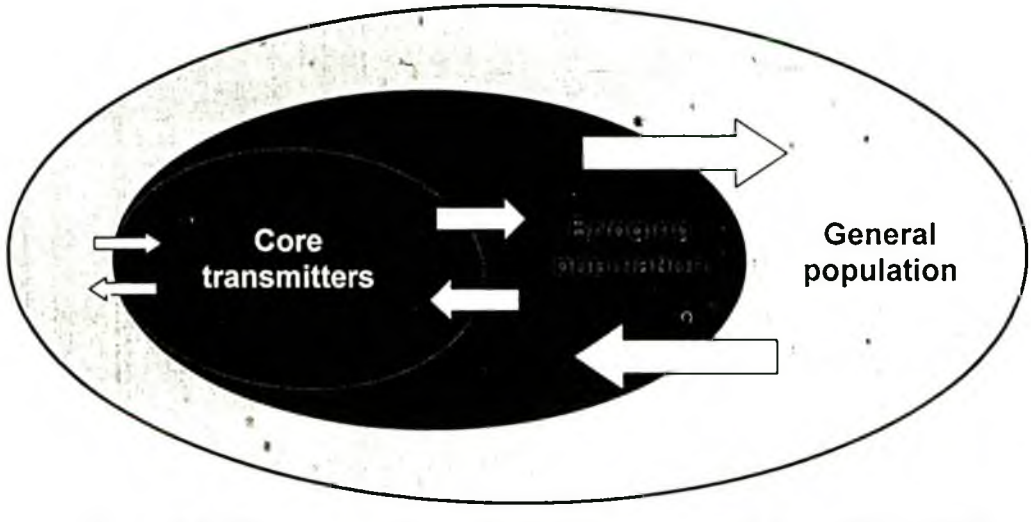


Figure 2.5: The concept of core transmitters (adapted from WHO, 2007)

Among the women, use of hormonal contraceptive methods was found associated with increased risk of gonococcal infection, while barrier methods such as condoms and diaphragms used with spermicidal foams and gels were found to exert a protective effect against the infection (Louv *et al.*, 1989).

The individuals at the economic and/or social margins in more developed or Western-style industrialised countries found to bear the burden of the disease. African and Hispanic Americans and those of Caribbean origin in the United Kingdom have disproportionately high disease rates (Lacey *et al.*, 1997; Winn Jr. *et al.*, 2006). In terms of economic marginalisation, the greatest burden of disease falls upon less developed countries so that the highest disease incidence and prevalence is found in Africa, South and East Asia and central and South America (WHO, 2001).

2.1.2d: Gonococcal infection among the MSMs

The gonococcal infection among the homosexual men (men who have sex with men, MSM) was found as a serious problem. The high incidence of the infection among the MSMs was found related to the variety of sexual practices, large numbers of sexual contacts, presence of infection at body sites with no evident symptoms and underutilization of existing public healthcare facilities that project negative attitudes toward homosexuality (Felman and Morrison, 1977).

In a study in Chicago, USA, 33.1% homosexual men were found infected with *N. gonorrhoeae* of which 18.5% were isolated from urethra, 16.3% from rectum and 5.6% from oropharynx (Janda *et al.*, 1980). However, the rates of gonococcal infection among the MSMs have been found to be reduced in the US along with other sexually transmitted diseases (STDs) followed the advent of HIV epidemic during the early 1980s (Judson, 1983; CDC, 1984; Fox *et al.*, 2001). On the other hand, during the 1990s as well as in the recent years, increases in rates of gonorrhoea and other STDs among MSMs were

documented (CDC, 1999, CDC, 2007b). In Australia, gonorrhoea was found most frequently among the HAMs (homosexually active men) or the MSMs (Donovan *et al.*, 2000). The highest incidence of gonorrhoea was observed in MSM in the UK recently (1834 per 100,000 population) with an overall incidence that was 20-times higher that in the heterosexual population (90 per 100,000 population) (Risley *et al.*, 2007).

The MSMs are believed to exist as isolated groups of population mostly residing in the cruising areas. But in a study in Kathmundu, Nepal found MSMs having sex with the people of the social and sexual network within the community who were intimately integrated among the general population (Boyce *et al.*, 2001).

2.1.2e: Gonococcal infection: Molecular epidemiology

The ability to differentiate reliably between unrelated gonococcal strains by molecular methods is essential for studies of the epidemiology of gonorrhoea. Although various phenotypic methods have facilitated the differentiation, methods with further discrimination using the molecular approaches have long been felt and recently applied in many studies.

The epidemiological relationship of the disease has been identified using many molecular techniques in the recent years including arbitrarily primed polymerase chain reaction (AP-PCR), amplified ribosomal-DNA restriction analysis (ADRA), opa-typing, pulsed-field gel electrophoresis (PFGE) and sequencing of *por* gene. Among them, Opa typing as described by O'Rourke *et al.*, PFGE using the restriction enzymes, and sequencing of entire *por* gene were found the best techniques with the highest discrimination for typing isolates of *N. gonorrhoeae* (van Looveren *et al.*, 1999; Palmer and Arnold, 2001).

In a genetic analysis of the *N. gonorrhoeae* isolates by PFGE identified two strains of the predominant serovars (IB-2 and IB-3) causing majority of the infections in homosexual men and young heterosexuals in Sweden (Unemo *et al.*, 2002). Gonococcal DNA was subjected to molecular fingerprinting by PCR amplification followed by *Taq1* digestion of their *opa* genes identified the endemic ciprofloxacin-resistant *N. gonorrhoeae* in the UK (Corkill *et al.*, 2003). Opa typing was found better discriminatory comparing with A/S phenotyping between unrelated isolates of *N. gonorrhoeae* (Howie *et al.*, 2004).

Finally, among the sequence-based methods, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) was found to be the most advantageous because it generates a simple numerical sequence type (ST) from combined alleles of two highly variable genes (*por* and *tbpB*). In the Scotland, investigators identified the heterogeneous ciprofloxacin-resistant gonococcal isolates using NG-MAST (Palmer *et al.*, 2005). Investigators also identified *N. gonorrhoeae* from a piece of clothing and established the relationship of gonococcal DNA from a child and an adult using genotyping by NG-MAST which resulted in conviction of the man for sexual assault (Martin *et al.*, 2007).

2.1.3: Gonorrhoea: Pathogenesis

The causative agent of gonorrhoea, the *N. gonorrhoeae* (gonococci) infects mucus-secreting columnar epithelial surfaces and the areas most frequently infected are the urethra, cervix, rectum, pharynx, and conjunctiva. Squamous epithelium, lining the adult vagina, is not susceptible to infection by the *N. gonorrhoeae*. However, the prepubescent vaginal epithelium, which has not been keratinized under the influence of oestrogen, may be infected (Todar, 2004).

The ability of the organism to attach to and multiply in the mucosal surfaces despite the flow of mucus and other body fluids is the essential first stage in the pathogenesis of gonorrhoea. Attachment of gonococci to mucosal cells is mediated in part by pili and by one of the outer membrane proteins (OMPs), protein II or opa protein (Ward *et al.*, 1974; Lambden *et al.*, 1979). There are 11 different opa proteins that help in adherence to different cell receptors on different types of human cells including macrophages and the strong adherence mediated by the opa proteins appeared to be essential for internalization of gonococci. This binding mechanism helps *N. gonorrhoeae* to bypass the classic phagocytic route into macrophages and prevents the microbes' destruction, allowing it to survive and grow inside the phagocyte (Makino *et al.*, 1991).

Following attachment to columnar epithelium, gonococci penetrate through or between cells to reach the subepithelial connective tissue. Transfer of another gonococcal major outer membrane protein, protein I (PI or por protein), into the host cell is supposed to initiate endocytosis by epithelial cell (Holmes and Morse, 1994). The gonococcal protein I serves as a porin in the outer membrane and can cross to the host cell membrane and bind to actin filaments. This may cause a remodelling of the host membrane and assist in invasion (Judd, 1989).

Gonococci attached to the surface of the polymorphonuclear leukocytes (PMN) resist phagocytosis and are not killed by the PMNs. The failure of the PMN to kill surface-attached gonococci appears to be a consequence of the failure of the PMN to enclose the virulent gonococci within a phagosome (Densen and Mandell, 1978). Gonococci bound to neutrophils may not be ingested, because gonococcal pili reduce ingestion of the organism perhaps by altering neutrophil membrane fluidity. Piliated gonococci stimulate neutrophil respiratory metabolism even though they are not ingested. This wastage of microbicidal activity is supposed to render these phagocytes less able to kill bacteria that they successfully ingest (Densen and Mandell, 1978; Britigan *et al.*, 1985)

An enzyme produced by pathogenic Nesisseria, IgA1 protease, inactivates IgA1 may interfere with IgA-mediated anti-adherence activity, resulting in an increased attachment (Holmes and Morse, 1994).

The endotoxin function of the lipooligosaccharide (LOS) causes damage to epithelial cells and disrupts complement activation that contributes to local cytotoxicity and inflammation as well as to fever and systemic toxicity in complicated infections. After release into the subepithelial space, gonococcal LOS may interact with serum and complement to generate chemotactic factors, leading to intense inflammatory reactions and brisk PMN response that characterizes acute gonorrhoea (Hook and Holmes, 1985). During infection, bacterial LOS and peptidoglycan are released by autolysis of cells. Gonococci also produce several proteases, peptidases and phospholipases which may play a role in pathogenesis (Todar, 2004).

In humans, most extracellular iron is bound by the major iron-binding proteins (transferrin and lactoferrin) and the resulting concentration of free iron is far too low to support gonococcal growth. Under these iron-limiting conditions, *N. gonorrhoeae* has several systems to obtain iron from the host including the receptor proteins that bind lactoferrin and transferrin. Gonococci only bind with human transferrin and lactoferrin, and this specificity explains the gonococci as obligate human pathogens (DeRocco and Cornelissen, 2007).

The asymptomatic cases of the disease are often associated with infection by certain Por IA gonococcal serovars and with arginine, hypoxanthine and uracil (AHU) and certain other auxotypes of *N. gonorrhoeae* (Knapp and Holmes, 1975; Knapp *et al.*, 1984; Brunham *et al.*, 1985). The fact that a substantial majority of the infections of the cervix, rectum and pharynx are asymptomatic might be explained if gonococci multiply at these sites without significant invasion of deeper tissues. However, asymptomatic infections of the urethra or cervix usually serve as focal sources for bacteremia (Rice *et al.*, 1986; Mehrany

et al., 2003) and there might be some tissue invasion since disseminated gonococcal disease is a more common complication of asymptomatic infections than of acute gonorrhoea.

Studies of isolates recovered from cases of disseminated gonococcal infection (DGI) have shown that these more invasive strains have unusual characteristics, including unique nutritional requirements (e.g. requirements for arginine, hypoxanthine and uracil for growth (AHU strains)), defined Por IA serovar classification, resistance to the bactericidal action of normal human serum, and exquisite susceptibility to penicillin (Eissenstein *et al.*, 1977; Knapp and Holmes, 1975; Schoolnik *et al.*, 1976). In parts of USA, the strains causing (DGI) were found to belong to a single auxotype and were resistant to killing by natural antibodies (Schoolnik *et al.*, 1976). With the decline in the prevalence of AHU/ Pr IA serovars in recent years, increasing numbers of DGI infections have been associated with other auxotype/ serovar classes (Brunham *et al.*, 1985; Tapsall *et al.*, 1992).

Strains producing symptomatic local infection preferentially activate the classical complement pathway, whereas those causing DGI (and often asymptomatic gonococcal infection) preferentially activate the alternative pathway (Densen *et al.*, 1982).

Spread of gonococci from the cervix to the endometrium and salpinges may be enhanced in women using intrauterine device (IUD) and was found associated with an increased frequency of salpingitis (Targum and Wright, 1974). Menstruation further increases the risk of intraluminal ascent from the cervix and also predisposes to gonococcal bacteremia. An alternative suggestion is that gonococci are transported to the fallopian tube on sperm (James *et al.*, 1976). The menstrual blood increases concentrations of transferrin and haeme at the mucosal surfaces, both of which can be used as a source of iron by all isolates of gonococci (Mickelsen and Sparling, 1981). 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 (Sparling and Handsfield, 2000).

Epidemiological data suggest that only one-third of men become infected after a single exposure to *N. gonorrhoeae*, and under experimental conditions, an inoculum of 10^3 organisms was found necessary to establish urethral infection in 50% of male volunteers (Holmes and Morse, 1994).

Gonococcal isolates from homosexual men tend to be more resistant to antimicrobials than the isolates from heterosexuals. This may be due to the fact that certain highly susceptible strains are rapidly killed by bile salts and fatty acids in faeces and these organisms rarely occur in homosexual men, while gonococci possessing a gene for multidrug resistance (*mtr*) are more resistant to bile salts and fatty acids and occur with increased frequency in homosexual men (Holmes and Morse, 1994).

The significant enhancement of sexual transmission of the human immunodeficiency virus (HIV) occurs in the presence of gonorrhoea. This is thought to be as a result of both an increase in the HIV viral load in semen or cervico-vaginal fluids of those infected with gonorrhoea, and an increase in the number of target cells for HIV in the inflammatory exudate which accompanies symptomatic STDs (Cohen *et al.*, 1997; Zhang *et al.*, 2005).

2.1.4: Gonorrhoea: Clinical presentations

Both symptomatic and asymptomatic genital as well as extragenital tract infections are caused by *N. gonorrhoeae* and there is a wide spectrum of clinical presentations of the gonorrhoea. There is also considerable overlap between these symptoms and those occurring in other STDs, most notably

those due to *Chlamydia trachomatis*. Therefore, it is quiet difficult to establish an aetiological diagnosis of gonorrhoea on clinical grounds alone.

2.1.4a: Symptomatic Uncomplicated Genital Gonorrhoea

The commonest uncomplicated gonococcal genital infection in men is an acute urethritis and usually presented by a urethral discharge in about 80% of the cases and burning on micturition (dysuria) in about half the time. The urethral discharge is purulent in 75% of the cases, cloudy in 20% and mucoid in about 5%. The consistency of the discharge at presentation is affected by the length of time that the infection has been incubating and whether the patient has recently urinated. The incubation period ranges from 1 to 14 days or longer, with an average of 2 to 7 days (Winn Jr *et al.*, 2006).

In females, the primary gonococcal infection is present in the endocervix, with concomitant urethral infection occurring in 70-90% of the cases. After an incubation period of 8-10 days, patients may present with cervicovaginal discharge, abnormal or intermenstrual bleeding and pelvic pain; the later suggests the presence of upper genital tract disease (Sweet, 1993). The presence of dysuria indicates significant urethral involvement.

Symptoms of uncomplicated endocervical infection often resemble those of other conditions, and the symptomatology of gonococcal endocervicitis is often clouded by co-infection with *Chlamydia trachomatis, Trichomonas vaginalis*, and/or *Candida albicans*. Although the genital tract may appear normal, careful endocervical examination often reveals areas of friable cervical mucosa that bleed on swabbing. Only 10-20% of infected women, however, will present with an obvious mucopurulent endocervical discharge (Winn Jr. *et al.*, 2006).

2.1.4b: Sexually transmitted extragenital gonococcal disease

N. gonorrhoeae may also cause pharyngeal and anorectal infections duly transmitted by sexual act with or without involving the genital organs.

The rates of pharyngeal gonococcal infection were found higher in homosexual men (20.9-25.0%) and heterosexual women (10.0%) than heterosexual men (3.2-7.0%) and were significantly associated with the practice of fellatio (oral sex performed upon penis) with men who have urethral infection, and occasionally in heterosexual men resulting from performing cunnilingus (act of using the mouth, lips and tongue to stimulate the female genitals) with an infected partner (Bro-Jorgensen and Jensen, 1973). While pharyngitis is a relatively common clinical manifestation of gonorrhoea, gonococcal stomatitis has also been reported (Schmidt *et al.*, 1961).

Anorectal gonorrhoea is often overlooked owing to the mildness or absence of symptoms. Two types of disease reported: primary anorectal gonorrhoea, usually commoner among the MSMs and secondary anorectal gonorrhoea, occurring chiefly in the females with urogenital gonorrhoea. Moreover, accidental anorectal gonorrhoea occurs due to insertion of thermometers, enema nozzles or gloves contaminated with gonococcal pus (Catterall, 1962). Some individuals with rectal gonococcal infection may experience acute proctitis with mild anorectal pain and itching, a mucopurulent discharge, bleeding, tenesmus, and constipation for 5 to 7 days after infection (Rompalo, 1999). Anoscopic examination of the anal canal usually reveals an oedematous and erythematous rectal mucosa and a purulent discharge associated with anal crypts (William *et al.*, 1981). Chlamydia infection, herpes simplex virus infection, and other sexually transmitted infections are included in the differential diagnosis of anorectal gonococcal infection (Winn Jr *et al.*, 2006).

2.1.4c: Non-sexually transmitted unusual gonococcal infections

Ocular gonococcal infections, seen primarily among neonates who acquired the organism during passage through an infected birth canal (ophthalmia neonatarum) have been also reported among adults who become infected via genital secretions (Wan *et al.*, 1986). Laboratory personnel working with cultures may also become accidentally infected if care is not taken to protect

the eyes (Malhotra *et al.*, 1998). Infection of the eye results in periorbital cellulites, a profuse purulent discharge, conjunctival injection, eyelid oedema and erythema, and epithelial and stromal keratitis. Inadequate treatment of eye infections can lead to ulcerative keratitis, corneal perforation and blindness (Wan *et al.*, 1986).

Rare reports of primary cutaneous gonococcal infection, resulting from inoculation on preexisting injury or lesion, have also been reported, including a case of gonococcal mastitis in a male (Scott and Scott, 1982; Bodsworth *et al.*, 1993).

2.1.4d: Asymptomatic gonococcal infections

Majority of the gonococcal infections in women (20%-80%) are supposed to be asymptomatic. Women with asymptomatic infections often manifest low-grade or no endocervical inflammation or discharge (Wilfert and Gutman, 1992; Knapp and Rice, 1995). About 10% of infections in men are found asymptomatic at the time of diagnosis, probably during the prodromal stages of the infection (Winn Jr. *et al.*, 2006).

Over 90% of oropharyngeal gonococcal infections are asymptomatic and are diagnosed by culture of the organism from the throat (Barlow, 1997). Although frequently remain asymptomatic, pharyngeal infection may produce exudative tonsillitis. Asymptomatic pharyngeal gonococcal infection usually clears spontaneously over several weeks, even without therapy (Holmes and Morse, 1994). In addition, gonococcal infections of anorectum (rectal gonorrhoea) are often asymptomatic (Caterrall, 1962).

2.1.4e: Complicated gonococcal disease and disseminated gonococcal infections (DGI)

Symptoms of gonococcal infection gradually resolve without treatment and often lead to complications. The complications are commoner in women and the newborn.

Among the infected women, an ascending gonococcal infection may occur in 10-20% of the cases and result in acute pelvic inflammatory disease (PID) manifested as salpingitis, endometritis and tubo-ovarian abscess, all of which can lead to scarring of the fallopian tubes, ectopic pregnancies, sterility and chronic pelvic pain (Schoolnik *et al.*, 1976; Sparling and Handsfield, 2000). Clinical features of Gonococcal PID include bilateral lower abdominal pain, abnormal cervical discharge and bleeding, pain on motion, fever, peripheral leukocytosis, elevated erythrocyte sedimentation rates and C-reactive protein in about two-thirds of the patients, while chills, nausea and vomiting are variable. The PID caused by *N. gonorrhoeae* generally occurs early and often during or shortly after the onset of menstruation. The development of gonococcal PID is influenced by many factors, including the use of oral contraceptives, IUDs, vaginal douching, characteristics of the infecting strains and the immune competence of the host. Obstruction of the fallopian tubes, leading to infertility, occurs in 10-20% of women following a single episode of acute gonococcal PID and in 50-80% after three or more episodes. Among women with ectopic pregnancies, up to 80% have a history of prior PID (Doyle *et al.*, 1991).

In women with salphingitis, a perihepatitis called the Fitz-Hugh-Curtis syndrome, characterized by direct extension of the organisms from the fallopian tube to the liver and peritoneum resulting in right, upper quadrant pain, the finding of adhesions between the liver and anterior abdominal wall often found on laparoscopy (Sweet, 1993; Sparling and Handsfield, 2000; Winn Jr *et al.*, 2006; Mahon *et al.*, 2007).

Endocervical gonorrhoea may also complicate pregnancy and is a recognized co-factor for spontaneous abortion, chorioamnionitis, premature rupture of membranes and premature delivery. First trimester abortion occurs in about 20% of the pregnant women with gonorrhoea. Infants born to women with genital tract infection are at risk for gonococcal conjunctivitis (ophthalmia neonatarum) or pharyngeal gonococcal infection (Winn Jr *et al.*, 2006).

Occasionally, in a small percentage (approximately 0.5-3%) of infected individuals, gonococci may invade bloodstream resulting in disseminated gonococcal infection (DGI). The DGI is characterized by low-grade fever (rarely above 39^0C), haemorrhagic skin lesions, tenosynovitis, migratory asymmetrical polyarthralgias, tenosynovitis and frank arthritis (arthritis-dermatitis syndrome). The knees are most frequently involved, followed in order by the elbows, ankles, wrists and the small joints of the hands and feet. Synovial effusion is demonstrable in about one-third of the patients (Holmes *et al.*, 1971). Infected synovial fluid frequently contains 50,000 to 200,000 cells/ mm^3, with 90% being PMNs (Flemming *et al.*, 1986). Gram stain and culture of joint aspirates are positive in only 10-30% of cases. Cultures for *N. gonorrhoeae* are usually negative from synovial fluid containing less than 20,000 leukocytes/ mm^3 and are usually positive from fluids containing 80,000 leukocytes/ mm^3 or more (Handsfield *et al.*, 1976). Polymerase chain reaction (PCR) has also been applied to the detection of gonococci in synovial fluid (Muralidhar *et al.*, 1994).

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 peripherally on the extremities (e.g. toes and fingers). On occasion, extensive macular, popular or petechial rashes may develop (Mehrany *et al.*, 2003).

Gonococcal endocarditis is a rare clinical entity developing in about 1-2% of patients with disseminated infections, usually involving the aortic valve and follows a rapid and destructive course (Weiss *et al.*, 1992). In 1996, Thompson and Brantley reported a case of gonococcal endocarditis in a 23-year old man that required prosthetic valve placement resulting from severe aortic insufficiency and a prolapsed aortic cusp (Thompson and Brantley, 1996). In 1997, another case of gonococcal endocarditis is association with systemic lupus erythematosus was diagnosed in a 24-year old woman who presented with non-specific symptoms of cough, chest pain, fever, and malaise without signs or symptoms of DGI (Tikly *et al.*, 1997). Transthoracic and transoesophageal echocardiograms showed large vegetation on the pulmonary valve, and blood cultures were positive for *N. gonorrhoeae*. Pericarditis and pericardial effusions are also complications of DGI, and adult respiratory distress syndrome has been reported in association with Gonococcal bacteremia (Belding and Carbone, 1991; Wilson *et al.*, 1990). Gonococcal meningitis is also a rare complication of disseminated infection that has features typical of meningitis caused by other organisms (Rice *et al.*, 1986).

Clinical entities that resemble DGI include Reiter's syndrome, pyogenic and crystal-induced arthritis, syphilitic and tuberculous arthritis, rheumatoid arthritis, Lyme disease, and rheumatic fever (Ross, 1996). Repeated bouts of DGI have also been observed in individuals with certain complement deficiencies (Ellison *et al.*, 1988).

2.1.5: Gonorrhoea: Laboratory diagnosis

The laboratory diagnosis of gonorrhoea depends on the demonstration of *N. gonorrhoeae* by direct visualization and/ or detection of the organism and/or its DNA in the clinical samples (Jephcott, 1997).

The gonorrhoea case definition includes any person meeting the laboratory criteria as follows: (a) *N. gonorrhoeae* has been isolated from a clinical specimen using culture, (b) *N. gonorrhoeae*-specific antigen or nucleic acid has been demonstrated in a clinical specimen, and/or (c) *N. gonorrhoeae* Gram-negative intracellular diplococci have been identified in a urethral smear from a symptomatic male (Velicko and Unemo, 2009).

2.1.5: Gonorrhoea: Treatment

2.1.5a: Introduction

A standard treatment regimen should be available in a community, because an effective treatment not only eradicates infection and prevents the development of complications; it also has an important public health benefit of shortening the duration of infection and thus decreasing transmission and eliminating the reservoirs of infection.

2.1.5b: Gonococcal infection: Treatment guidelines

An accepted definition of gonococcal treatment efficacy requires a cure rate of over 95% and a change in the treatment regimen is recommended when the prevalence of antimicrobial resistance exceeds 5% for a specific antibiotic (CDC, 1987; Tapsall, 2001).

The current recommended regimens for uncomplicated gonococcal infections of the cervix, urethra, and rectum include injection (Inj.) ceftriaxone 125 mg intramuscularly (IM) in a single dose or capsule (Cap.) cefixime 400 mg orally in a single dose or 400 mg by suspension (200 mg/ 5ml) plus treatment for Chlamydia, if chlamydial infection is not ruled out. Alternative regimens include Inj. spectinomycin 2.0 gm in a single IM dose or single-dose cephalosporin regimens including Inj. ceftizoxime 500 mg IM; or Inj. cefoxitin 2.0 gm IM, administered with probenecid 1.0 gm orally; or Inj. cefotaxime 500

mg IM. Some evidences indicate that cefpodoxime 400 mg and cefuroxime axetil 1.0 gm might be oral alternatives (CDC, 2006).

Regimens for uncomplicated gonococcal infections of the pharynx include Inj. ceftriaxone 125 mg IM in a single dose plus treatment for chlamydia if chlamydial infection is not ruled out. Regimen for DGI includes Inj. ceftriaxone 1 g IM or IV (intravenously) every 24 hours. Alternative regimens include Inj. cefotaxime 1.0 gm IV every 8 hours, or Inj. ceftizoxime 1.0 gm IV every 8 hours, or Inj. spectinomycin 2.0 gm IM every 12 hours (CDC, 2006).

Treatment should be continued for 24–48 hours after clinical improvement, at which time therapy may be switched to one of the following regimens to complete at least 1 week of antimicrobial therapy: cefixime 400 mg orally twice daily, or cefixime suspension 400 mg by suspension (200 mg/5ml) twice daily, or cefpodoxime 400 mg orally twice daily (CDC, 2006).

2.1.6: Gonorrhoea: Preventive measures

There is no effective vaccine to prevent gonorrhoea. Candidate vaccines consisting of PilE protein or Por are of little benefit. The development of an effective vaccine has been hampered by the lack of a suitable animal model and the fact that an effective immune response has never been demonstrated.

Control of gonorrhoea (and other STDs) requires a complex, integrated and comprehensive strategy of education, counselling, diagnosis, treatment and case-finding. Key elements are prevention through promotion of safer sexual practices and the availability of health care services. Condoms were found effective in preventing the transmission of gonorrhoea (Todar, 2004).

The control measures for gonococcal infection listed in the STD treatment guidelines by the CDC include the following: (i) education and counselling of persons at risk on ways to avoid gonorrhoea through changes in sexual behaviours and includes abstinence and reduction of sex partners, and use of condoms during sexual act; (ii) identification of asymptomatically infected persons and of symptomatic persons unlikely to seek diagnostic and treatment services can be implemented by mass screening of the individuals having high-risk sexual behaviours like CSWs, MSMs and contacts with CSWs; (iii) effective diagnosis and treatment of infected persons include an established surveillance system equipped with the personnel and logistics to carry out effective diagnosis of the cases of gonorrhoea. For development of an effective treatment regimen, a well organized antimicrobial surveillance system is essential; and (iv) evaluation, treatment, and counselling of sex partners of persons who are infected with gonorrhoea can be implemented by partner notification by the infected persons seeking treatment (CDC, 2006).

Partner notification is the process by which providers or public health authorities learn from persons with gonorrhoea about their sex partners and help to arrange for the evaluation and treatment of sex partners. Providers can seek this information and help to arrange for evaluation and treatment of sex partners, either directly or with assistance from the available local healthcare centres.

2.2: Characterization of *Neisseria gonorrhoeae*

2.2.1: *N. gonorrhoeae*: Taxonomy

The genus Neisseria belongs to the family Neisseriaceae which has undergone many taxonomic changes. The genus was assigned to the family Coccaceae until 1948, when it was reassigned as the type genus in the family Neisseriaceae (Murray and Branham, 1948).

Now, the family Neisseriaceae includes four genera: Neisseria, Moraxella, Kingella and Acinetobacter. The genus Neisseria consists of 11 human species that are considered as "true Neisseriae" including the *N. gonorrhoeae* and 3 animal species collectively called the "false Neisseriae (Vedros, 1984). The taxonomic positions of some Neisseria species are currently being debated.

In 1988, using several new molecular techniques, *N. gonorrhoeae* has been included under the family Neisseriaceae of the new β-Proteobacteria class (Stackebrandt *et al.*, 1988). Application of molecular techniques resulted in several proposed changes in the taxonomy of the family Neisseriaceae. The true Neisseriae, Kingella species and *Moraxella urethralis* were assigned to the β-subgroup of the Proteobacteria and recommendations were made to remove Acinetobacter, Moraxella, Branhamella and the false Neisseriae from the family Neisseriaceae (Winn Jr. *et al.*, 2006).

2.2.2: *N. gonorrhoeae:* Habitat and general characteristics

Most Neisseria species are non-pathogenic and are normal inhabitants only of the oro- and nasopharyngeal mucous membranes of humans (Knapp and Hook III, 1988). The organism *N. gonorrhoeae* are always pathogenic, infects the humans only, is highly adapted to the genital tract, colonizing less efficiently to other mucous membranes of the body like oral, conjunctival and rectal mucosa and survive poorly outside the natural host (Knapp and Rice, 1995).

N. gonorrhoeae is a Gram-negative non-motile diplococcus, 0.6 to 1.0 μm in diameter. Individual cocci are kidney-shaped and when organisms occur in pairs, the flat or concave sides are adjacent. The organism is typically found associated with or intracellularly within PMNs (neutrophils) of the purulent discharge of Gonorrhoea (Brooks *et al.*, 2004).

The microbe is fastidious requiring a number of growth factors and has a very narrow temperature range for growth of 35-37^0 C. It grows well in an atmosphere of 3-10% added CO_2 (Wilfert and Gutman, 1992; Freingold and Peacocke, 1999). Strains of *N. gonorrhoeae* are variable in their cultural requirements so that media containing haemoglobin, Nicotine Adenine Dinucleotide (NAD), yeast extract and other supplements are needed for isolation and growth of the organism.

The *N. gonorrhoeae* and other members of the genus Neisseria are highly susceptible to adverse environmental conditions such as temperature extremes, drying, chilling, and exposure to unfavourable pH or to ultraviolet light (Wilfert and Gutman, 1992). Drying kills the organism in 1 to 2 hours, but can survive for several hours on clothing and other surfaces, and bodily fluids seem to have a preservative effect. For example, it has been known to survive for 6 to 7 weeks in dried pus (The world of microbes, 2009).

2.2.3: *N. gonorrhoeae*: Antigenic structures and virulence factors

The antigenic ultrastructure of *N. gonorrhoeae* is composed primarily of two sets of components: (a) the outermost surface structures including the pili; and (b) the trilaminar outer membrane containing several proteins named as outer membrane proteins (OMPs), phospholipids and lipooligosaccharide (LOS). Almost all of the antigenic structures of *N. gonorrhoeae* contribute to the virulence in establishing the gonococcal infection in a complex series of molecular interactions with the host cells.

Attachment of gonococci to human epithelial cells is the essential first step of the infection and is thought to be mediated by pili and protein II (opa protein). Transfer of protein I (por protein) from gonococcal outer membrane to the

membrane of nonciliated epithelial cells is supposed to initiate invasion of epithelial cells. Gonococcal LOS is directly cytotoxic (for example, to the ciliated cells of the fallopian tube epithelium) and could interact with natural IgM serum antibody and complement in the subepithelial tissues to initiate inflammation. Additionally, the IgA1 protease may destroy IgA1 antibody, which tend to block the gonococcal attachment.

2.2.3a: Gonococcal outermost surface structure: Pili

The outermost surface structure of *N. gonorrhoeae* are pili or fimbriae, the filamentous hair-like proteins composed of repeating peptide subunits (pilin) with molecular weights of approximately 17,000 to 21,000 Daltons (Robertson *et al.*, 1977; Brooks *et al.*, 2004). Each of the pilin subunits is composed of about 165 amino acids (Schoolnik *et al.*, 1984) and exists in association with some other pilus-associated proteins which in together are exposed on the surface and extend several micrometers outside the bacterial cell (Danielsson, 1986; Nassif *et al.*, 1999; Brooks *et al.*, 2004).

The gonococcal pilin molecules can be divided immunologically into three imaginary regions: (i) a constant region- composed of a high percentage of hydrophobic amino acids, is always conserved (does not differ); (ii) a semivariable middle region- in which many amino acid substitutions are found, serves in attachment to host cells and is less prominent in immune response; and (iii) a highly variable region- at the carboxy terminal that in addition to amino acid substitutions, contains insertions and deletions of one to four codons. This part is immunodominant and determines the antigenic heterogeneity of pili in *N. gonorrhoeae* (Schoolnik *et al.*, 1984; Wilfert and Gutman, 1992; Brooks *et al.*, 2004).

Although all gonococcal strains are capable of producing pili, the structure and antigenicity of these components vary from strain to strain. (Brooks *et al.*, 2004) *N. gonorrhoeae* pili thus undergo high-frequency phase and antigenic

variations. Phase variation refers to a reversible switch between alternative states of pilus expressing (P^+) or non-expressing (P^-) and occurs at high frequencies (about 10^{-5} or 10^{-4}). Phase variation is easily recognized by consequent changes in colony morphology on solid clear medium. Pilus antigenic variation also occurs at high frequencies as determined by *in vitro* observations as well as by comparing pilus antigens from different clinical isolates. A single gonococcal cell has been found possessing the inherent genetic capacity to produce at least eight antigenically variant pili (Wilfert and Gutman, 1992).

When gonococci are freshly isolated from a patient, the majority of colonies are small and shiny, and some others are larger and dull-surfaced (Jephcott and Reyn, 1971; Kellog *et al.*, 1963). The *N. gonorrhoeae* in small and shiny colonies, designated types 1 or 2, produce pili, are virulent and found in fresh isolates from genitourinary tract infections, whereas the organisms in larger colonies (types 3 and 4) are non-piliated, avirulent and appear on subculture (Kellog *et al.*, 1963; Jephcott and Reyn, 1971; Swanson *et al.*, 1971). Many strains from extragenital sites also are found to be non-piliated even in fresh isolates (Wilfert and Gutman, 1992). The ability of the type 1 or 2 phenotypes to produce pili is lost upon nonselective subculture in the laboratory. A population of type 3 or 4 colonies can give rise to occasional piliated (Pil^+) variants, but a much lower frequency than the Pil^+ to Pil^- transition. A population composed predominantly of piliated gonococci can be maintained in the laboratory by selective passage of colonies with the appropriate phenotype (Cannon and Sparling, 1984).

The gonococcal pili enhance attachment to host cells and confer resistance to phagocytosis by human PMNs (Swanson, 1973; James-Holmquest *et al.*, 1974; Johnson *et al.*, 1977; Blake and Swanson, 1975; Dilworth *et al.*, 1975; Brooks *et al.*, 2004). Pili are associated with the initial adhesion of *N. gonorrhoeae* to human epithelial cells by binding to the protein CD46, a human-specific

membrane co-factor receptor protein expressed by all nucleated cells (Kallstrom *et al.*, 2001). Perhaps the adhesion occurs with phase-variable adhesion proteins PilC and/or Pil E located at the tip of the pilus fibre, similar to the tip-located pilus proteins of uropathogenic *Escherichia coli* (Lund *et al.*, 1988; Kallstrom *et al.*, 1997; Jonsson *et al.*, 1994; Rudel *et al.*, 1995a; Nassif *et al.*, 1999; Kallstrom *et al.*, 2001; Rytkonen *et al.*, 2001). Expression of mature pili or at least the major subunits of pilus fibre, PilE and PilC are essential for a high-level competence for transformation of extrachromosomal DNA (Rudel *et al.*, 1995b; Nassif *et al.*, 1999).

However, non-piliated bacteria may also colonize humans and cause mild urethritis. Pili also found to confer twitching motility (Cohen and Cannon, 1999).

2.2.3b: Gonococcal outer membrane structures

N. gonorrhoeae possesses a typical Gram-negative outer membrane composed of proteins, phospholipids and lipopolysaccharide (LPS). However, LPS of the organism is distinguished from enteric LPS by its highly-branched basal oligosaccharide structure and the absence of repeating O-antigen subunits. For these reasons, LPS of *N. gonorrhoeae* is referred to as lipooligosaccharide (LOS). The outer membrane of *N. gonorrhoeae* also bears several protein antigens that have been studied in considerable detail. Three proteins are present in large amounts and these have been named proteins I, II, and III (PI, PII, and PIII) (Swanson, 1981).

2.2.3b.i: Protein I (PI or Por protein)

The protein I (PI) or Por protein extends through the gonococcal cell membrane and is the predominant protein of the gonococcal outer membrane accounting for about 60% of the total weight and appears to consist of a single polypeptide chain (Johnston *et al.*, 1976; Wilfert and Gutman, 1992; Murphy, 1994). The protein I is located in the outer membrane in such a way that most of the

molecule, including both the N- and C-terminal ends, is within the membrane and only a small part of the central portion of the molecule is exposed on the surface (Wilfert and Gutman, 1993; Brooks *et al.*, 2004).

The PI occurs as trimeric pore-forming transmembrane proteins (porins) are being aggregated with protein III. The PI molecules act as porins (hence called Por proteins) allowing small essential hydrophilic solutes and anions to diffuse through the otherwise hydrophobic outer membrane (Douglas *et al.*, 1981; Heckels, 1981; Young and Davis, 1983; Blake and Gotschlich, 1983; van der Ley, 1991; Wilfert and Gutman, 1992; Derrick *et al.*, 1999). It has a molecular weight of 32 to 36 KDa and is present in the same submolecular weight in gonococci from each of the different colony types from any one strain (Heckels, 1981).

Each strain of *N. gonorrhoeae* has a single genetically stable class of PI, but it has been shown to vary among different gonococcal strains, both in primary structure and apparent molecular weight as well as in antigenicity. (Johnston *et al.*, 1976; Cannon and Sparling, 1984) The antigenic differences in different strains form the basis for several different serotyping systems for gonococci (Johnston, 1980; Buchanan and Hildebrandt, 1981; Sandstrom *et al.*, 1982; Tam *et al.*, 1982; Blake and Gotschlich, 1983).

Immunological and biochemical data have demonstrated that there are two distinct structural variants of the porin protein, IA and IB, which have different orientations in the outer membrane. The PIAs tend to be smaller than the PIBs (Blake *et al.*, 1981; Sandstrom *et al.*, 1982; Barrera and Swanson, 1984). The porin proteins in each variant have been further subclassified into serovars based on reactivity to a panel of Por-specific monoclonal antibodies (Knapp *et al.*, 1984; Tam *et al.*, 1982). The antigenic expression of PorB within any strain is stable; however, diversities between strains exist and these form the basis for serological group (serogroup) and serovar determination with monoclonal

antibodies, e.g., by using co-agglutination technique (Sandstrom and Danielsson, 1980; Knapp *et al.*, 1984).

The PI resembles the porins of *Escherichia coli* in molecular weight, trimeric configuration, and estimated pore size (Gotschlich *et al.*, 1987b) and readily translocates from living gonococci to foreign membranes, artificial or natural (Lynch *et al.*, 1984). The PI molecules have been shown to rapidly move from gonococcal outer membranes to the more fluid cytoplasmic membrane of human cells. This process may initiate endocytosis of the gonococcus, representing the first step in gonococcal invasion of the epithelium. Purified PI also has profound effects on the physiology of human PMNs, markedly interfering with degranulation but having no significant effect on superoxide generation (Haines *et al.*, 1988).

A correlation has been found between the molecular weight of PI and the type of gonococcal infection. The *N. gonorrhoeae* strains with a low molecular weight PI usually are resistant to complement-mediated killing by normal serum and were found associated with disseminated infection (DGI), whereas the strains with a high molecular weight PI are highly serum-sensitive and were found in symptomatic genital infections (Cannon *et al.*, 1983; Brunham *et al.*, 1985; Wilfert and Gutman, 1992).

2.2.3b.ii: Protein II (PII or Opa protein)

N. gonorrhoeae can produce another type of a series of one or several outer membrane proteins called Opa proteins (PII) having subunit molecular weights ranging from 24 to 32 KDa. Because they were initially described as proteins present in the outer membrane of opaque colony variants and generally absent in transparent variants of the same strain, they have been referred to as "opacity associated protein" or Opa proteins (Nachamkin *et al.*, 1981; Swanson, 1982; Brooks *et al.*, 2004). One portion of the Opa molecule is within the gonococcal outer membrane, and the rest is exposed on the surface (Brooks *et al.*, 2004).

These are heat-modifiable proteins and expression of PII proteins by a gonococcal strain is variable, being subject to phase variation with a frequency of about 10^{-3} per cell division (Swanson, 1978; Stern *et al.*, 1986). This variation is believed to contribute to the evasion of host immune responses and may account in part for recurrent gonococcal infections. The presence of monoclonal antibodies specific for unique epitopes in individual proteins emphasizes the extensive intrastrain and interstrain variability in their antigenic structure (Wilfert and Gutman, 1992). 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.

The protein II functions in adhesion of gonococci within colonies and in attachment of gonococci to host cells, especially cells that express carcinoembryonic antigens (CD66). The PII^+ cells are characterized by clumping (autoagglutination) and by adherence to mucosal cells and neutrophils (Bessen and Gotschlich, 1987). Organisms expressing these proteins usually are isolated from localized infections, whereas organisms that lack PIIs were recovered from disseminated infections (Wilfert and Gutman, 1992).

There are indirect evidences that possession of different PII proteins may be of significance in different stages of gonococcal infection. Differences in colony opacity of different isolates correlate with differences in site of isolation or type of infection (Draper *et al.*, 1980; James and Swanson, 1978). In general, gonococci, those form transparent colonies and those do not possess PII in the outer membrane, are believed to be more virulent or invasive (Salit and Gotschlich, 1978; Virji and Everson, 1981).

2.2.3b.iii: Protein III (PIII or Rmp)

Protein III (MW about 33,000), also known as reduction modifiable protein (Rmp) is another outer membrane protein found in all strains of *N.*

gonorrhoeae occurring in complex with PI to form porins. This protein is antigenically conserved in all gonococci and does not undergo antigenic variation and is found in a complex with Por and LOS (Brooks *et al.*, 2004). It shares partial homology with the OmpA protein of *Escherichia coli.* The structural gene of PIII has been cloned which shows sequence homology with the enterobacterial OmpA proteins (Gotschlich *et al.*, 1987a). Antibodies to Rmp, induced either by a Neisserial infection or by colonization with *E. coli,* tend to block bactericidal antibodies directed against Por and LOS. The anti-Rmp antibodies may thus increase susceptibility to infection by *N. gonorrhoeae,* because the PIII appears to be the major binding site for IgG blocking antibody, which interferes with complement-mediated killing of serum-resistant *N. gonorrhoeae* by immune serum (Wilfert and Gutman, 1992; Toders, 2004).

2.2.3b.iv: Transferrin binding proteins (tbp)

With the exception of a few species, most microorganisms require iron as an essential nutrient for growth and metabolic processes, including RNA synthesis and electron transport (Briat, 1992). The *N. gonorrhoeae* can acquire iron by direct interaction with human iron-binding proteins, including the serum glycoprotein (transferrin). Iron internalization from host transferrin requires the expression of a bacterial receptor, which specifically recognizes the human form of transferrin. Two gonococcal transferrin-binding proteins have been implicated in transferrin receptor function, TbpA and TbpB. These receptors are induced under low-iron conditions and are able to extract iron directly from transferrin and lactoferrin, respectively. The proteins can also extract iron from haeme and haemoglobin (DeRocco and Cornelissen, 2007).

2.2.3b.vi: Protein H8

The protein H8 is a recently detected antigen found in all strains of *N. gonorrhoeae* and *N. meningitidis* tested, but not in saprophytic Neisseria species. Within a given strain, the H8 antigen appears to be antigenically

homogeneous irrespective of piliation or opacity colony type, and all strains have in common one or more surface-exposed epitopes of this antigen.

Monoclonal antibodies to the H8 protein are bactericidal for gonococcal strains that are susceptible to killing by normal human serum. Because of its surface location and widespread distribution among all pathogenic Neisseria, the H8 antigen has been suggested as a possible candidate for a vaccine against both Gonococcal and meningococcal infections (Wilfert and Gutman, 1992).

2.2.3b.vii: Lipooligosaccharide (LOS)

The gonococcal lipooligosaccharide (LOS) has the molecular weight of 3000–7000 Daltons (Sparling, 1999; Brooks *et al.*, 2004). In a form of molecular mimicry, gonococcal LOS structurally resembles human cell membrane glycosphingolipids.

The terminal galactose of human glycosphingolipids is often conjugated with sialic acid (also called *N*-acetylneuraminic acid, NANA). Gonococci do not make sialic acid but do make a sialyltransferase that functions to take NANA from the human nucleotide sugar cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA) and place the NANA on the terminal galactose of a gonococcal acceptor LOS (Brooks *et al.*, 2004). The sialylation of the gonococcal LOS inhibits normal bactericidal activities of the antibodies, phagocytosis by the neutrophils and complement activation by factor H binding and may consequently result in evasion of the immune system by serum resistance (Elkins *et al.*, 1992; Wetzler *et al.*, 1992; Rest and Frangipane, 1992; Ram *et al.*, 1999).

2.2.3b.viii: IgA1 Protease

Gonococci and certain other mucosal pathogens such as *N. meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae* release IgA1 protease, which cleaves IgA1 antibodies at the hinge region between the Fc and Fab

portions of the molecule (Mulks and Plaut, 1978). Inactivation of secretory IgA1 may be an initial step in the pathogenesis of the gonococcal infection. Koomey and associates (Koomey *et al.*, 1982) have successfully cloned the genes for IgA1 protease and constructed gonococcal recombinants deficient in this enzyme that could be tested for pathogenicity in experimental infections. Unfortunately only humans and chimpanzees have proved susceptible to mucosal gonococcal infections, greatly limiting the pace of such research.

2.2.3b.ix: Gonococcal plasmids

Plasmids are the extrachromosomal bacterial DNA capable of replicating independently. Several different types of both transmissible and non-transmissible plasmids had been identified in the *N. gonorrhoeae* including a small (2-6 MDa) cryptic plasmid with no detectable function, penicillinase-encoding PPNG plasmids, high-level tetracycline-resistance-determining TRNG plasmids as well as 24.5/ 25.4 MDa conjugative plasmids. Although almost all of the *N. gonorrhoeae* were found to contain either one or more of the plasmids, it has been determined that the isolates of proline-, citrulline-, and uracil-requiring auxotype were plasmid free (Dillon and Pauze, 1981). Plasmid-borne virulence determinants in *N. gonorrhoeae* are associated with antimicrobial resistance.

Cryptic plasmids

The cryptic plasmids are the smallest being 2.6 MDa (approximately 4.2 kb) and were first described in 1972 (Engelkirk and Schoenhard, 1972). In an initial survey, 96% of clinical isolates of *N. gonorrhoeae* was found to carry the cryptic plasmid (Roberts *et al.*, 1979). Their function is still unknown and is found not to correlate with the virulence of the gonococcal strains. (Roberts, 1989) The use of cryptic plasmid-derived probes for DNA hybridization was thought to have limited application for the detection of gonococci in clinical samples. Investigators subsequently identified the *cppB* gene in the cryptic plasmid pJD1 as well as in the gonococcal chromosome of all tested strains

(Hagblom *et al.*, 1986). Subsequently a PCR protocol was developed to identify gonococci in the clinical specimens using primers derived from the integrated regions of the *cppB* gene (Korch *et al.*, 1985; Ho *et al.*, 1992).

ß-lactamase (penicillinase)-producing plasmids

Penicillinase production in the isolates of *N. gonorrhoeae* (PPNG strains) is mediated by a family of six related beta-lactamase-producing plasmids (PPNG plasmids). These plasmids are distinguished by their size determined in agarose gel electrophoresis (Gouby *et al.*, 1986; Dillon and Yeung, 1989; Brett, 1989). The plasmids have been named by their first geographic origin as Asia type (7426 bp/ 4.9 MDa), Africa type (5599 bp/ 3.1 MDa), Toronto type (5154 bp/ 2.6 MDa), Rio type (5154 bp/ 2.6 MDa), Nimes type (6798 bp/ 4.0 MDa) and New Zealand type (9309 bp/ 6.0 MDa). Three of them, the Asia, Africa and the Toronto-type plasmids have been associated with epidemic outbreaks, while the others have been isolated sporadically (Dillon and Yeung, 1989).

The *N. gonorrhoeae* strains carrying the Asia-type plasmid were first isolated in the USA in 1976 (Ashford *et al.*, 1976) and named according to their epidemiological origin in the Far East. The strains carrying the Africa-type plasmid were isolated firstly in the United Kingdom in 1976 (Percival *et al.*, 1976; Phillips, 1976). The Africa-type plasmid has a 2.1 kb deletion when compared with the Asia-type. Two other deletion derivatives were detected in 1984 by separate research groups and designated the Toronto- (Yeung *et al.*, 1986) and the Rio-type (van Embden *et al.*, 1985) plasmids. Due to similarity in size of these two plasmids, they are not differentiated in most epidemiological surveys and all plasmids of 2.9-3.05 MDa were reported by some authors as Toronto type (Hamett *et al.*, 1997; Sarafian *et al.*, 1994; Reiman *et al.*, 1992; Chu *et al.*, 1992) and by others as Rio-type plasmids (vaz Pato *et al.*, 1988). Strains harbouring the Asia, Africa and Rio/ Toronto-type PPNG plasmids occur throughout the world and other plasmids, including the Nimes (Gouby *et al.*, 1986), the New Zealand (Brett, 1989) and a single isolate

carrying an unnamed variant (3.9 MDa) isolated in the Philippines (Knapp *et al.*, 1997) have been reported.

The incidence of PPNG infections in Singapore increased alarmingly from 3 cases in 1976 to 1,976 cases (19.2%) in 1979. Of these PPNG infections, female prostitutes contributed 72.7% in 1979 (Rajan *et al.*, 1981). The PPNG strains first appeared in Taiwan in late 1976, rose to 37.8% in 1982 and has remained high (50%-62%) since 1983 (Chu *et al.*, 1992). In Bangkok, Thailand, 17.8% of the *N. gonorrhoeae* isolates were PPNG (Knapp *et al.*, 1997). In African country Zaire, 67% *N. gonorrhoeae* isolates were PPNG (van Dyck *et al.*, 1995). In Durban, South Africa, the PPNG strains increased from 16.4% to 19.0% in the period from 1990 through 1993 (Chenia *et al.*, 1997). In Kigali, Rwanda, the prevalence of PPNG remained stable at a rate of 39% during 1985-91 and increased to 61% in 1992-93 (Bogaerts *et al.*, 1998). In a study in Mumbai, India, 48.5% (16/33) of the *N. gonorrhoeae* isolates were resistant to Penicillins and 46.7% (15/33) were penicillinase producers (PPNG) of which majority (71.4%) were Asia type (Divekar *et al.*, 1999). On the contrary, all of the PPNG plasmids (100%) were reported Africa type in Bangladesh (Bhuiyan *et al.*, 1999). (Table 2.2)

Table 2.2: Worldwide reports of PPNG plasmids and their types (modified from Alam, 2008)

Reporting year	*Country*	*No. of PPNG reported*	*Type of PPNG plasmid*	*Rate (%)*	*Authors*
1982	Far East	27 (75%)	Africa Asia	00 100.0	Ng *et al.*
1986	Rwanda	54 (51.9%)[§]	Africa Asia Both	70.0 25.0 5.0	Bogaerts *et al.*
1987	Jamaica	20[¶]	Africa Asia	70.0 30.0	Dillon *et al.*
1988	Munich, Germany	23[¶]	Africa Asia	70.0 30.0	Abeck *et al.*
1991	London, UK	649 (40.8%)	Africa Asia	66.1 33.9	Ison & Easmon
1992	Taiwan	-	Africa Asia Toronto	00 82-95 5-18	Chu *et al.*
1997b	Bankok, Thailand	26 (25.7%)	Africa Asia	96.2 3.8	Knapp *et al.*
1998	Bandung, Indonesia	26 (52%)	Africa Asia	00 100.0	Djajakusumah *et al.*
1999	Mumbai, India	14 (42.4%)	Asia Africa	71.4 26.6	Divekar *et al.*
1999	Bangladesh	22 (23.4%)	Africa Asia	100.0 00	Bhuiyan *et al.*
2001	Caribbean countries	90 (31.9%)	Asia Africa Toronto	67.8 1.1 31.1	Dillon *et al.*

[§]Only 20 of the PPNG isolates examined for types of plasmid
[¶]Percentage not available

Tetracycline-resistance determining plasmids

High-level tetracycline resistance (MIC ≥16μg/ml) in *N. gonorrhoeae* is mediated by the *Tet-M* determinant carried on a 40 kb (25.2 MDa) conjugative plasmid (Morse *et al.*, 1986) and the isolates carrying this plasmid has been called tetracycline resistant *N. gonorrhoeae* (TRNG). The 25.2 MDa conjugative Tet-M Neisseria plasmid was first isolated in 1983 in the USA

(CDC, 1985b) followed by isolates reported from The Netherlands (Roberts *et al.*, 1988). Restriction endonuclease analyses of the plasmids carried by TRNG isolates from the USA and The Netherlands revealed two different plasmids, American type (1600 bp) and Dutch type (700 bp) (Morse *et al.*, 1986; Xia *et al.*, 1995). The TRNG isolates have been also reported from all over the globe including the countries in Europe, Asia, Africa and South America (Ison *et al.*, 1988; van Dyck *et al.*, 1992; Gascoyne-Binzi *et al.*, 1993).

Conjugative Plasmids

Two types of conjugative plasmids have been identified in *N. gonorrhoeae*: the 24.5 MDa and the 25.2 MDa plasmids.

The 24.5 MDa conjugative plasmid was first described in 1974 (Mayer *et al.*, 1974). In a survey of five geographical locations within the United States, the number of strains carrying the 24.5 MDa plasmid varied from 10% to 53%. In some cases, the 24.5 plasmid is the only plasmid species present; however, it can coexist with the 2.6 MDa and the gonococcal β-lactamase plasmids (Roberts *et al.*, 1979). The plasmid species in *N. gonorrhoeae* isolates from six Far East countries found that 93% of the PPNG and 22% of the non-PPNG isolates contained the 24 MDa plasmid (Ng *et al.*, 1982). In Zurich, Switzerland, 65.1% of the PPNG isolates were found to contain 24.6 MDa transfer plasmids (Eichmann and Eugster, 1987). Analysing PPNG strains in Singapore it was found that 56.5% of the Asia type plasmids were accompanied by 24.5 MDa transfer plasmid, whereas 14.3% of the non-PPNG isolates also contained the 24.5 MDa transfer plasmid (Poh *et al.*, 1991).

The 24.5 MDa plasmid carries no detectable markers for antibiotic resistance, or resistance to heavy metal or ultraviolet rays. However, it efficiently mobilizes itself and the smaller β-lactamase plasmids between strains of *N. gonorrhoeae* (Roberts and Falkow, 1979). When short mating times are used, only the β-lactamase plasmids are transferred to the recipient, whereas after

overnight mating, many of the *N. gonorrhoeae* transconjugants carry both the β-lactamase and 24.5 MDa plasmids and can transfer both plasmids to other *N. gonorrhoeae* strains. The host range of the 24.5 MDa plasmid is very limited even under laboratory conditions and other than *N. gonorrhoeae*, only certain species of *N. cinerea* maintain it (Genco *et al.*, 1984).

The 25.2 MDa Tet-M conjugative plasmid was first identified in a strain of *N. gonorrhoeae* isolated in 1982 (Knapp *et al.*, 1987). By 1985, a number of clinical isolates in North America and Europe carried this plasmid. At present, the 25.2 MDa plasmid appears to be endemic in North America and has been found in Great Britain and the Netherlands. Most of the strains in The Netherlands and a few from North America carry the 3.2 MDa β-lactamase in addition to the 25.2 MDa and 2.6 MDa plasmids (Knapp *et al.*, 1987). Recently, the 24.5 MDa conjugative Gonococcal plasmids have been shown to share >60% of their DNA sequences with 25.2 MDa plasmids (Morse *et al.*, 1986). These data have led to the hypothesis that the 25.2 MDa plasmid was formed by the transposition of the *Tet-M* determinant encoding Tetracycline resistance onto the 24.5 MDa plasmid. The 25.2 MDa plasmids have characteristics which differ from those of the 24.5 MDa ancestral plasmids, but they have maintained the ability to move themselves and the β-lactamase plasmids to recipient strains.

2.2.4: *N. gonorrhoeae:* Genetics and antigenic heterogeneity

Gonococci have evolved mechanisms for frequently switching over from one antigenic form to another antigenic form of the same molecule. The frequency of this switching is an extremely rapid rate of change for a bacterium, taking place in one in every $10^{2.5}$-10^{3} gonococci (Brooks *et al.*, 2004).

The switching mechanism for pilin has been the most thoroughly studied and this is different from the mechanism for Opa. Gonococci possess multiple

genes that code for pilin, but only one gene is inserted into the expression site at a time. The organism 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 for Opa involves, at least in part, the addition or removal from the DNA of one or more of the pentameric coding repeats preceding the sequence that codes for structural Opa gene. The switching mechanism for LPS is not known (Cannon and Sparling, 1984).

The high degree of antigenic heterogeneity makes pili as well as Opa proteins and LOS ineffective targets for the immune system of the host and may explain the rapid adaptation of the bacteria for adherence to different types of cells in different ecological niches of the human body.

The genetic analysis of pilus production in the gonococcus has shown that pilus expression is regulated at two expression loci on the chromosome, although many other regions contain silent pilin information. When the cell is in the P^+ state, either one or both expression sites contain an intact pilin gene. In most cases, a P^+ to P^- switch results in the deletion of pilin information from either one or both of the expression sites. The generation of a complete pilin gene within the expression loci is the result of multiple recombination events. At the DNA level, the mixing and matching of individual semivariable and hypervariable gene segments by recombination increases the number of new pilin epitopes any one cell is capable of producing (Wilfert and Gutman, 1992).

N. gonorrhoeae is capable of exchanging genetic information by transformation and conjugation. The organisms are competent for transformation by either chromosomal or plasmid DNA throughout their growth cycle and take up naked DNA with subsequent incorporation into the chromosome at regions of nucleotide sequence homology (Rudel *et al.*, 1995a). Neisseria species exchange DNA *in vivo* by transformation from nucleic acids

released from autolysed cells. Transformation may result in altered phenotypic characteristics. It has been suggested that transformation might be the principal means of exchange of chromosomal DNA between gonococci in nature (Graves *et al.,* 1982), inasmuch as there are no known gonococcal bacteriophage and gonococcal conjugal plasmids fail to detectably mobilize chromosomal genes (Norlander *et al.,* 1979; Biswas *et al.,* 1980).

2.2.5: *N. gonorrhoeae :* Phenotypic characteristics

Characterization of clinical isolates of *N. gonorrhoeae* might play significant roles in formulation of effective control measures for gonococcal infection. Among the various phenotypic characteristics of the organism, antimicrobial susceptibility determines the treatment regimen for the circulating strains of the organism. Plasmid profile supports the assumption regarding level of resistance among the clinical isolates and together with serotypes and serovar determination provides important clues of the transmission chain and level of virulence of the isolates.

2.2.5a: *N. gonorrhoeae* : Antimicrobial Susceptibility

2.2.5a.i: Antimicrobial Susceptibility Surveillance

A number of industrialized countries have developed gonococcal resistance surveillance programmes including the United Kingdom, the United States, Canada, Australia, Sweden and The Netherlands.

In the United Kingdom, the gonococcal resistance to antimicrobials surveillance programme (GRASP) is the national sentinel surveillance programme, was established in June 2000 with the aims to establish a sustainable, cost-effective, national sentinel surveillance system for monitoring antimicrobial resistance to *N. gonorrhoeae* in England and Wales. The system combines laboratory and clinical data on gonococcal isolates obtained from

purposely selected laboratories, and covers two distinct geographical regions within the UK: London and outside London (Paine *et al.*, 2001).

In the United States in 1986, the Center for Disease Control and Prevention (CDC) established the gonococcal isolate surveillance project (GISP). Data from GISP have documented important patterns of antimicrobial resistance, and have steered CDC national guidelines for gonorrhoea treatment. Regional laboratories under GISP determined the susceptibilities of the *N. gonorrhoeae* isolates to penicillin, tetracycline, spectinomycin, ceftriaxone, ciprofloxacin and azithromycin. The values of the minimum inhibitory concentrations (MICs) were interpreted according to criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (CLSI, 2007). Due to the emergence of decreased susceptibility to cefixime, the antibiotic (cefixime) was discontinued in 2007 from the GISP antimicrobial susceptibility panel (CDC, 2009).

In Australia, the Australian gonococcal surveillance programme (AGSP) was established in 1979 to monitor the susceptibility to antibiotics of gonococci isolated throughout the country. The AGSP is actually a component of the national Neisseria network of Australia and comprises participating laboratories in each State and Territory (AGSP, 2002).

In Sweden, gonorrhoea is a notifiable disease and the Swedish reference laboratory is regularly monitoring the antimicrobial resistance of the *N. gonorrhoeae* isolates (Velicko and Unemo, 2009).

The World Health Organization (WHO) established a surveillance programme at different regions of world known as the gonococcal antimicrobial surveillance programme (GASP). The GASP in Australia is the WHO Western Pacific region gonococcal antimicrobial surveillance programme (WHO WPR GASP) is continuing including many countries in the region (15 countries were

participating in 2004) (WHO WPR GASP, 2006). The WHO-Southeast Asia region (SEAR) is working in India since 1999 to monitor antimicrobial susceptibility of the *N. gonorrhoeae* isolates in this region (Bala *et al.*, 2007).

2.2.5a.ii: Antimicrobial resistance in gonococci

The clinical impact of resistance is immense, characterized by increased cost, length of hospital stay and mortality (Niederman, 2001; Toubes *et al.*, 2003; Cosgrove and Carmeli, 2003; Overbye and Barrett, 2005). The antimicrobial resistance often occurs as a result of inappropriate initial antimicrobial therapy (Toubes *et al.*, 2003; Harbarth *et al.*, 2003; Masterton *et al.*, 2003).

Bangladesh and Southeast Asian countries

In Bangladesh, *N. gonorrhoeae* isolates from female commercial sex workers showed 11.7% resistance to ciprofloxacin in 1998 (Bhuiyan *et al.*, 1999), followed by 37.8% in 1999 (Rahman *et al.*, 2001) and turning to almost ineffectiveness (90.5% resistance) in 2002 (ICDDR,B, 2003a). To penicillin, the rates of resistance were variable: 62% in 1997 (Bhuiyan *et al.*, 1999) and 68.7% in 1998 (Alam *et al.*, 2002). In another report, penicillin resistance was 50% in 1997, 51% in 1998 and decreasing to 28% in 1999 (Rahman *et al.*, 2002). To tetracycline, the rates of resistance were: 57% in 1997 (Bhuiyan *et al.*, 1999), 87.9% in 1998 (Alam *et al.*, 2002) and 94.7% in 2002 (ICDDR,B, 2003a).

In India, 47.4% *N. gonorrhoeae* isolates were found resistant to penicillin during 2002 to 2006 showing a significant rise from 53.8% in 2002 to 68.4% in 2003, followed by a significant fall to 18.2% in 2006. Similarly, ciprofloxacin resistance increased significantly from 80.7% in 2002 to 97.2% in 2004 followed by a significant decrease to 83.6% in 2006. All isolates were susceptible to spectinomycin and 5.5% ceftriaxone less susceptible strains were identified in 2006 (Bala *et al.*, 2007).

In Sri Lanka, resistance of the *N. gonorrhoeae* to ciprofloxacin was 10.3% in 1996 and after withdrawal of the antibiotic, reported resistance was 8.2%. Resistance to penicillin continues to be a problem. No resistance was reported for ceftriaxone and spectinomycin. Resistance to cefuroxime was reported in 0.5% of the strains (WHO, 2002).

Western Pacific region

World health organization (WHO) has been monitoring antimicrobial resistance in *N. gonorrhoeae* by the Western Pacific region gonococcal antimicrobial surveillance programme (WPR GASP) since its inception in 1994. The WHO WPR GASP examined approximately 10,000 *N. gonorrhoeae* isolates from 15 countries including Australia, China, Japan, Korea, New Caledonia, New Zealand, Papua New Guinea, the Philippines, Singapore and Vietnam in 2004 for resistance to antibiotics. Treatment option in the region was found limited by persistence of high rates of resistance to penicillins and quinolones. There were infrequent instances of spectinomycin resistance and the presence of gonococci with decreased susceptibility to third generation cephalosporins was noted again in several centres (WHO WPR GASP, 2006).

Latin America and the Caribbean

The gonococcal antimicrobial surveillance programme (GASP) in the Americas and the Caribbean was established in the Ottawa, Canada for confirmation of the MIC, plasmid content and *tet-M* type. In a report from three Caribbean countries published in 2001, high percentages of penicillin and/or tetracycline resistance were observed in *N. gonorrhoeae* isolates from Guyana (92.9%), St. Vincent (44.1%) and Trinidad (42.4%). Isolates from all three countries were susceptible to ceftriaxone, ciprofloxacin and spectinomycin. Most of the PPNG isolates (61/90, 67.8%) were found to carry Africa-type plasmids, 28 of 90 (31.1%) carried Toronto-type plasmids and a single isolate (1.1%) carried an Asia-type plasmid. The *tet-M* determinant in tetracycline resistant *N.*

gonorrhoeae isolates were predominantly of the Dutch type (68/91, 74.7%) (Dillon *et al.*, 2001).

African nations

In a recent cross-sectional study at Maputo, Mozambique to ascertain the effectiveness of kanamycin for treatment of gonorrhoea found that 22 (40%) *N. gonorrhoeae* isolates were intermediate and 4 (7%) were resistant to kanamycin; 42 (77%) displayed high level resistance to tetracycline (MIC $\geq$ 16 mg/L); 34 (65%) were penicillinase producers (PPNGs), and 52 (95%) had spectinomycin MICs of 64 mg/L. All isolates were susceptible to ciprofloxacin (MIC $\leq$0.06 mg/L), ceftriaxone (MIC $\leq$0.015 mg/L) and cefixime (MIC $\leq$ 0.015 mg/L) (Teke *et al.*, 2009).

2.2.5a.iii: Mechanisms of antimicrobial resistance

There are three mechanisms which describe how bacteria can develop resistance to antimicrobial agents: (i) alteration of the antibiotic target, (ii) restriction of drug access to the target, and (iii) inactivation or degradation of the drug. In case of *N. gonorrhoeae*, these mechanisms are also found to be equally effective in developing resistance to the antimicrobial agents.

The development of antibiotic resistance in the gonococcus may involve primarily either chromosomal or extrachromosomal (plasmid-mediated) mechanisms and for some antibiotics like penicillin and tetracycline, both mechanisms are implicated. Chromosomally-mediated resistance to penicillins giving rise to the strains termed CMRNG, results from alterations to the target site (for example, penicillin binding protein or PBP in the cell wall) and bring about changes in access of the antibiotic to this target site when porins in the outer cell membrane are modified (Shafer and Folster, 2006). The alteration in the target structure is also associated with multi-drug resistance (MDR). Additionally, gonococcal acquisition of one of the several types of plasmids

which contains genetic elements coding for production of a TEM-type beta lactamase led to the appearance of the PPNG strains (Ashford *et al.*, 1976).

Chromosomally-mediated resistance of gonococci to certain antibiotics (erythromycin, penicillin and tetracycline) has been reported due to the mutation in the PBP-encoding genes, termed as *mtr* (multiple transferable resistances) as well as due to replacement of the major outer membrane porin protein (por) (Spratt, 1994; Gill *et al.*, 1998). The *mtr* is effective due to the action of mtrC-mtrD-mtrE efflux pump that exports hydrophobic agents by an energy-dependent process. Mutations in the mtr-related genes (*mtrR*) can decrease susceptibility of gonococci to penicillin by 2-fold, whereas, with a co-resident *penA* (wild-type gene encoding PBP-2) mutation resistance to penicillin increases by 8- to 10-fold (Hagman *et al.,* 1995; Gill *et al.*, 1998). In gonococcal strains harbouring *penA*, *penB* (porIB-encoding gene) and *mtrR* mutations increase resistance to penicillin nearly 66-fold (Veal *et al.*, 2002).

Antimicrobial resistance in *N. gonorrhoeae* may also arise by selection of resistant mutants from endogenous flora produced by spontaneous chromosomal mutations or it can be acquired from other bacteria by plasmid-mediated transfer or DNA transformation and recombination.

The potency of the fluoroquinolones is determined by their ability to reach and act on their target enzymes, DNA gyrase and topoisomerase IV, which are both required for bacterial DNA replication (Wolfson and Hooper, 1985). In *N. gonorrhoeae*, three mechanisms of fluoroquinolones resistance have been reported: (i) the development of mutations in the DNA gyrase subunit A (Gyr A) encoded by the *gyrA* gene; (ii) mutation in the DNA topoisomerase IV encoded by the *parC* gene; and (iii) reduced accumulation of quinolone in the cells (Belland *et al.*, 1994; Tanaka *et al.*, 1998). Double mutations in the *gyrA* along with additional mutations in the topoisomerase gene, *parC* have been

found significantly associated with high-level resistance of Ciprofloxacin in the *N. gonorrhoeae* isolates (Deguchi *et al.*, 1996; Su and Lind, 2001).

Types of N. gonorrhoeae by resistance mechanism

The gonococcal isolate surveillance project (GISP) in the USA uses six mutually exclusive categories of resistance for describing chromosomally- and plasmid-mediated resistance to penicillin and tetracycline: (1) penicillinase producing *N. gonorrhoeae* (PPNG): β-lactamase-positive and tetracycline MIC <16.0 μg/ml; (2) plasmid-mediated tetracycline resistant *N. gonorrhoeae* (TRNG): β-lactamase-negative and tetracycline MIC ≥16.0 μg/ml; (3) PPNG-TRNG: β-lactamase-positive and tetracycline MIC ≥16.0 μg/ml; (4) chromosomally-mediated penicillin-resistant *N. gonorrhoeae* (PenR): non-PPNG and penicillin MIC ≥ 2.0 μg/ml and tetracycline MIC <2.0 μg/ml; (5) chromosomally-mediated tetracycline-resistant *N. gonorrhoeae* (TetR): non-PPNG and penicillin MIC <2.0 μg/ml and tetracycline MIC 2.0-8.0 μg/ml; and (6) chromosomally- mediated resistance to both penicillin and tetracycline (CMRNG): non-PPNG and penicillin MIC ≥ 2.0 μg/ml and tetracycline MIC 2.0-8.0 μg/ml (Fox *et al.*, 1997).

2.2.5b: N. gonorrhoeae: Auxotypes

Auxotyping is the determination of a strain's growth requirements and since a defined medium capable of supporting the growth of most gonococcal isolates for nutritional requirements was developed (Catlin, 1973), auxotyping became the most accepted method for differentiating between strains of *N. gonorrhoeae*. Auxotyping determines the type of *N. gonorrhoeae* strains by nutritional requirements for proline (Pro), arginine (Arg), hypoxanthine (Hyp), uracil (Ura), methionine (Met) and histidine (His) using modified Heckels' medium (Copley and Egglestone, 1983). During the 1990s, the most widely employed method for differentiation of *N. gonorrhoeae* strains was based on auxotyping and serological (A/S) characterization (van Looveren *et al.*, 1999).

But A/S classification system is complicated, labourious and time-consuming as well as not providing sufficient discrimination.

N. gonorrhoeae isolates recovered from cases of DGI have unique nutritional requirements for arginine, hypoxanthine and uracil for growth (AHU strains) (Eisenstein *et al.*, 1977; Knapp and Holmes, 1975; Schoolnik *et al.*, 1976). The AHU strains are generally unable to remove iron from lactoferrin, which could explain the tendency of such strains to cause asymptomatic mucosal infections leading to eventual DGI (Mickelsen and Sparling, 1981; Holmes and Morse, 1994).

2.2.5c: N. gonorrhoeae: Serotypes and serovars

Serological classification of *N. gonorrhoeae* isolates is done by coagglutination method using the monoclonal antibodies against the outer membrane protein, protein IA and protein IB, of the organism (Knapp *et al.*, 1984).

The serological classification of *N. gonorrhoeae* by coagglutination was first developed by Sandstrom and Danielsson who were able to divide *N. gonorrhoeae* strains into three antigen classes: W, J, and M using selectively absorbed polyclonal rabbit antisera (Sandstrom and Danielsson, 1980). Only the W antigens were found to be suitable for serological classification and the gonococci were then divided into three serogroups: WI, WII and WIII. Serogroups WI and WII/WIII correspond to two mutually exclusive forms of outer membrane protein I, protein IA and protein IB as well as serotype A and serotype B respectively (Sandstrom *et al.*, 1982).

The serological classification of the *N. gonorrhoeae* isolates were performed by coagglutination against gonococcal outer membrane protein I, using the Ph panel of monoclonal antibodies of the Phadebact GC serovar test (Boule Diagnostics AB, Huddinge, Sweden) and the nomenclature of Bygdeman and co-workers (Bygdeman *et al.*, 1983).

Most of the first Greek TRNG isolates were non-PPNG strains of various IB serovars and the only PPNG/TRNG strain encountered was of serovar "Arst" carried the African-type plasmid. A cluster of MDR isolates belonging to the newly emerged serovar "Bropyst" exhibited relatively increased MIC values to norfloxacin and ciprofloxacin (Tzelepi *et al.*, 1997).

In a study to see the gonococcal serovar patterns in Stockholm observed that the majority of the serovars belonged to serogroup WII/WIII (serotype B). A total of 7 serovars included 62% of all isolates investigated. Three of these serovars (Arost, Bropt and Brpyust) accounted for 49% of all isolates in the study. The remaining four serovars (Arst, Bpyvut, Bropyst and Bpyust) accounted for 13% of all isolates (Ruden, 1994).

Arost (71%) was the most common among the type A serovars and Brpyust (20%) and Bropt (19%) was the most common among the type B serovars in the Gonococcal strains isolated in Stockholm, Sweden (Bygdeman *et al.*, 1993).

Among the clinical isolates of *N. gonorrhoeae* at the Edinburgh Royal Infirmary, majority (63%) were serogroup WII/WIII (serotype B) with Arost being the predominant serovar among serotype A and Bropyt being the predominant serovar among the serotype B (Coghill and Young, 1987). Over a 5-year period in Edinburgh, 32 different serovars were found associated with homosexually acquired gonococcal infections with five predominating serovars of Av, Bropyt, Brpyut, Brpyust and Brpyut (Young *et al.*, 1991).

2.2.6: *N. gonorrhoeae* : Genotypic characteristics

2.2.6a: Introduction

The molecular typing methods have been widely accepted because of their better discrimination among the isolates. A genotypic method is based on a target gene that accumulates genetic variation quickly, so that individuals that are sexual contacts or part of a short transmission chain demonstrate an indistinguishable type, while individuals that are unlinked have different types. The *N. gonorrhoeae por* and *opa* gene, encoding the porin and opacity proteins respectively have been found helpful for genotyping the isolates (Howie *et al.*, 2004; O'Rourke *et al.*, 1995; Rahman *et al.*, 2001).

2.2.6b: Opa types

The molecular types identified applying DNA amplification (PCR) of *opa* gene of an isolate, followed by digestion of the amplicons with restriction enzymes and finally resolving the restriction fragments by polyacrylamide gel electrophoresis.

The Opa typing was initially reported by O'Rourke and colleagues in 1995 and proposed a genotyping scheme based on nucleotide sequence variation in the gonococcal *opa* gene family, the Opa-types (O'Rourke *et al.*, 1995). Subsequently, other workers have both modified and validated the technique (Camarena *et al.*, 1995; van Looveren *et al.*, 1999; Viscidi *et al.*, 2000; Palmer *et al.*, 2001).

2.2.6c: MLST types

The sequence types identified applying multilocus sequence typing (MLST) is based on the principles of multilocus enzyme electrophoresis, but characterizes the alleles present at multiple housekeeping genes directly by nucleotide sequencing rather than indirectly by the electrophoretic mobility of their gene products (Maiden *et al.*, 1998). The advantages of MLST compared to typing methods that involve comparison of DNA fragments in gels are that the data

are unambiguous, can be shared between laboratories and lend themselves to analyses of the genetic relationships among isolates.

The MLST schemes have been developed for several bacterial species, demonstrating the broad applicability of the method and MLST-generated data have been used to address aspects of the population genetics and evolutionary biology of bacterial species. Application of MLST for *N. gonorrhoeae* have not been reported widely yet.

2.2.6d: NG-MAST genotypes

Are the sequence-based genotypes using *N. gonorrhoeae* multi antigen sequence typing (NG-MAST). For this molecular typing, internal regions of *por* and *tbpB* genes of *N. gonorrhoeae* are amplified by PCR (polymerase chain reaction) and both strands of DNA are sequenced. Sequence data are then aligned with internationally accessible data using different software, edited and trimmed to a fixed length from the conserved position. A 2/3 digit allelic profile is then obtained for each gene by submitting the corresponding sequence data into the NG-MAST database available at www.ng-mast.net as a single-locus query from the drop down box. The website software checks for the correct length and/ or any unrecognized character(s) and checks again against all existing alleles for the particular gene. The software reports whether the sequence data matches an existing allele providing allele number or assigns a new allele number if sequence has not been found previously. The allele numbers thus obtained from the two loci (*por* and *tbpB*) in combination provides a sequence type (ST) (Martin *et* al, 2004; Martin *et al.*, 2007).

2.2.7: *N. gonorrhoeae*: Laboratory methods for characterization

2.2.7a: Isolation and Identification

The laboratory diagnosis of gonococcal infection depends on the demonstration of *N. gonorrhoeae* or detection of its DNA in the samples. There are a number of techniques available for identification of *N. gonorrhoeae* from clinical specimens as well as from cultured colonies. Presumptive identification of the *N. gonorrhoeae* is based on growth on selective GC agar, Gram stain morphology of the growth and 'oxidase' test positivity (Knapp and Rice, 1995). The presumptive identification is regarded as sufficient for diagnosis of the organism in many settings, especially grown from genital tract sites.

Confirmatory identification is more important especially for isolates from extragenital sites. In order to obtain a confirmatory diagnosis of *N. gonorrhoeae*, the presumptively identified isolate needs to be confirmed by any of the species confirmative assay. For example, carbohydrate utilization assays, coagglutination tests, biochemical and rapid enzyme substrate tests, DNA probe hybridization assays, nucleic acid amplification tests (NAATs), or monoclonal fluorescent-antibody tests can be used (Knapp, 1988; Knapp and Koumans, 1999; van Dyck *et al.*, 1999). However, despite the high sensitivity and specificity of these assays, a few auxotypes or individual strains may not be identified as *N. gonorrhoeae* and some other species, especially commensal Neisseria or *N. meningitidis* can be misidentified as *N. gonorrhoeae* (Jephcott, 1997; Johnson *et al.*, 2002; Knapp, 1988; Knapp and Koumans, 1999).

2.2.7a.i: Specimens for isolation and identification

Specimens for the isolation of *N. gonorrhoeae* may be obtained form the genital tract, urine, anal area, oropharynx, conjunctiva, Bartholin's gland, fallopian tubes, endometrium, blood, joint fluid, skin lesions or gastric contents of neonates (Knapp and Rice, 1995).

As for all diagnostic tests, the collection and transport of appropriate samples are often critical determinants of success or failure in demonstrating the presence of *N. gonorrhoeae*. For example, gonococci in the sexually active girls and women reside in the endocervical glands and Endocervical specimens are appropriate among them, in preference to a vaginal swab.

2.2.7a.ii: Specimen Collection and Transport

Urethral specimens should be collected at least 1 hour after the patient has urinated. Anorectal swabs that are contaminated with faeces should be discarded and a second specimen should be obtained (Knapp and Rice, 1995).

The best method for preserving viable organism is to inoculate specimens directly onto a nutritive medium and inoculate them at 35-37^{0}C in a CO_2-enriched atmosphere immediately after collection.

If the specimens must be transported to a local laboratory and it is more important to place the inoculated plates in a CO_2-enriched atmosphere than to incubate them at 35^{0}C to 37^{0}C. The inoculated media may be held at room temperature in a CO_2-enriched atmosphere in either candle extinction jars or commercial CO_2-generating systems such as JEMBEC plates (Martin and Jackson, 1975) for up to 5 hours without appreciable loss of viability (Knapp and Rice, 1995).

If specimens must be transported to a distant town, various transport systems are available including the non-nutritive swab transport system using Stuart's or Amies's buffered semisolid transport media (Farhat *et al.*, 2001) and culture media transport systems using transport of specimens on culture media. For the culture media transport system, the inoculated media should be incubated for 18-24 hours before being transported and the specimen should arrive within 48 hours (Knapp and Rice, 1995; Winn Jr. *et al.*, 2006).

2.2.7a.iii: Gram's staining for N. gonorrhoeae

Microscopy of Gram-stained smears, especially of urethral specimens from men and women is used widely for a presumptive diagnosis of gonorrhoea. Although, the microscopy is rapid, inexpensive and specific but comprises a much lower sensitivity for cervical specimens. Pharyngeal and rectal specimens are not suitable for microscopical identification of *N. gonorrhoeae* due to contaminating commensal bacteria. Gram stain diagnosis of gonorrhoea from other sites such as conjunctiva and skin is suboptimal. Furthermore, early and asymptomatic infection as well as some auxotypes and serological variants of *N. gonorrhoeae* may be less likely to be detected by microscopy (Bignell, 2001; Chernesky, 1999; Knapp and Koumans, 1999; Manavi *et al.*, 2003; van Dyck *et al.*, 1999).

Microscopy involves the identification of *N. gonorrhoeae* as Gram-negative diplococci in the PMNs in stained smears using light microscopes. It is easy and quick to perform by suitably trained personnel, usually yielding results within half an hour. However, examination of a Gram-stained smear requires availability and maintenance of a good quality microscope and the services of a competent Microscopist.

When compared with culture, the sensitivity of urethral smears from symptomatic male exceeds 95% with a specificity of 98%, but the sensitivity in women, asymptomatic men and rectal smears is highly variable and drops to 40-70% at best (Ison, 1990).

2.2.7a.iv: Culture

Introduction

Culture-based systems provide high specificity (virtually 100% if definitive identification procedures are applied), but are expensive and require personnel trained in the handling the fastidious gonococcus. On the contrary, culture

method allows antibiotic susceptibility testing (AST) and characterization of the isolated strains.

Culture identification of *N. gonorrhoeae* is the gold standard for definitive diagnosis of gonorrhoea (Bignell and WHO, 2001; van Dyck *et al.*, 1999). Maximai recovery of the gonococci is obtained when specimens are plated directly onto growth medium immediately after collection. If immediate culturing is not possible, an effective transportation medium for the specimens and a short transportation time are required. In addition, especially for extragenital samples, the use of a selective culture medium combined with a non-selective medium, optimized inoculation and incubation are crucial. Use of non-selective media is important because few *N. gonorrhoeae* strains are sensitive to the concentrations of trimethoprim or vancomycin used to make the medium selective (Jephcott, 1997; Johnson *et al.*, 2002; van Dyck *et al.*, 1999 Chernesky, 1999). Direct inoculation of genital specimens onto selective culture media (direct plating) followed by CO_2 incubation yields sensitivities of 90-95%. Samples appropriately stored in transport medium and inoculated within 24 hours give good isolation rates, while suboptimal sampling, storage or transport conditions will have a dramatic impact yielding sensitivities of 40-70% (Ison, 1990; Davies *et al.*, 1998).

Culture media for N. gonorrhoeae

There are two basic requirements for reliable gonococcal culture media, namely, an enriched medium to support the growth of the fastidious Gonococcus (for example chocholate agar medium) and a selective medium (such as modified Thayer- Martin medium) (Thayer and Martin, 1966; Martin *et al.*, 1974) to allow the isolation of the organism from specimens with a wide and numerous normal flora.

A variety of enriched selective media for culture of *N. gonorrhoeae* are available and include modified Thayer Martin (MTM) medium, Martin-Lewis

(ML) medium, GC-Lect medium (BD Biosciences) and New York City (NYC) medium. The MTM, ML and GC-Lect are chocolate agar based media that are supplemented with growth factors (for example Isovitalex®, Becton Dickinson, USA) for fastidious microorganisms, whereas NYC medium is a clear peptone-cornstarch-based medium containing yeast dialysate, citrated horse plasma and lysed horse erythrocytes (Faur *et al.*, 1973; Jephcott, 1997). Antibiotics are incorporated to make the medium selective for Gonococci. The usual antibiotics added are Vancomycin (2-3 μg/ml), Colistin (6-7.5 μg/ml), Nystatin (12.5 μg/ml) and Trimethoprim (1.5-8 μg/ml) (VCNT supplement, Oxoid, UK) (Martin *et al.*, 1974; Jephcott, 1997). Lincomycin (1 μg/ml) may be replaced for Vancomycin and Amphotericin B (1 μg/ml) may be replaced for Nystatin (Young, 1978).

Inoculation of specimens

Media for isolation of *N. gonorrhoeae* should be at room temperature before inoculation and should not be excessively dry or moist. Specimens collected on swabs should be firmly rolled in a "Z" pattern on selective media and cross-streaked with a bacteriological loop.

Incubation conditions

The presence of CO_2 is required to initiate gonococcal growth and an incubation temperature of 35^0C- 37^0C as well as a humid atmosphere is necessary. These requirements can be met reliably by modern CO_2 incubators. The CO_2 level of the incubator should be 3-7%; higher CO_2 concentrations may actually inhibit growth of some strains. Candle extinction jar is another good alternative and achieves a CO_2 level of 3-5%. The atmosphere should be moist and with candle jars, moisture evaporating from the medium during incubation is usually sufficient for organism growth. The CO_2 incubators that are not equipped with humidifiers may be kept moist by placing a pan of water on the lower shelf (Winn Jr. *et al.*, 2006).

The CO_2 incubators are desirable if large numbers of specimens must be processed. Alternative methods for providing a CO_2-enriched atmosphere (candle extinction jars or commercial CO_2-generating systems) may be used when specimens must be transported to a laboratory or when only small numbers of specimens are processed. It is important (i) to use a non-scented candle (the vapour from scented candles may be toxic), and (ii) to relight the candle if the jar is opened to add more plates (Knapp and Rice, 1995).

In case if it is not possible to immediately incubate the inoculated media, it more important to place the inoculated plates in a CO_2-enriched atmosphere than to incubate them at 35^0C-37^0C. Inoculated media may be held at room temperature in a CO_2-enriched atmosphere in candle extinction jars for up to 5 hours without appreciable loss of viability (Knapp and Rice, 1995).

Identification of N. gonorrhoeae colonies

After overnight incubation, typical colonies of *N. gonorrhoeae* of 0.5-1mm in diameter appear and may vary from grey to white, transparent to opaque and raised convex to flat. Frequently a mixture of different colony types appears on a plate. Colonies exhibiting characteristic morphology are confirmed by Gram stain and oxidase reaction for preliminary identification. Subsequent confirmation by carbohydrate utilization tests are done for the full identification of *N. gonorrhoeae* (van Dyck *et al.*, 1989; Davies *et al.*, 1998).

All isolates of oxidase-positive, Gram-negative diplococci that are recovered from urogenital sites and that grow on selective media are presumptively identified as *N. gonorrhoeae*. The superoxol test also provides an additional presumptive test for identifying the isolates. However, confirmatory identification tests are recommended for all isolates and are required for identification of isolates from extra-genital sites like throat, rectum, blood, joint fluid and CSF (Winn Jr. *et al.*, 2006).

2.2.7a.v: Serology/ Immunological Diagnosis

Serological tests are of limited value for the diagnosis of gonococcal infection because of gonococci to react poorly with the antibodies and further non-Gonococcal isolates were often found to cross-react with the gonococcal antibodies. Currently immunological identification tests include fluorescent antibody and coagglutination tests.

Fluorescent Antibody (FA) test

Older FA tests employed fluorescein-conjugated polyclonal anti-gonococcal antibodies raised in rabbits. Non-gonococcal isolates often cross-reacted with these reagents and some gonococci reacted weakly or not at all (Knapp, 1988). The FA procedures are now commercially available (*N. gonorrhoeae* culture confirmation Test; Syva Co., Palo Alto, Calif, USA) uses monoclonal antibodies that recognize epitopes on Protein I of *N. gonorrhoeae*.

To perform the test, a light suspension of the organism is dried and heat fixed on an FA slide, overlaid with the FA reagent, and incubated for 15 minutes. After the smear is rinsed, dried and mounted with a cover slip, it is examined with a fluorescent microscope. *N. gonorrhoeae* organisms appear as apple green fluorescent diplococci. This FA reagent is highly sensitive and specific (Laughon *et al.*, 1987; Welch and Cartwright, 1988); however, some gonococcal strains may be weakly fluorescent or fail to fluoresce (Walton, 1989). The advantages of the test are that it is rapid, colonies can be tested directly from selective media, and only a small amount of growth is needed.

Coagglutination methods

Protein A-enriched *Staphylococcus aureus* cells sensitized with monoclonal antibodies against *N. gonorrhoeae* are used as the reagent for confirmatory identification. Three kits are commercially available: the Gonogen I test (New Horizons Diagnostics, Columbia, Md, USA), the Phadebact GC OMNI test

(Bactus AB, Sweden) and the Meritec GC test (Meridian Diagnostics, Ohio, USA). Test performances are similar for all of the three test kits.

A standardized suspension of the organism is prepared and boiled for 5-10 minutes. After cooling, the suspension is reacted with single drops of the test and the control reagents included in the kit. After the contents are mixed for a prescribed length of time, agglutination in the test reagent is compared with that in the control. In general, the coagglutination tests are reliable for identifying *N. gonorrhoeae* (Carlson *et al.*, 1987; Dillon *et al.*, 1988).

2.2.7a.vi: Enzyme Immunoassay

Tests using antigen detection and enzyme immunoassay as the signalling mechanism was developed and extensively trialled. However, after evaluation in low and high prevalence settings as well as in symptomatic and asymptomatic men and women it was concluded in a meta analysis that "there are a few to no situation for which this assay is recommended" (Koumans *et al.*, 1998).

2.2.7a.vii: Molecular methods

Difficulties in specimen collection, transport and culture-based diagnosis of gonorrhoea have accelerated the development and application of nucleic acid detection tests, with or without amplification. The molecular technology has been used to develop tests with a high sensitivity and specificity. For the diagnosis of urethritis and endocervicitis by the molecular techniques, a non-invasive first voided urine sample usually suffices. Thus the molecular techniques providing with a highly reliable result, minimum collection difficulties and less demanding in terms of transport requirements is now increasingly being introduced.

Numerous DNA/RNA-based non-cultural molecular methods for identification of *N. gonorrhoeae* have been developed. Some are based on probe

hybridization (Modarress *et al.*, 1999) and many others are nucleic acid amplification tests (NAATs) for detection of *N. gonorrhoeae* specific DNA/RNA sequences located on the chromosome or the cryptic plasmid of the organism. In general, the hybridization methods have a high specificity but a somewhat lower sensitivity than the NAATs (Fredlund *et al.*, 2004).

In nucleic acid hybridization tests (probe tests), a specific probe binds to any complementary nucleic acid present in the sample. The nucleic acid is not amplified, but measuring an attached chemiluminescent label enhances estimation of the bound probe. A commercial version is available that provides specimen collection kits and a lytic transport medium that stabilises the preparation for one month. The test is now combined with a hybridisation test for *Chlamydia trachomatis* as well. The sensitivity of the test is about 85% and the specificity 99%. These data were derived from a review of a number of studies (Koumans *et al.*, 1998) where the sensitivity was as low as 54% in some evaluations (Beltrami *et al.*, 1998). This technology can also be used for confirmation of cultures of *N. gonorrhoeae,* but is expensive.

Over the years, the NAATs based on polymerase chain reaction (PCR) (Farrell, 1999), ligase chain reaction (LCR) (Carroll *et al.*, 1998), nucleic acid sequence-based amplification (NASBA) (Mahony *et al.*, 2001), transcription-mediated amplification (TMA) (Gaydos *et al.*, 2003) and real-time strand displacement amplification (SDA) (Little *et al.*, 1999) have been described.

Advantages of the NAATs are the usually very high sensitivity and specificity, fastness, non-invasive sampling, lack of requirement for viable organisms, potential for using home-obtained samples such as urine or tampons (Knox *et al.*, 2002), the eventual possibility of cost-efficient pooling of specimens, and the opportunity for simultaneous detection of several agents, e.g., *N. gonorrhoeae* and *Chlamydia trachomatis* (Jephcott, 1997; Johnson *et al.*, 2002; Tapsall, 2001). On the other hand, disadvantages include: (a) specificity (i.e.,

false positive results) and in some cases, sensitivity problems (i.e., false negative results) with some of the methods mainly due to existence of amplification inhibitors (Mahony *et al.*, 1998) or use of an unsuitable target gene, e.g., genes on the cryptic plasmid (Palmer *et al.*, 2003); (b) the need of expensive equipment; (c) the risk of contamination by previously amplified DNA/RNA; (d) the lack of effective and licensed protocols for some specimens (e.g., pharyngeal, rectal, conjunctival and blood); and (e) that no viable bacteria are isolated for antibiotic susceptibility testing and strain characterization (Jephcott, 1997; Johnson *et al.*, 2002; Tapsall, 2001).

In most West-European countries, it is presently not recommended to use NAATs as the sole method for the routine diagnosis of gonorrhoea. This is due to the fact that these methods have been unable to detect any antibiotic susceptibility patterns or give important epidemiological information about the heterogeneous strain populations that circulate in the societies. However, for screening (sub)populations or for use in developing countries with problems concerning transportation of viable organisms in specimens or culturing, the NAATs may be more suitable.

2.2.7b: Storage of the *N. gonorrhoeae*

2.2.7b.i: Storage in culture media

An isolate of *N. gonorrhoeae* usually survives no longer than 48 hours in culture, although some isolates may survive for 72 to 96 hours. Isolates should be subcultured every 18-24 hours to maintain maximum viability. An 18-24 hours culture should be used to inoculate any diagnostic test that requires viable organisms (Knapp and Rice, 1995).

2.2.7b.ii: Frozen storage

Long-term storage of gonococcal isolates is achieved with a -20^{0}C/-80^{0}C freezer, liquid nitrogen (-196^{0}C), or freeze-drying. To store frozen isolates, dense suspensions of 18-24-hours pure cultures are prepared in Glycerol broth

(Tryptic Soy broth containing 20% Glycerine). Frozen preparations of gonococcal isolates should not be repeatedly thawed and refrozen, because as many as 99% of the cells in a suspension may be destroyed during the freezing and thawing of the preparations. The loss of viability of gonococci in frozen storage is due to physical destruction (shearing) of cells by crystals of the suspending medium that forms during the freezing and thawing processes. The loss of viability during freezing may be minimized by flash freezing the specimen in an Acetone or Alcohol bath containing dry ice. Some isolates may survive storage at -20^{0}C for up to 2 weeks. The use of freezing media containing Glycerine results in a softer product from which a sample may be taken with a sterile bacterial loop without thawing the preparation (Knapp and Rice, 1995).

2.2.7b.iii: Lyophilization of N. gonorrhoeae

This is a freeze-drying storage mechanism of the organism. To prepare lyophilates, 18-24-hours cultures of the isolates are suspended in special lyophilization media and distributed in small aliquots (usually 0.25 to 0.5 ml) in lyophilization ampoules. Skimmed milk is not recommended as a suspending medium for the lyophilization of *N. gonorrhoeae* (and other Neisseria species), because fatty acids in the milk may be toxic for some organisms and it is impossible to determine the density of the organism.

For the purpose of lyophilization, the suspensions of the isolates are frozen at -70^{0}C or in an Ethanol-dry ice bath and dried in a vacuum evaporator for 18-24 hours until the moisture has evaporated. The dried preparation should be powdery and if the preparation is syrupy, the vial should be discarded. One ampoule should be opened immediately to ascertain that the preparation is viable and pure, and to verify the identity of the organism and important characteristics, e.g., antimicrobial susceptibilities. Ampoules are best stored at 4-10^{0}C or -20^{0}C; storage of the ampoules at room temperature is not recommended. Oxygen may diffuse slowly into the ampoule through the thin

seal, particularly with thin-walled ampoules. Thus one ampoule should be opened every 1 to 2 years to confirm that the preparation is viable. If the preparation is not viable after 48 hours of inoculation, new ampoules must be prepared (Knapp and Rice, 1995).

2.2.7c: Antimicrobial Susceptibility tests (AST)

2.2.7c.i: Disk diffusion method of AST

In the disk-diffusion susceptibility tests, disks containing known amounts of an antimicrobial agent are placed over the surface of an agar plate of non-selective medium that has been inoculated with a suspension of a strain of *N. gonorrhoeae* to give a confluent lawn of growth. The antimicrobial agent diffuses into the medium, causing a zone of inhibition of growth of the strain around the disk corresponding to the susceptibility of the organism to the antibiotic. Interpretative inhibition zone diameters have been established for susceptibility test results to permit classification of the *N. gonorrhoeae* isolate as being susceptible, intermediate (or exhibiting decreased susceptibility) or resistant to an antimicrobial agent.

Agar dilution method

Susceptibility testing by agar dilution methods are used to determine the minimum inhibitory concentration (MIC) usually expressed in micrograms per milliliter of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are usually tested at $\log_2$ (two-fold) serial dilutions, and the lowest concentration that inhibits visible growth of an organism is recorded as the MIC (Sahm and Washington II, 1991).

The concentration range used may vary with the drug, organism identification or site of infection. Ranges should include concentrations that allow determinations of the interpretive categories (i.e. susceptible, moderately susceptible or intermediate and resistant) and also include the acceptable ranges for quality control reference strains. For antibiotics such as penicillin and

tetracycline, wide variations exist in susceptibility patterns of gonococcal isolates from different countries. To reduce the number of concentrations that should be tested, an approximation of the local susceptibility patterns for such antibiotics is required. Where appropriate, the concentrations tested should include the critical concentrations at which significant treatment failures may be expected, when using recommended dosages. Generally, it is more relevant and important to know the upper rather than the lower limits of the MIC values (van Dyck *et al.*, 1989).

Any drug available in powder form may be used for MIC determination. Antibiotic powders of reagent grade yielding around 100% purity should be used and the concentration used must be based on the activity or potency (active ingredients per mg) of the antibiotic as specified by the manufacturer. The stock solution of the antibiotic to be tested should be prepared first and the necessary serial dilutions should be prepared diluting the stock solution. The stock solutions should be prepared by dissolving the antibiotic in appropriate solvent and sterilized by membrane filtration (0.22μm filter). The stocks may be stored in aliquots in tightly sealed vials at -20^{0}C or below for up to six months. The stock solutions should be used immediately when thawed and they should never be re-frozen for later use (van Dyck *et al.*, 1989).

Two types of media have been recommended for *N. gonorrhoeae* for preparation of agar dilution plates: (1) GC agar base with defined growth supplement (e.g. Isovitalex, Oxoid, UK); and (2) Diagnostic sensitivity test (DST) agar (Oxoid, UK) with growth supplement. Different concentrations of the antibiotic should be prepared from stock solution and solidified media plates should be stored in sealed plastic bags at 4^{0}C for up to 1 week. However, for Penicillin, which is less stable, the plates are recommended to use within 3 days (van Dyck *et al.*, 1989).

Variations in inoculum size may substantially affect MIC determinations; therefore, careful preparation is required to obtain accurate results. The recommended final inoculum for agar dilution is 10^4 CFU per spot.

For interpretation and reporting of results, a single colony or a faint haze left by the initial inoculum should not be regarded as growth. If two or more colonies persist at antimicrobial concentrations beyond an otherwise obvious endpoint or if there is no growth at lower concentrations but growth at higher concentrations, the isolate should be subcultured to confirm purity and the test should be repeated (NCCLS, 1995).

E-test

The E-test is performed using a procedure almost similar to that described for disk-diffusion method. The E-test is a new technique for quantitative antimicrobial susceptibility testing using strips containing a known gradient of an antimicrobial agent and calibrated to give results as MICs of the agents (Baher *et al.*, 1991). E-test strips are placed on the surface of a 150-mm plate (containing non-selective medium inoculated as in the disk diffusion method) in a radial pattern with the lowest concentration of the agent toward the centre of the plate and the highest concentration of the agent toward the edge of the plate (Yeung *et al.*, 1993).

The E-test has been compared with different recommended gold standard reference methods for antimicrobial susceptibility determinations with panels of Gram-positive and Gram-negative microorganisms, including the fastidious organisms and found E-test as an attractive alternative to the agar dilution method for Gonococcal AST (Yeung *et al.*, 1993). Because of the difficulties with agar dilution method, E-test is recommended by many authors as a simpler, reproducible method to determine MICs for gonococcal isolates.

2.2.7d: Serotyping and serovar determination of the *N. gonorrhoeae*

The Pharmacia (Ph) panel of serological classification of *N. gonorrhoeae* include monoclonal coagglutination reagents consisting of five WI (corresponding to protein IA of *N. gonorrhoeae*) reagents of Ar, Ao, As, At and Av as well as nine WII/WIII (corresponding to protein IB of *N. gonorrhoeae*) of Br, Bo, Bp, By, Bu, Bv, Bs, Bt, and Bx. A serovar of *N. gonorrhoeae* was defined as the pattern of reactivity of a test strain with a given set of monoclonal antibodies specific for either the WI (protein IA) or WII/WIII (protein IB) serogroup. Each serovar was, therefore, depicted by an upper case letter "A" if it belonged to serogroup WI (protein A) and a "B" for WII/WIII (protein IB) isolates and then followed by lower case letters representing positive reactions with the corresponding coagglutination reagents (Coghill and Young, 1987).

2.2.7e: Identification of plasmids of the *N. gonorrhoeae*

Large and small plasmid DNAs of the *N. gonorrhoeae* have been conventionally extracted by alkaline lysis method (Birnboim and Doly, 1979; Birnboim, 1983; Bhalla *et al.*, 1998; Bhuiyan *et al.*, 1999). The method is simple enough to permit the analysis of plasmid DNA by gel electrophoresis. The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Bacteria are lysed by treatment with a solution containing sodium dodecyl sulfate (SDS) and NaOH (SDS denatures bacterial proteins, and NaOH denatures chromosomal and plasmid DNA). Adequate pH control is accomplished without using a pH meter. Upon neutralization with potassium acetate, most of the reannealed chromosomal DNA and bacterial proteins precipitate (as does the SDS, which forms a complex with potassium) and are removed by centrifugation. The reanneal plasmid DNA from the supernatant is then concentrated by ethanol precipitation (Ausubel *et al.*, 1995b).

Another alternative to extract plasmid DNA is the boiling miniprep protocol, by which bacteria that contain plasmid DNA are broken open by treatment with lysozyme, Triton (a nonionic detergent) and heat. The chromosomal DNA remains attached to the bacterial membrane and is pelleted to the bottom of a centrifuge tube during a brief spin. Plasmid DNA is precipitated from the supernatant with isopropanol (Holmes and Quigley, 1981). This procedure is recommended for preparing small amounts of plasmid DNA from 1 to 24 cultures. It is extremely quick, but the quantity of DNA produced is lower than that from the alkaline lysis miniprep (Ausubel *et al.*, 1995a).

Soon primers were developed to identify plasmids in *N. gonorrhoeae* applying the PCR (polymerase chain reaction) methods. Among the PCR protocols, detection of the 25.4 MDa Tet-M plasmids for Tetracycline-resistance plasmids (TRNG plasmids) (Xia *et al.,* 1995) and assay for discriminating β-lactamase-producing plasmids (PPNG plasmids) (Dillon *et al.*, 1999) are mention worthy. In the TRNG plasmids PCR protocol, oligonucleotide primers were designed to amplify from within the *tet-M* gene through the downstream region. One oligonucleotide primer RM4 (5'-CCAAATCCTTTCTGGGCT-3') was from nucleotide 3292 to 3309 in the *Ureaplasma urealyticum tet-M* gene (Genbank access no. U08812) and the second oligonucleotide primer G1 (5'-ATCACTCACAGTTAAT-3') was within sequences shared by the 24.5 MDa Gonococcal plasmid (Xia *et al.,* 1995).

A PCR assay was also developed for the β-lactamase-producing epidemic plasmids (viz., Asia, Africa and Toronto types with corresponding Genbank accession numbers U20374, U20375, and U20419, respectively) after aligning primary DNA sequences from Genbank and using manual alignments coupled with the Primer Designer Computer program (Scientific and Education Software). The DNA sequences for the primers developed were: JDA (5′-TACTCAATCGGTAATTGGCTTC-3′) and JDB (5′-CCATATCACCGTCGGTACTG-3′). Based on the DNA sequence of the

gonococcal PPNG plasmids, the predicted sizes of the amplicons for strains containing Asia, Africa or Toronto/ Rio-type plasmids were 4.9, 3.1 and 2.6 kb, respectively (Dillon *et al.*, 1999).

Plasmids can be maintained for a short period (up to 1 month) in bacterial strains simply by growing on selective plates and storing at 4^0C. For permanent storage, bacteria harbouring the plasmid should be grown to saturation in the presence of the appropriate selective agent. An equal volume (1-2 ml) of bacteria should be added to sterile 100% glycerol or a DMSO-based solution and frozen at -70^0C in sterile vials. Cells taken from storage should again be grown on a selective plate and the plasmid DNA should be checked by restriction analysis (Ausubel *et al.*, 1995b).

Plasmid DNA can be stored in TE buffer at 4^0C for several weeks or preserved for several years by storing at -20^0C or -70^0C. Most investigators prefer to store plasmids as frozen DNA, due to the widely held belief that plasmids stored in bacteria are sometimes lost, are rearranged, or accumulate insertion sequences and transposons during storage or on revival (Ausubel *et al.*, 1995b).

2.2.7f: Genotyping of the *N. gonorrhoeae* by NG-MAST (Martin *et al.*, 2004)

For preparation of *N. gonorrhoeae* DNA, bacterial suspensions are made in phosphate buffered saline (PBS) (pH 7.3) and pelleted by centrifugation at 2000 rpm for 5 minutes, washed once, resuspended in PBS and boiled for 5 minutes. The lysate is centrifuged again at 2000 rpm for 5 minutes and the supernatant stored at -20^0C for next use in sequencing the internal fragments of *por* and *tbpB*.

The PCR amplification of *por* gene fragment was performed in reaction volumes of 50 µl containing 50 pmol of each primer, 1x buffer, 2.5 Units of Taq polymerase, 2µl of DNA lysate, 0.2 mmol/l each dNTP and water. The

PCR cycle involved an initial denaturation of 4 minutes at 95^0C, followed by 25 cycles of 30 seconds at 95^0C, 30 seconds at 58^0C for *por* and at 62^0C for *tbpB*, and 1 minute at 72^0C, followed by a final extension of 10 minutes at 72^0C and cooling to 4^0C. The PCR amplification of the *tbpB* gene fragment was performed with the *tbpB* primers by use of the same method, but with an annealing temperature of 69^0C. The amplicon for *por* was 737 bp, and for *tbpB* was 589 bp (Martin *et al.*, 2004).

The amplified DNA fragments were precipitated with 20% polyethylene glycol 8000 and 2.5 mol/l sodium chloride, washed twice in 70% ethanol, dried and resuspended in sterile water. The DNA fragments are sequenced on each strand with the primers used in initial PCR amplification by use of BigDye terminator cycle sequencing kit and applied to a DNA sequencer. The trace files from forward and reverse sequencing reactions are analyzed and edited by use of Sequence navigator software and trimmed to the correct length.

For *por*, a sequence of 490 bp was used to define alleles starting at a conserved sequence of TTGAA and for *tbpB*, a sequence of 390 bp is selected for defining alleles starting at a conserved region CGTCTGAA. The edited and trimmed *por* and *tbpB* sequences were initially compared with each other using a non-redundant database available at http://www.mlst.net and each different sequence at *por* and *tbpB* was assigned a new allele number in the order that the alleles were discovered. A 2-locus allelic profile was assigned to each isolate by use of the assigned allele numbers at *por* and *tbpB* (e.g., 44-56 has the *por*-44 allele combined with the *tbpB*-56 allele) and each allelic profile was assigned as a sequence type (ST), to provide a convenient descriptor for the strain.

The sequences of the known alleles at *por* and *tbpB* and all known STs were then entered into a publicly accessible database on the NG-MAST web site available at http://www.ng-mast.net. The edited sequences of *por* and *tbpB*

from new isolates were subsequently assigned as either known alleles or new alleles and their STs were assigned by use of the interrogation software available at the ng-mast web site.

Chapter 3: Materials and Methods

3.1: STUDY PERIOD: July, 2001 to December, 2003 and November, 2006 to July, 2009.

3.2: STUDY SITES AND LABORATORIES

3.2.1 Sylhet:

3.2.1a: Department of Microbiology, Sylhet MAG Osmani Medical College (SOMC), Sylhet. The SOMC is one of the government-owned post-graduate Medical Colleges, located at Sylhet City in Bangladesh (Appendices XIII and XIV); and

3.2.1b: The SOMC study site includes: (i) skin and venereal diseases (VD) outpatients Department (OPD) of SOMC Hospital (SOMCH), Sylhet for selection of the male-OPD cases; as well as (ii) laboratories at the Department of Microbiology, SOMC for data management, specimens collection and processing; and (iii) the medical centre for the MSM (male sex for male) of an non-government organization Bandhu Social Welfare Society (BSWS) at Sylhet City for data and specimen collection for the MSM cases.

3.2.2 Dhaka:

3.2.2a: Department of Microbiology, National Institute of Preventive and Social Medicine (NIPSOM), Mohakhali, Dhaka for data management.

3.2.2b: Laboratories (listed on next page) under the Laboratory Sciences Division (LSD), International Center for Diarrhoeal Diseases Research in Bangladesh (ICDDR,B), Mohakhali, Dhaka (www.icddrb.org) for characterization of the *Neisseria gonorrhoeae* isolates.

(i) Former RTI (reproductive tract infection) / STI (sexually transmitted infection) Laboratory (later converted into Food Microbiology Laboratory); and

(ii) Molecular and Serodiagnostic Laboratory of the Clinical Laboratory Services.

3.3: STUDY POPULATION

A total of 1025 male cases in two groups of populations including male-OPD (n=371), SOMCH and MSM (n=654) were enrolled for sociodemographic characteristics with the inclusion and exclusion criteria described below.

In addition, few predetermined characteristics of 441 individuals with high-risk sexual behaviour were included from which 441 *N. gonorrhoeae* isolates were included for characterization in the present study. These individuals were: (i) floating female commercial sex workers based in Dhaka (CSW-floating) (n=322); (ii) brothel-based female CSWs from Gualanda, Faridpur (n=20); (iii) male-truckers based in Dhaka (n=35); and (iv) male-OPD, DMCH (Dhaka Medical College Hospital) based in Dhaka (n=64).

3.3.1: Inclusion criteria

3.3.1a: Inclusion criteria for the male-OPD, SOMCH:

The symptomatic male individual, attending the Skin VD OPD of SOMCH with complaints suggestive of genital gonococcal infections like urethral discharge and/or dysuria.

3.3.1b: Inclusion criteria for the MSMs:

The MSM individual, with or without anogenital symptoms, attending the medical centre for the MSMs.

3.3.2: Exclusion criteria (in both groups)

Individuals who had taken any antibiotic during previous one week of enrollment were not included in the study.

3.4: ETHICAL PERMISSION

As the study was funded and supported by the then RTI /STI Laboratory, LSD, ICDDR,B, the ethical permission was obtained from the Ethical Review Committee of the ICDDR,B through Dr. Motiur Rahman, the then Scientist and Head, RTI/STI Laboratory. (Appendix V)

3.5: WRITTEN INFORMED CONSENT FROM THE CASES

Each of the enrolled cases was first informed about the study and the procedure of consent giving. The cases, who could read, were then offered the consent form printed in Bangla (the national language) (Appendix VI) and the form was read out to the illiterates to make them understand the contents. Both the literate (educated) and illiterate enrolled persons made their unbiased consents by putting signature or thumb impression over consent form.

3.6: DATA COLLECTION BY INTERVIEW

Sociodemographic information related to gonococcal infection like age, education status, level of education, occupation, marital status, pre-marital sex, extra-marital sex, sexual exposure with female CSWs and condom use during sex of the individuals giving consent was collected by face-to-face interview in a pre-tested questionnaire (Appendix VII) and the data were entered into a statistical software (SPSS version 16.0) file for analysis.

3.7: SPECIMEN COLLECTION

Specimens of urethral discharge were collected from male-OPD cases and urethral and/or anal discharges were collected from the MSM cases. Specimens were collected in sterile cotton swabs and for each of the specimens, two such swabs were collected: one for culture and the other for microscopic identification of *N. gonorrhoeae*.

3.7a: Specimen collection from male-OPD population

After giving consent and interview for data collection, specimens were collected from each of the enrolled cases.

Urethral discharge from the male-OPD cases were collected in the sample collection room provided with good light, ventilation and sufficient privacy within the "Research Laboratory" at the Department of Microbiology, SOMC. The individuals were requested to hold urine for at least 1 hour before specimen collection, because micturition is supposed to wash out the existing exudates at the urethra, if any (Knapp and Rice, 1995).

Purulent urethral discharge was expressed by stripping the penis anteriorly and specimens from cases with no visible urethral discharge, the tips of the sterile cotton swabs were introduced into anterior urethra for about 2-3 cm, rolled in 360 degrees to ensure adequate collection of any discharge.

One of the collected swabs was inoculated immediately over the culture plate and the other was smeared over a clean glass slide for Gram's staining. After proper application, the swabs were placed into a discard basket and finally incinerated.

3.7b: Specimen collection from MSM population

After giving consent and interview for sociodemographic data, specimens were collected from each of the enrolled MSM cases.

Urethral specimens were collected from each of the MSM cases and in addition, anorectal swabs were collected from the MSMs having anal complaints. All specimens from the MSMs were collected at a collection room within a medical centre for the MSMs by the BSWS at Mirabazar, Sylhet City.

Urethral specimens from the MSMs were collected similarly as described for the male-OPD cases. For collection of anorectal specimens, the MSM individuals were requested to lie over the bed on the side with legs flexed at hips and knees. A sterile cotton swab was inserted into the anal canal for about 3-5 cm, rotated for few seconds to collect exudates (if any) from anal crypts just inside the anal ring (van Dyck *et al.*, 1989; Winn Jr. *et al.*, 2006) and two such anorectal swabs were collected.

3.8: MICROSCOPIC EXAMINATION OF THE SWAB SMEARS

3.8a: Microscopy to identify Neisseria gonorrhoeae

For this purpose, one of the urethral as well as anorectal swab specimens was smeared over a properly labeled clean glass slide. The smear was air dried, fixed with methanol by applying few drops over the dried smear, and stained by Gram's staining procedure.

The Gram-stained smears were then examined under a Microscope to see Gram-negative intracellular diplococci within pus cells / polymorphonuclear neutrophils (PMNs) (Figure 3.1) and the results were recorded properly for each of the cases.

3.8b: Microscopy to identify cases of urethritis

Microscopic finding of the Gram-stained urethral smear showing $\geq$ 5 PMNs per high power field (HPF) was considered as a case of urethritis (Haddow *et al.*, 2004; Iser *et al.*, 2005; Vesic *et al.*, 2007).

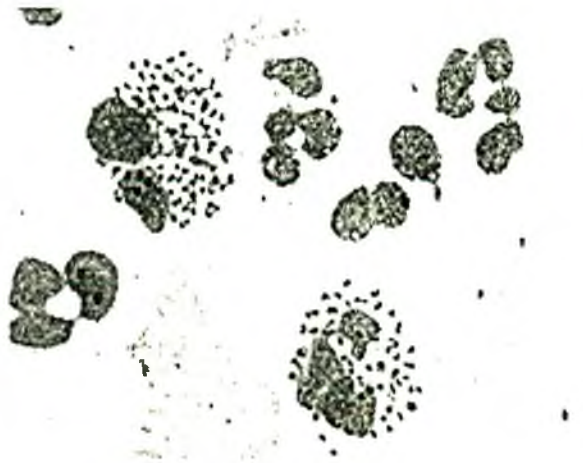


Figure 3.1: Gram-stained smear of urethral swab showing Gram-negative intracellular diplococci and multilobed nuclei of the polymorphs.
(Magnification: 100x15)

3.9: CULTURE OF THE SPECIMENS FOR *N. GONORRHOEAE*

3.9a: Media preparation

Modified Thayer Martin medium (MTM) with GC agar base (Oxoid, UK), saponin-lysed sheep blood, growth supplement and antibiotic supplement was prepared following standard protocols. (WHO, 1989) (Appendix II.1) The plates of the medium were stored in a refrigerator within sealed plastic bags labeled properly for a maximum of 4 weeks. Before use, the plates were taken out and warmed in the incubator at 37^0C for 30 minutes.

3.9b: Inoculation of specimens

For the male-OPD cases, collection room was within the laboratory and specimens were inoculated directly onto the culture plates immediately after collection. For the MSMs, the properly warmed MTM plates were carried to the collection centre for MSMs for inoculation of specimens at the site of collection and kept into CO_2 extinction jars (Appendix IV.1) for 3-5 hours before transferring into the incubator (Knapp and Rice, 1995) at Department of Microbiology, SOMC.

3.9c: Incubation

The inoculated plates were kept into a CO_2 extinction jar with a burning white, non-scented candle (because the vapour from coloured, scented candles may be toxic) (Knapp and Rice, 1995) and lid of the jar was closed immediately. The candle inside the jar was re-lighted and the lid kept closed each time new inoculated plates were added (Knapp and Rice, 1995). After completing the inoculation of all collected specimens in a day, the jar was placed inside the incubator at 37^0C for next 18-24 hours.

3.9d: Growth observation and identification of N. gonorrhoeae

After proper incubation, the plates were checked for any growth of colonies. Colonies, if any over the plates, were identified as those of *N. gonorrhoeae* if satisfied the standard criteria (van Dyck *et al.*, 1989). (Table 3.1)

Table 3.1: Identifying criteria for *N. gonorrhoeae* grown on culture media

Test procedure	*Identifying criteria for N. gonorrhoeae*
Colony characteristics observation	Small, glistening, raised, and colourless dew-drop colonies
Gram's staining[1]	Gram-negative diplococci
Superoxol test[2]	Positive
Oxidase test[3]	Positive
Sugar fermentation test[4]	Ferments Glucose only

[1]Grams's staining standard procedure (Cheesbrough, 2000); [2]Superoxol test (Saginur *et al.*, 1982): procedure described in Appendix III.1; [3]Oxidase test (van Dyck *et al.*, 1989): procedure described in Appendix III.2; [4]Sugar fermentation test (Brown, 1974): procedure described in Appendix III.3.

3.10: STORAGE OF THE *N. GONORRHOEAE* ISOLATES

For the purpose of storage, a heavy inoculum of the identified *N. gonorrhoeae* isolate was made by subculture over a MTM plate. After proper incubation for 18-24 hours with CO_2, the heavy growth of the isolate was scrapped off using a sterile cotton swab and mixed well into a 20% glycerol broth (Appendix II.3) vial labeled with *N. gonorrhoeae* isolate identification (ID) number, date of storage, and date of isolation of the organism.

The suspension prepared for each of the *N. gonorrhoeae* isolate was then kept into a -20^{0}C freezer in the Department of Microbiology, SOMC for next use.

3.11: TRANSFER OF THE *N. GONORRHOEAE* ISOLATES TO ICDDR,B, DHAKA

A subcultured replica of each of the *N. gonorrhoeae* isolates in Sylhet was transferred to the then RTI/ STI Laboratory, ICDDR,B, Dhaka for further study of characterization of the isolates. For this purpose, each *N. gonorrhoeae* isolate was inoculated over a MTM plate, incubated at 37^{0}C in a CO_2 extinction jar for 6-8 hours and transported overnight to the ICDDR,B. At RTI/ STI Laboratory of the ICDDR,B, each of the transferred isolates was stored in 20% glycerol broth at -80^{0}C after prior identification and subculture properly.

3.12: IDENTIFICATION OF THE CASES OF GONOCOCCAL INFECTION

3.12a: Case definition

The cases of male individuals showing following laboratory criteria were considered as cases of gonococcal infection (Jephcott, 1997; Velicko and Unemo, 2009).

(i) Isolation of typical Gram-negative, oxidase positive diplococci (presumptive *N. gonorrhoeae*) in urethral or anogenital specimen by culture on MTM medium; or

(ii) Observation of Gram-negative intracellular diplococci within PMNs (polymorphonuclear neutrophils) in the Gram-stained smear of urethral / anal swab; or

(iii) Both.

3.13: MEASURES OF VALIDITY OF MICROSCOPY FOR IDENTIFYING GONOCOCCAL INFECTION

Measures of validity like sensitivity, specificity, positive predictive value, negative predictive value and predictive accuracy of microscopy for identifying gonococcal infection were calculated using the two-by-two formula (Hanrahan and Madupu, 1994) where a= true positive, b= false positive, c= false negative and d= true negative values of microscopy and comparing with culture results as "gold standard". (Table 3.2)

Table 3.2: The two-by-two table for calculating measures of validity

Test result	*Infection*	*No infection*	*Total*
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	a + b+ c+ d

Sensitivity= a/ a + b

Specificity= d/ b + d

Positive predictive value= a/ a + b

Negative predictive value= d/ c + d

Predictive accuracy= a + d/ a+ b+ c+ d

3.14: FREE TREATMENT FOR THE CASES OF GONOCOCCAL INFECTION

The identified cases of gonococcal infection were provided with free treatment by a single dose of cap. cefixime (400 mg) (cap. Roxim®, Fisons, Bangladesh) on prescription by the Venereologists (Appendix VIII) following recommendation by the Centers for Disease Control and Prevention (CDC) treatment guideline (CDC, 1993).

3.15: SELECTION OF THE *N. GONORRHOEAE* ISOLATES FOR CHARACTERIZATION

A total of 495 *N. gonorrhoeae* isolates were included for characterization for the phenotypes like antimicrobial resistance, serotype and serovar, and plasmid infection as well as for genotype (sequence type, ST). The selected *N. gonorrhoeae* were isolated from six different high-risk populations in three areas of Bangladesh. (Table 3.3)

3.15a: Selection of the N. gonorrhoeae

(i) A total of 54 of the *N. gonorrhoeae* strains isolated in 2003 from Sylhet; and

(ii) Another 441 of the *N. gonorrhoeae* isolated during 2003-2006 were supplied by the then RTI/ STI Laboratory.

3.16: SUBCULTURE OF THE *N. GONORRHOEAE* ISOLATES

Selected *N. gonorrhoeae* strains were sub-cultured from the master stocks on pre-warmed GC agar medium with 1% growth supplement (Appendix II.2) and incubated overnight in a CO_2 incubator (Appendix IV.3) for next investigations.

Table 3.3: Source populations and sites of isolation of the *N. gonorrhoeae* selected for characterization (n=495)

Source population	*Site of Isolation of N. gonorrhoeae*	*Number of N. gonorrhoeae isolates*	*Percent*
Female CSW[¶]-floating	Dhaka	322	65.1
Male-OPD[§], DMCH[¶§]	Dhaka	64	12.9
Male-Truckers	Dhaka	35	7.1
Male-OPD, SOMCH[¶¶]	Sylhet	46	9.3
MSM	Sylhet	8	1.6
Female CSWs- Brothel based	Gualanda, Faridpur	20	4.0
	Total	495	100.0

[¶]CSW- Commercial Sex Worker, [§]OPD- Out-patient's Department, [¶§]DMCH- Dhaka Medical College Hospital, [¶¶]SOMCH- Sylhet MAG Osmani Medical College Hospital, MSM- Male individuals having Sex with Male

Note: The *N. gonorrhoeae* isolated at sites other than Sylhet were isolated and supplied for characterization by the ICDDR,B

3.17: RE-CONFIRMATION OF THE *N. GONORRHOEAE* ISOLATES

Subcultured isolates showing growth over defined media plates after proper incubation was re-confirmed as *N. gonorrhoeae* following the standard criteria as gram-negative diplococci, oxidase positive and superoxol positive, as described previously (van Dyck *et al.*, 1989; Knapp and Rice, 1995).

3.18: NEW PERSONAL STOCKS MADE

The fresh heavy growth from a secondary subculture plate of MTM of the selected *N. gonorrhoeae* isolates were preserved in vials of 20% glycerol broth (Appendix II.3) following standard protocol (van Dyck *et al.*, 1989) for personal works and these stocks were identified as 'perstock' and kept preserved in -80 degrees celsius for the study of characterization.

3.19: PAPER ACIDOMETRIC TEST FOR BETA-LACTAMASE

All selected strains (n=495) of *N. gonorrhoeae* were tested for β-lactamase (penicillinase) production (PPNG, penicillinase producing *N. gonorrhoeae*) by paper acidometric method using penicillin solution with bromocresol purple indicator (Appendix I.8) (van Dyck *et al.*, 1989).

3.19a: Test procedure

Blotting paper, labeled with the tested IDs (identification numbers) of the *N. gonorrhoeae,* was saturated with the penicillin solution with bromocresol purple in a petriplate. Fresh colonies from subculture of the organism were rubbed over blotting paper freshly soaked with penicillin solution.

A colour change from blue to yellow (usually was visible within 3-5 minutes) were considered as positive for PPNG, and negative tests remained unchanged. The results were recorded properly for interpretation.

3. 20: ANTIMICROBIAL SUSCEPTIBILITY TEST (AST) BY AGAR DILUTION MIC METHOD

All of the *N. gonorrhoeae* isolates were subjected to AST using agar dilution methods for determination of minimum inhibitory concentrations (MICs) for six antimicrobial agents, namely penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone and cefixime.

Susceptibility result of each of the isolates to see whether it was "susceptible", "intermediate" or "resistant" was determined following the standardized criteria/ breakpoint described previously. (Table 3.4)

3. 20a: Antibiotic solutions for agar dilution MIC test

The reagent grade antibiotic powders of the selected agents were procured from Upjohn, USA and stock solutions of the antibiotics were prepared following standard protocols (van Dyck *et al.*, 1989; NCCLS, 1995, NCCLS, 2001) (Appendices I.6) and kept at -20^{0}C for next use. All of the *N. gonorrhoeae* isolates were tested for different sets of two-fold concentrations of the antimicrobial agents prepared following a standard scheme of dilutions. (Table 3.5, Table 3.6)

The concentrations of the antimicrobial agents tested against each *N. gonorrhoeae* isolate were considered by taking into account the lowest and the highest available data. Solvents and diluents for each of the antimicrobial agents were used following standard recommendations (van Dyck *et al.*, 1989; NCCLS, 1995; NCCLS, 2001). (Table 3.6)

Table 3.4: Minimum Inhibitory Concentration (MIC) interpretive standards criteria (µg/ml) for *N. gonorrhoeae* (NCCLS, 2001)

Antimicrobial agent	MIC (µg/ml)		
	Resistant	Intermediate	Sensitive
Penicillin	≥ 2.0	0.125-1.0	≤ 0.06
Tetracycline	≥ 2.0	0.5-1.0	≤ 0.25
Spectinomycin	≥ 128.0	64.0	≤ 32.0
Ceftriaxone	-*	-*	≤ 0.25
Cefixime	-*	-*	≤ 0.25
Ciprofloxacin	≥ 1.0	0.125-0.5	≤ 0.06

* Criteria not available

Table 3.5: Diluents, solvents and range of concentrations used for the antimicrobial agents in agar dilution AST

Antimicrobial agent	*Origin*	*Solvent*	*Diluent*	*Range of concentrations*
Penicillin G	Upjohn, USA	PBS*	Water	0.03-128.0 µg/ml
Tetracycline	Upjohn, USA	Water	Water	0.03-128 µg/ml
Ciprofloxacin	Upjohn, USA	Water	Water	0.03-128.0 µg/ml
Spectinomycin	Upjohn, USA	Water	Water	16.0-128.0 µg/ml
Ceftriaxone	Upjohn, USA	PBS*	Water	0.004-2.0 µg/ml
Cefixime	Upjohn, USA	PBS*	Water	0.004-2.0 µg/ml

*PBS- 0.1 M Phosphate buffer solution (PBS), pH 6.5 (Appendix I.5)

Table 3.6: Scheme for preparing dilutions of antimicrobial agents tested for determination of MIC of the *N. gonorrhoeae* isolates (adapted from NCCLS, 2001)

Step	*Primary conc.(µg/ml)*	*Volume (ml)*	*Distilled Water (ml)*	*Intermediate conc. (µg/ml)*	*Dilution agar media preparation*		
					Antibiotic soln (ml)	*Media (ml)*	*Final conc. in media (µg/ml)*
1	5120	-	-	5120	2	18	512
2	5120	2	2	2560	2	18	256
3	2560	2	2	1280	2	18	128
4	1280	2	2	640	2	18	64
4	640	2	2	320	2	18	32
5	320	2	2	160	2	18	16
6	160	2	2	80	2	18	8
7	80	2	2	40	2	18	4
8	40	2	2	20	2	18	2
9	20	2	2	10	2	18	1
10	10	2	2	5	2	18	0.5
11	5	2	2	2.5	2	18	0.25
12	2.5	2	2	1.25	2	18	0.125
13	1.25	2	2	0.625	2	18	0.06
14	0.625	2	2	0.3125	2	18	0.03
15	0.3125	2	2	0.156	2	18	0.016
16	0.156	2	2	0.08	2	18	0.008
17	0.08	2	2	0.04	2	18	0.004

3. 20b: Media preparation for agar dilution AST

For the purpose of agar dilution AST, plates of GC agar medium with 1% defined growth supplement (Appendix II.2) incorporated with required concentrations of the selected antibiotics (Appendices I.6) were prepared, and kept into refrigerators until next use within 1 week.

3. 20c: Inoculation for Agar dilution AST for MIC

The test antibiotic incorporated media were taken out from the refrigerator and kept into the drier to make them at room temperature. Suspensions of the *N. gonorrhoeae* isolates (usually 6-12 isolates were taken at an occasion) were made into 0.5 ml sterile PBS/ Tryptic Soy broth in an equivalent to 0.5 McFarland standard (Appendix I.7) giving a concentration of about 10^8 colony forming units (CFU) per ml. The suspension was homogenized carefully and diluted 1:10 to obtain 10^7CFU/ ml.

An amount of 100 µl of each of the test bacterial suspensions was dispensed into the corresponding cubicle of the multipoint inoculator. The cubicles were arranged properly in an ascending order of the isolate ID numbers. Probes of the inoculator were fitted rightly and the machine was started for single inoculations over a plate. As the multipoint inoculator (Appendix IV.4) delivered approximately 1µl, therefore, final concentration delivered over medium plate was approximately 10^4 CFU per inoculum.

The inocula were allowed to dry within the safety hood (Appendix IV.2), plates were then inverted and incubated in the CO_2 incubator at 37^0C for next 18-24 hours.

3. 20d: Reading susceptibility results of Agar dilution AST

The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of an antimicrobial agent yielding no growth. The MIC was expressed as µg/ml of the antibiotic.

3. 20e: Multi-drug Resistant (MDR) N. gonorrhoeae isolates

N. gonorrhoeae isolates showing resistance to penicillin, tetracycline and ciprofloxacin were considered as multi-drug resistant (MDR) isolates (Wang *et al.*, 2003).

3.21: SEROTYPE AND SEROVAR DETERMINATION

3.21a: N. gonorrhoeae isolates selection

Because of constraints in availability of reagents, 239 isolates of *N. gonorrhoeae* were selected randomly for serotyping and serovar determination. Randomization was done by selecting every alternate ID numbers among the total available isolates.

3.21b: Antigen preparation

The 18-24 hours colonies, grown on GC agar medium with 1% growth supplement (Isovitalex) (Appendix II.2), were first identified as *N. gonorrhoeae* by the standard identifying criteria as described previously. The colonies were harvested using a sterile cotton swab into 1.5 ml phosphate buffered solution (PBS) (Appendix I.5) at pH 7.2 in eppendorf tubes to make a smooth milky suspension of the organism. The suspension was then boiled for 10 minutes using floating racks and after boiling was allowed to cool at room temperature before testing. The prepared antigen suspension was stored at 4^0C for up to two weeks when could not be tested immediately.

3. 21c: Monoclonal antibody reagents

Serotype and serovar of each of the selected *N. gonorrhoeae* isolate was determined using Phadebact panel of monoclonal antibodies (Boule Diagnostics AB, Huddinge, Sweden) consisting of five *N. gonorrhoeae* protein IA-specific reagents (Ar, Ao, As, At and Av) (Figure 3.2) and nine *N. gonorrhoeae* protein IB-specific reagents (Br, Bo, Bp, By, Bu, Bv, Bs, Bt and Bx). (Figure 3.3) The monoclonal antibody reagents were kept in a refrigerator at 4-8^0C.

3. 21d: Test procedure

Monoclonal antibody reagents were taken out from the refrigerator and antigen suspension of the *N. gonorrhoeae* isolate to be tested were mixed well before testing. The test was carried out by adding 20µl of each of the reagents and

20µl of the test isolate on glass plates for agglutination. Plates were then rocked gently for 2 minutes and coagglutination reactions were read using a dark background. Control wells were run mixing 20µl of normal saline with each of the test isolates.

3. 21e: Scoring results

Agglutination reactions of each of the monoclonal antibody reagents with each test isolate was observed and was scored as negative when showed a smooth milky background and positive when showed a granular or clumping reaction with background clearing. (Figure 3.4)

3. 21f: Assigning serotype and serovars

Serotype of a strain of *N. gonorrhoeae* was defined as the type of reagent with which it reacted, for example when reacted with protein IA reagents only was considered as *N. gonorrhoeae* serotype A and when reacted with protein IB reagents only was considered as *N. gonorrhoeae* serotype B and when reacted with both protein IA and protein IB reagents was considered as *N. gonorrhoeae* mixed serotype.

A serovar of *N. gonorrhoeae* was assigned as the pattern of reactivity of the test isolate with a given set of monoclonal antibodies specific for protein IA or protein IB or both. (Coghill and Young, 1987) For example, a *N. gonorrhoeae* isolate reacting with protein IA set of "r", "s", and "t" was assigned as of serovar "Arst"

Figure 3.2: Phadebact panel of protein IA antisera reagents (Ao, Ar, As, At, & Av) (Source: Bactus AB, SE-14144 Huddinge, Sweden)

Figure 3.3: Phadebact panel of protein IB antisera reagents (Bo, Bp, Bs, Bt, Bu, Bv, Bx & By) (Source: Bactus AB, SE-14144 Huddinge, Sweden)

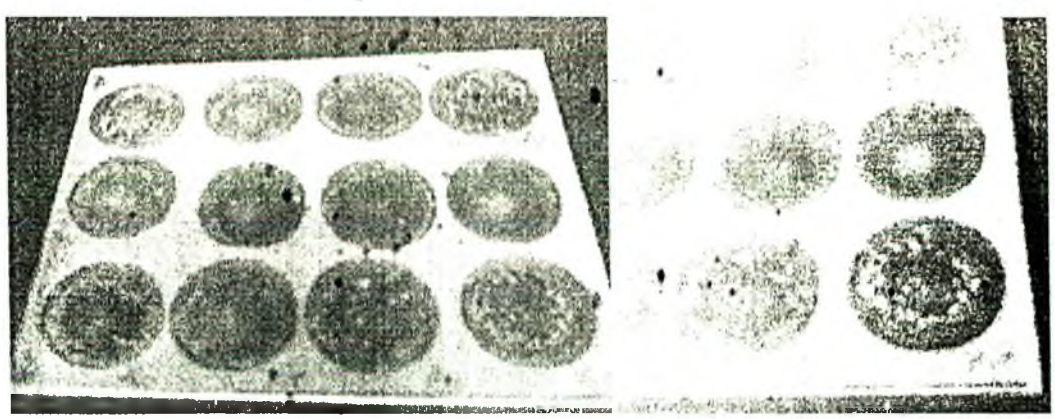


Figure 3.4 : Phadebact panel coagglutination test showing positive & negative results

3.22: DNA EXTRACTION BY BOILING (Ausubel *et al.*, 1995b)

One-fourth of an MTM medium plate was inoculated heavily for subculture with a selected strain of *N. gonorrhoeae* and incubated overnight with added CO_2. Growth of the organism was scrapped off with moistened sterile cotton swabs and suspended in 500 µl sterile 1x PBS (Appendix I.5) in a properly labeled eppendorf tube kept into a floating rack and boiled for 5 minutes.

The suspension in the eppendorf was cooled to room temperature after boiling and centrifuged at 13,000 rpm in a high speed ultracentrifuge (Centrifuge 5415D, Eppendorf, USA) for 2 minutes to prepare a clear supernatant fluid over the killed bacterial pellet.

An amount of 200 µl of the undisturbed aqueous supernatant was separated for each isolate in a separate eppendorf with proper labeling for plasmid profile and molecular typing. The tubes were then stored at -20^{0}C for next use.

3.23: POLYMERASE CHAIN REACTION (PCR) FOR PPNG PLASMIDS

3.23a: Selection of N. gonorrhoeae strains

A total of 261 *N. gonorrhoeae* isolates showing penicillin MIC ≥2.0 µg/ml (n=255) as well as those with penicillin MIC ≤ 2.0µg/ml, but showing PPNG-positive results by paper acidometric method (n=6) were selected for PPNG plasmid identification by PCR.

3.23b: Procedure of PCR for PPNG plasmids

Extracted DNA of the selected *N. gonorrhoeae* (usually 10 t0 15 strains in a batch) along with one known positive, and one known negative was thawed over ice and spinned at 13,000 rpm for 2 minutes.

Reaction mixture for PPNG PCR was prepared at a volume of 25 µl for each isolate with the recommended reagents according to the protocol described previously for detecting epidemic plasmids (Appendix I.9) (Dillon *et al.*, 1999) and taken into properly labeled PCR tubes. The DNA sequences of the PPNG-PCR primers were:

JDA 5'-TACTCAATCGGTAATTGGTTC-3' and

JDB 5'-CCATATCACCGTCGGTACTG-3'

One of the *N. gonorrhoeae* isolates, previously known to produce Africa type PPNG plasmid, was used as "positive" control and ddH_2O as "negative" control for amplification with each batch of PCR.

The PCR tubes were then loaded into the PCR machine (Appendix IV.5) for PPNG with the parameters of initial denaturation at 94^0C for 3.0 minutes, cycle denaturation at 94^0C for 15 seconds, annealing at 60^0C for 15 seconds, extension at 72^0C for 5 minutes for 30 cycles, and a final extension at 72^0C for 5 minutes. After completion of 30 cycles, the products were removed from the PCR machine, and subjected to gel-documentation.

3.23c: Electrophoresis of the PPNG PCR Products

Gels (0.8% agarose in 0.5x TBE stained with ethidium bromide) were prepared with a suitable comb. After solidification, the gel was placed over the gel tank (BioRad, Sub-cell GT Wide Mini, Italy) keeping the buffer slightly over the gel. The PCR products (3-4 µl) were mixed with loading dye (5-8 µl) and loaded into the gel wells with a molecular weight marker (1kb). The electrophoresis power was switched on at 55 volts for about 2 hours.

After an adequate transfer of the products, power was switched off and gel was placed over a transilluminator (Appendix IV.6). Images were captured with appropriate software and saved properly. The images were printed to see the

bands of the PPNG plasmids and the results shown on images were recorded properly.

The results of the PPNG bands were interpreted following molecular weight markers. Bands around 3 kbp were considered "Africa type" PPNG plasmids. For a few strains producing indefinable double bands, images were sent to Professor Jo-Anne R. Dillon for interpretation. (Appendix IX) (Professor Dillon is the principal investigator developing PPNG-PCR primers and protocol, available at: http://www.usask.ca/biology/dillon/).

3.24: POLYMERASE CHAIN REACTION (PCR) FOR TRNG PLASMIDS

3.24a: Selection of N. gonorrhoeae

A total of 396 *N. gonorrhoeae* isolates showing tetracycline MIC $\geq$ 8.0 µg/ml were subjected to TRNG plasmids identification by PCR method as recommended by the investigators (Xia *et al.*, 1995).

3.24b: Procedure of TRNG PCR

Extracted DNA of the selected *N. gonorrhoeae* isolates (usually 18 t0 22 strains in a batch) was thawed over ice and spinned at 13,000 rpm for 2 minutes. The reaction mixture for TRNG was prepared at a volume of 25 µl for each isolate with the recommended reagents (Xia *et al.*, 1995) (Appendix I.10) and taken into properly labeled PCR tubes. The DNA sequences of the oligonucleotide primers used were:

RM4 5'-CCAAATCCTTTCTGGGCT-3' and

G1 5'-ATCACTCACAGTTAAT-3'

N. gonorrhoeae isolate previously known to produce TRNG plasmid was used as "positive" control and ddH_2O as "negative" control for amplification with each batch of TRNG PCR.

The PCR tubes were then loaded into the PCR machine (Appendix IV.5) for TRNG with following parameters of initial denaturation at 95^0C for 5 minutes, cycle denaturation at 94^0C for 30 seconds, annealing at 60^0C for 30 seconds, extension at 72^0C for 3 minutes for 30 cycles and a final extension at 72^0C for 7 minutes. After completion of 30 cycles, the products were removed from the PCR machine and subjected to gel documentation. If gel documentation could not be done immediately, the PCR products were kept in a refrigerator for next use.

3.24c: Electrophoresis of the TRNG PCR Products

Gels (0.8% agarose in 0.5x TBE stained with ethidium bromide) were prepared with a suitable comb. After solidification, the gel was placed over the gel tank (BioRad, Model no. Sub-cell GT Wide Mini, Italy) keeping buffer slightly over the gel.

The PCR products (3-5 µl) were mixed with loading dye (5-8µl) and loaded into the gel wells with known positive and negative controls and a marker (100 bp DNA ladder, Invitrogen, USA). The electrophoresis power was switched on at 55 volts for 2 hours. After an adequate transfer of the products, power was switched off and gel was placed over a transilluminator. (Appendix IV.6) Images were captured with appropriate software, saved and printed to see the bands of the PPNG plasmids. Bands around 600 bp were considered "Dutch type" and around 1600 bp were considered the "American type" TRNG plasmids (Xia *et al.*, 1995).

3.25: CLASSIFICATION OF THE *N. GONORRHOEAE* ISOLATES BY RESISTANCE MECHANISM

For the purpose of analysis, the *N. gonorrhoeae* isolates were classified into six different groups according to the mechanism of resistance to penicillin and tetracycline. (Table 3.7)

Table 3.7: Types of *N. gonorrhoeae* isolates by mechanism of resistance to penicillin and tetracycline

Resistant groups of N. gonorrhoeae	*Criteria*	*Resistance type*
PPNG	*N. gonorrhoeae* isolates showing positive result by PPNG-PCR and negative or not applicable with TRNG-PCR	Plasmid-mediated resistance to penicillin
TRNG	*N. gonorrhoeae* isolates showing positive result by TRNG-PCR and negative or not applicable with PPNG-PCR	Plasmid-mediated resistance to tetracycline
PPNG-TRNG	*N. gonorrhoeae* isolates showing positive results with both PPNG-PCR and TRNG-PCR	Plasmid-mediated resistance to both penicillin and tetracycline
Non-PPNG	*N. gonorrhoeae* isolates with Penicillin MIC ≥2.0 µg/ml showing negative results by PPNG-PCR and not applicable to TRNG PCR	Chromosomally-mediated resistance to penicillin
Non-TRNG	*N. gonorrhoeae* isolates with tetracycline MIC ≥2.0 µg/ml showing negative results by TRNG-PCR and not applicable to PPNG PCR	Chromosomally-mediated resistance to tetracycline
Non-PPNG-non-TRNG	*N. gonorrhoeae* isolates with penicillin MIC ≥ 2.0 µg/ml showing negative result with PPNG-PCR and tetracycline MIC ≥ 2.0 µg/ml showing negative result with TRNG-PCR	Chromosomally-mediated resistance to both penicillin and tetracycline

3.26: GENOTYPING OF THE *N. GONORRHOEAE* BY NG-MAST

3.26a: Isolate selection

Due to constraint of funds and logistics, 30 of the multi-drug-resistant (MDR) *N. gonorrhoeae* isolated from floating (street-based) female CSWs were selected and analyzed for genotyping using NG-MAST as described previously (Martin *et al.*, 2004; Palmer *et al.*, 2005).

3.26b: Primary PCR of DNA extract for por/ tbpB gene

Polymerase chain reaction (PCR) were performed in 50µl reaction volumes and carried out using a MBS 0.2G Thermohybrid amplification machine. Primers were having following sequences:

por forward: 5′- CAA GAA GAC CTC GGC AA-3′

por reverse: 5′- CCG ACA ACC ACT TGG T-3′

tbpB forward: 5′- CGT TGT CGG CAG CGC GAA AAC -3′

tbpB reverse: 5′- TTC ATC GGT GCG CTC GCC TTG -3′

Each PCR contained 50 pmol of each primer, 1x PCR buffer (Qiagen), 2.5 U Taq Polymerase (Qiagen), 2µl of DNA lysate, 0.2 mmol/L of each dNTP (Applied Biosystems), and water to a volume of 50µl.

The PCR parameters were with an initial denaturation at 95^0C for 4 minutes, followed by 25 cycles of 95^0C for 30 seconds, 58^0C for 30 seconds for *por* and 62^0C for *tbpB*, 72^0C for 1 minute, followed by a final extension at 72^0C for 10 minutes and cooled to 4^0C and held. The primers were designed for amplified fragments of 737 bp for *por* gene and 589 bp for *tbpB*.

3.26c: Agarose gel electrophoresis of PCR products

The PCR products (5µl each) were run in an agarose gel electrophoresis (1% agarose stained with 2µl ethidium bromide) to check for the target amplified products and recorded properly using gel documentation system (Appendix IV.6).

3.26d: Column purification

The remaining amplified product (45µl) of either of the genes (*por* or *tbpB*) were carefully poured over filter of the sequencing columns (Qiagen/ Amplicon) using a micropipette. The column placed in an appropriate eppendorf was centrifuged at 5000 rpm for 12 minutes and the eppendorfs containing lysates were discarded.

The column was then placed upside down in another eppendorf and centrifuged again at 10,000 rpm for 3 minutes to get purified products into the eppendorfs. An amount of 5µl of 1x TE buffer was added to each purified product and calculated in a DNA calculator (Appendix IV.7) in 1:100 dilution for calculating the amount of DNA present. The column purified DNA was then diluted by PCR water to make 10-20 ng/µl appropriately if necessary.

3.26e: Cycle sequencing

The purified products were then amplified again in a concentration of 10-20 ng/µl with a reaction volume of 20µl containing Bigdye-4.0µl, one of the primers (either *por*-F, or *por*-R, or *tbpB*-F or *tbpB*-R)- 1µl (3.2 pmol), purified DNA- 1µl.

The program for cycle sequencing was as follows: Initial denaturation at 96^0C for 1 minute followed by 25 cycles of 96^0C for 20 seconds, 50^0C for 5 seconds, 60^0C for 3 minutes and followed by a final extension at 60^0C for 1 second and cooled to 4^0C and held.

3.26f: Precipitation, washing and drying of amplified products

The PCR products were taken out and added 95% ethanol in double volume (40µl) and 3M sodium acetate (pH 4.6) (Appendix I.11) in $1/10^{th}$ volume (2µl). Each of the tube was then votexed for 30 seconds and incubated at room temperature for 18 minutes to precipitate the extension products. The mixture was then centrifuged at 13,000 rpm for 18 minutes keeping the PCR tubes in appropriate holders, and supernatant was aspirated carefully.

The pellet was then washed with 200µl 70% ethanol by washing the walls of the tubes, votexed for 10 seconds, spinned at 13,000 rpm for 7 minutes and supernatant was discarded. The washing of the pellet was repeated once and finally dried perfectly for about 2 hours keeping tubes at room temperature.

3.26g: Final sequencing

An amount of 15µl formamide was added to each dried pellet and denatured at 95^0C for 2 minutes. The denatured product was kept into ice immediately for 5 minutes and finally transferred to a sequencing tube submitted to an ABI 3700 DNA sequencer for final sequencing.

3.26g: Genotyping

The sequenced chromatogram (Appendix X.1-X.14) and the WordPad (Appendix X.15-X.16) files from the forward and reverse sequence data were analyzed and edited by use of Chromas software (version 2.13, Technelysium Pty Ltd, Australia), and trimmed for *por* from the conserved region of TTGAA up to 490 bp and for *tbpB* from the conserved region of CGTCTGAA up to 390 bp (Martin *et al.*, 2004; NG-MAST, 2009).

The edited and trimmed *por* and *tbpB* sequences data were submitted for single locus allele to ng-mast website (www.ng-mast.net) for allele numbers. The numbers of the identified alleles at *por* and *tbpB* then entered for sequence types (ST) for each isolate. (Appendices XI.1-XI.13)

3.26h: Assignment of new alleles and sequence types

When the submitted sequenced data for either *por* or *tbpB* genes of the tested *N. gonorrhoeae* isolate were not matching >70% with any of the previously identified alleles, the auto-generated result called the allele a new one showing the exact percent of matching with a known allele. In such cases, the data were submitted again into the private pages allocated for this purpose with a username and a password by the website administration/ curator. After submission into the private pages, a new allele number was assigned by the website. Similarly, after submission of the two allele numbers for *por* and *tbpB* genes of a *N. gonorrhoeae* isolate, a new genotype (sequence type) number was assigned as the alleles did not match with any of the previously identified sequence type.

Chapter 4: Results

4.1: GONOCOCCAL INFECTION AND SOCIODEMOGRAPHIC CHARACTERISTICS AMONG THE CASES

4.1.1 Case enrollment

A total of 1025 male individuals were included in the present study to find out sociodemographic characteristics of which 371 (36.2%) were male-OPD, SOMCH and 654 (63.8%) were MSMs. (Table 4.1)

Throughout the three years of study during January, 2001 to July, 2003, the MSM cases were enrolled in higher numbers than the male-OPDs and this rate of enrollment among the MSMs was the highest (358, 34.9%) in 2003. (Figure 4.1)

4.1.2: Gonococcal infection among the cases

4.1.2a: Rate of gonococcal infection

Among the total 1025 individuals enrolled, 171 (16.7%) were found to have gonococcal infection. The rates of infection among the two groups of populations were: male-OPD, SOMCH- 143 (38.5%) and the MSMs- 28 (4.3%), showing a significantly higher rate among the male-OPD (chi-square test, p value <0.001). (Table 4.2)

Almost all of these infections were urethral (170, 99.4%) and only 1 (0.6%) was rectal. The rectal gonococcal infection was found in an MSM, who was a 20-years old illiterate, married young engaged in commercial receptive anal sex (MSM) for 8 years, complained of tenesmus and no neutrophil or gonococci was found in the smear of anal swab, but yielded growth of *N. gonorrhoeae* in the selective culture of anal specimen.

4.1.2b: Year-wise rate of gonococcal infection

The highest rate of gonococcal infection among the cases was found in 2002 (21.6%) and declined significantly in the following year of 2003 (14.3%) (Chi-square test value 8.2 at df=1, p value <0.01). (Table 4.3)

4.1.3: Identification of gonococcal infection

4.1.3a: Identification of gonococcal infection by microscopy and culture

The gonococcal infections were identified by finding *Neisseria gonorrhoeae* in the specimens using both microscopy and culture methods. Among the total 171 gonococcal infections, 26 (15.2%) were identified by microscopy alone (yielded no growth in culture), 5 (2.9%) were identified by culture alone (microscopy was negative) and remaining 140 (81.9%) infections were identified by both microscopy and culture. (Table 4.4)

4.1.3b: Validity measures of microscopy

Considering culture method as the 'gold standard', the measures of validity of microscopy for identification of gonococcal infection were calculated. Of the total 145 infections identified by culture method, microscopy identified 140 of the culture-positive infections (TP, true positive=140) and all 26 of the culture-negative infections (FP, false positive=26). On the other hand, 5 among the culture-positive infections were negative by microscopy (FN, false negative=5) and of the 880 culture-negative infections, 854 were negative by microscopy (TN, true negative=854). Thus the measures of validity of microscopy for identification of gonococcal infection were: sensitivity=96.6%, specificity=97.0%, positive predictive value=84.3%, negative predictive value=99.4% and predictive accuracy=97.0%. (Table 4.5)

Table 4.1: Distribution of the enrolled cases in two population groups in Sylhet

Population group	*Number enrolled*	*Percent*
Male-OPD, SOMCH[¶]	371	36.2
MSM[§]	654	63.8
Total	1025	100.0

[¶] Male-OPD, SOMCH: male individuals attending skin and venereal diseases outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM: male individuals having sex with male attending a medical centre for the MSMs at Sylhet City.

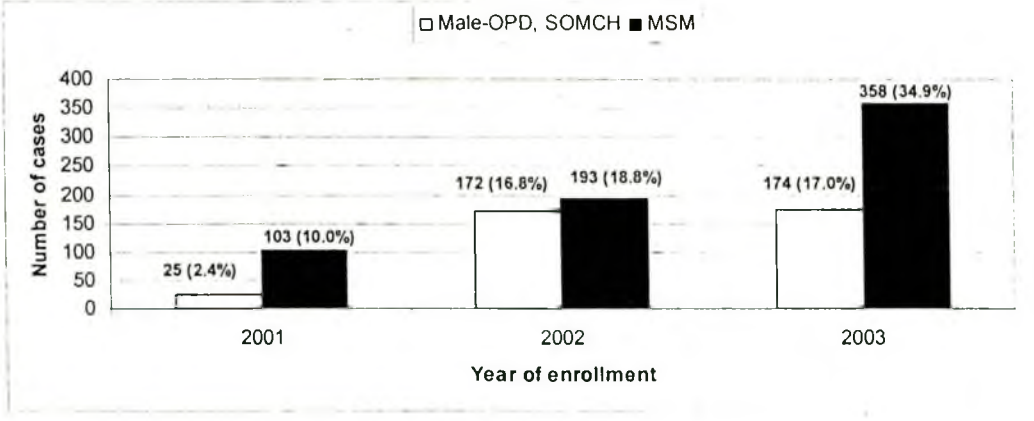


Figure 4.1: Bar diagram showing year-wise enrollment of the cases (n=1025) in two groups of populations

Table 4.2: Distribution of the gonococcal infection among the cases in Sylhet

Population groups	*Number of cases*	*Gonococcal infections*	
		Number	*Percent*
Male-OPD, SOMCH[¶]	371	143	38.7
MSM[§]	654	28	4.3
Total	1025	171	16.7

[¶] Male-OPD, SOMCH: male individuals attending skin and venereal diseases outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM: male individuals having sex with male attending a medical centre for the MSMs at Sylhet City;

Test of significance between infections among the male-OPD, SOMCH and MSMs: χ^2 (chi-square) test value= 199.9, at df=1 yielding p value <0.001

Table 4.3: Year-wise distribution of the gonococcal infection among the cases

Year of enrollment	*Enrolled*	*Gonococcal infection*	*Percent of infection*
2001	128	16	12.5
2002	365	79	21.6
2003	532	76	14.3
Total	1025	171	16.7

Test of significance of rate of gonococcal infection in 2002 and 2003:

χ^2 (chi-square) value= 8.2, at df=1 yielding p value <0.01

Table 4.4: Methods of identification of gonococcal infection among the cases

Methods of identification	*Number of gonococcal infection*	*Percent*
Microscopy alone (culture negative)	26	15.2
Culture alone (microscopy negative)	5	2.9
Both microscopy and culture	140	81.9
Total	171	100.0

Table 4.5: The two-by-two table to calculate validity of microscopy for identification gonococcal infection (comparing with culture method as gold standard)

Identification by microscopy	*Identification by culture*		*Total number of cases*
	Yes	*No*	
Yes	140 (a)	26 (b)	166 (a+b)
No	5 (c)	854 (d)	859 (c+d)
Total	145 (a+c)	880 (b+d)	1025 (a+b+c+d)

a= True positive (TP), b=False positive (FP), c=False negative (FN), and d=True negative (TN)

Measures of validity of microscopy (details on methods section, page- 100):

Sensitivity (a/a+c) = 96.6%

Specificity (d/b+d) = 97.0%

Positive predictive value (a/a+b) = 84.3%

Negative predictive value (d/c+d) = 99.4%

Predictive accuracy (a+d)/(a+b+c+d) = 97.0%

4.1.4: Isolation and preservation of the *N. gonorrhoeae* isolates

Of the 171 gonococcal infections, 145 (84.8%) yielded growth of *N. gonorrhoeae* in culture. Among these 145 isolates, 11 (7.6%) were lost due to technical errors and the remaining 134 (92.4%) were preserved at the Department of Microbiology, Sylhet MAG Osmani Medical College, Sylhet. (Table 4.6)

All of the 134 isolates of *N. gonorrhoeae* in Sylhet were also transferred to the then RTI/STI laboratory, ICDDR,B, Dhaka for further study. A few of these isolates (16, 11.9%) failed to grow after transfer and the remaining 118 (88.1%) strains were preserved in the RTI/STI laboratory, ICDDR,B, Dhaka. (Table 4.6)

Among the 145 *N. gonorrhoeae*, majority (74, 50.7%) was isolated in 2003 and 68 (93.3%) of which were transferred to ICDDR,B and 54 (79.4%) of them could be stored at ICDDR,B. Finally, this 54 *N. gonorrhoeae* isolates from Sylhet were later included at the ICDDR,B laboratories for characterization. (Table 4.6)

Table 4.6: Year-wise isolation and storage of the *N. gonorrhoeae* in Sylhet

Year of isolation	*Number (%) of N. gonorrhoeae*					
	at SOMC[¶]			*at ICDDR,B*[§]		
	Isolated	*Lost*	*Stored*	*Transferred*	*Failed to grow*	*Stored*
2001	16 (11.3)	3 (18.8)	13 (81.2)	13 (100.0)	0 (0)	13 (100.0)
2002	55 (38.0)	2 (0)	53 (96.2)	53 (100.0)	3 (27.8)	50 (72.2)
2003	74 (50.7)	6 (66.7)	68 (93.3)	68 (100.0)	14 (20.6)	54 (79.4)
Total	145 (100.0)	11 (7.6)	134 (92.4)	134 (100.0)	16 (11.9)	118 (88.1)

[¶] at SOMC: at Department of Microbiology, Sylhet MAG Osmani Medical College, Sylhet

[§] at ICDDR,B: at RTI/STI Laboratory, International Centre for Diarrhoeal Disease Research in Bangladesh

4.1.5: Anogenital complaints by the cases

Anogenital complaints presented by the cases were: dysuria (365, 35.6%), non-purulent urethral discharge (316, 30.8%), purulent/ mucopurulent urethral discharge (154, 15.0%), non-purulent anal discharge (90, 8.8%) and tenesmus (62, 6.1%). The highest among the complaints by the male-OPD, SOMCH cases was dysuria (36.4%), followed by purulent/ mucopurulent urethral discharge (35.8%) and non-purulent urethral discharge (27.8%). The highest among the complaints by the MSM cases was dysuria (34.9%), followed by non-purulent urethral discharge (32.3%), non-purulent anal discharge (14.2%), tenesmus (9.6%) and purulent/ mucopurulent urethral discharge (3.2%). There were a few asymptomatic cases (38, 5.8%) among the MSMs as well. (Table 4.7)

4.1.5a: Relationship between the complaints and gonococcal infection

The purulent/ mucopurulent urethral discharge of the cases was found related with the highest rate gonococcal infection (92.9%), followed by non-purulent urethral discharge (7.3%), tenesmus (1.6%) and dysuria (1.1%). (Table 4.8)

No case among with non-purulent anal discharge (n=93) or having no complaint (asymptomatic cases) (n=38) was found to be infected and these cases were included among the MSMs. (Table 4.8)

The gonococcal infection among the symptomatic cases in two groups of populations showed that the rate is still significantly high among the male-OPD cases. (38.7% among male-OPD versus 4.5% among symptomatic MSMs) (chi-square test p value <0.001). (Table 4.9)

Table 4.7: Anogenital complaints by the cases in two groups of populations enrolled in Sylhet

Anogenital complaints	*No. (%) of cases*		
	Total	*Male-OPD, SOMCH* ¶	*MSM* §
Non-purulent urethral discharge	316 (30.8)	103 (27.8)	213 (32.6)
Purulent/ mucopurulent urethral discharge	154 (15.0)	133 (35.8)	21 (3.2)
Dysuria	365 (35.6)	135 (36.4)	230 (35.2)
Tenesmus	62 (6.1)	0 (0)	62 (9.4)
Non-purulent anal discharge	90 (8.8)	0 (0)	90 (13.8)
None	38 (3.7)	0 (0)	38 (5.8)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

¶ Male-OPD, SOMCH cases: male individuals attending skin and venereal diseases outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

§ MSM cases: male individuals having sex with male attending medical centre for the MSMs in Sylhet City

Table 4.8: Relationship between the anogenital complaints and gonococcal infection among the cases (n=1025)

Anogenital complaints	*Number of cases*		*Percent of gonococcal infection*
	Total	*Found infected*	
Non-purulent urethral discharge	316	23	7.3
Purulent/ mucopurulent urethral discharge	154	143	92.9
Dysuria	365	4	1.1
Tenesmus*	62	1	1.6
Non-purulent anal discharge*	90	0	0
None (asymptomatic)*	38	0	0
Total	1025	171	16.7

* Presented by the MSMs only

Test of significance for rate (percent) of gonococcal infection with complaints:

χ^2 (chi-square) value= 751.4, at df=5 yielding p value <0.001

Table 4.9: Comparison between rates of gonococcal infection among symptomatic cases in two groups of populations

Population groups	*Number (%) of symptomatic cases*	*Gonococcal infection*	
		Number	*Percent*
Male-OPD, SOMCH[¶] (n=371)	371 (100.0)	143	38.7
MSM[§] (n=654)	616 (94.2%)	28	4.5

[¶] Male-OPD, SOMCH: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital;

[§] MSM: male individuals having sex with male attending a medical centre for the MSMs in Sylhet City;

Test of significance for difference in rates of infection:

χ^2 (chi-square) value= 187.6, at df=1 yielding p value <0.001

4.1.6: Urethritis among the cases

A total of 228 (22.2%) among the cases had urethritis (≥5 PMNs /HPF) and majority of the cases of urethritis (179, 78.5%) were among the male-OPD. (Table 4.10)

Different rates of urethritis were detected among the patients with presenting complaints. Thus, all (100.0%) of the patients with the complaint of purulent/ mucopurulent urethral discharge had urethritis. On the other hand, only 15.2% of the patients with non-purulent urethral discharge had urethritis. Similarly, only 6.8% of the patients presenting with dysuria had urethritis and 1 (2.6%) of the cases presenting with no complaints also had urethritis. (Table 4.11)

Majority of the cases of urethritis (74.6%) were due to gonococcal infection (gonococcal urethritis) and remaining one-fourth (25.4%) had no gonococcal infection (non-gonococcal urethritis). The rate of gonococcal infection among the urethritis cases is significantly higher than non-gonococcal aetiology (chi-square test p value <0.001). (Table 4.12)

Table 4.10: Distribution of the urethritis cases among the study populations

*Urethritis**	*No. (%) of cases*		
	Total	*Male-OPD, SOMCH* ¶	*MSM* §
Yes	228 (22.2)	179 (78.5)	49 (21.5)
No	797 (77.8)	192 (51.8)	605 (92.5)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

* Urethritis: cases showing ≥ 5 polymorphonuclear leukocytes (neutrophils) per high power field in the smear of urethral discharge;

¶ Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

§ MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.11: Relationship between complaints of the cases and urethritis

Major complaints	*Number of cases*		*Percent of urethritis*
	Enrolled	*Urethritis*	
Purulent/ mucopurulent urethral discharge	154	154	100.0
Non-purulent urethral discharge	316	48	15.2
Dysuria	365	25	6.8
None	38	1	2.6
Others (extra-urethral)	152	0	0
Total	1025	228	22.2

Table 4.12: Relationship between urethritis and gonococcal infection among the cases

Urethritis by microscopy*	*No. (%) of cases*		
	Enrolled	*Gonococcal infection*	*No gonococcal infection*
Yes	228 (22.2)	170 (74.6)	58 (25.4)
No	797 (77.8)	01 (0.1)	796 (99.9)
Total	1025 (100.0)	171 (16.7)	854 (83.3)

* Urethritis: cases showing ≥5 neutrophils per high power field in smears of urethral discharge

χ^2 (chi-square) value= 707.7, at df=1 yielding p value < 0.001

4.1.7: Relationship between sociodemographic characteristics and gonococcal infection of the cases

4.1.7a: Age distribution of the cases

Ages of the patients were found as low as 13 years up to the highest of 60 years with a mean age of 25 years and median of 24 ±6.31 years.

Distributing ages of the cases into 5 different classes with interval of 10 years, it was found that majority of enrollments was included in the 16-25 years age group (640, 62.4%), followed by 26-35 years group (317, 30.9%). The percent of case enrollments in the age groups of each group of study population was almost equal. (Table 4.13)

The rate of gonococcal infection was found to be the highest among older age group (above 45 years) (3/14, 21.4%), followed by 16-25 years group (112, 17.5%) and 36-45 years age group (8, 17.4%). The differences in rate of gonococcal infection among the age groups were tested by chi-square and found to have no significant difference (chi-square test, p value >0.10). (Table 4.14)

Table 4.13: Age groups of the cases distributed into two populations

Age groups	*No. (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Up to 15 years	8 (0.8)	4 (1.0)	4 (0.6)
16-25 years	640 (62.4)	224 (60.4)	416 (63.7)
26-35 years	317 (30.9)	116 (31.3)	201 (30.7)
36-45 years	46 (4.5)	21 (5.7)	25 (3.8)
> 45 years	14 (1.4)	6 (1.6)	8 (1.2)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.14: Relationship between age groups and gonococcal infection of the cases

Age groups	*No. (%) of the cases*	
	Enrolled	*Gonococcal infection*
Up to 15 years	8 (0.8)	1 (12.5)
16-25 years	640 (62.4)	112 (17.5)
26-35 years	317 (30.9)	46 (14.5)
36-45 years	46 (4.5)	9 (17.4)
Above 45 years	14 (1.4)	3 (21.4)
Total	1025 (100.0)	171 (16.7)

χ^2 (chi-square) value= 5.7, at df = 4 yielding p value >0.10

4.1.7b: Education level of the study population

Frequencies of formal education (literacy) among the 665 available cases showed that majority were educated (561, 84.4%). (Table 4.15)

The levels of education among 561 educated individuals showed that the highest number of cases were educated in the range of classes VI- X (39.1%), followed by up to class V (32.1%) and there were even many cases having post-graduate level of education (6.2%). The level of education in two groups of population was also found almost similar. (Table 4.16)

The rate of gonococcal infection was found higher among the illiterate (19.2%) than the literate (educated) (16.4%) and the difference in rate of infection is not significant (chi-square test, p value >0.10). (Table 4.17)

The rate of gonococcal infection was found to be the highest among the cases having post-graduate level of education (28.6%), followed by individuals having education up to class V (19.4%). The differences in rate of gonococcal infection among the cases with different levels of education is found significant (chi-square test p value <0.01). (Table 4.18)

Table 4.15: Education status of the two groups of populations

Education status	*No. (%) of cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Educated (literate)	561 (84.4)	173 (75.9)	388 (88.8)
Illiterate	104 (15.6)	55 (24.1)	49 (11.2)
Total	665 (100.0)	228 (100.0)	437 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.16: Level of education of the two groups of populations (n=561)

Level of education	*No. (%) of educated cases*		
	Total	*Male-OPD*	*MSM*
Up to Class V	180 (32.1)	54 (31.2)	126 (32.5)
Class VI to X	219 (39.1)	60 (34.7)	159 (41.0)
S.S.C.*	23 (4.1)	17 (9.8)	6 (1.5)
H.S.C.**	60 (10.7)	14 (8.1)	46 (11.9)
Graduation	44 (7.8)	4 (2.3)	40 (10.3)
Post-graduation	35 (6.2)	24 (13.9)	11 (2.8)
Total	561 (100.0)	173 (100.0)	388 (100.0)

* S.S.C.: secondary school certificate ** H.S.C.: higher secondary certificate

Table 4.17: Relationship between education status and gonococcal infection of the cases (n=665)

Education status	*No. (%) of cases*	
	Enrolled	*Had gonococcal infection*
Educated (literate)	561 (84.4)	92 (16.4)
Illiterate	104 (15.6)	20 (19.2)
Total	665 (100.0)	112 (16.8)

χ^2 (chi-square) value= 0.50, at df=1 yielding p value >0.10

Table 4.18: Relationship between level of education and gonococcal infection among the educated cases (n=561)

Level of education	*No. (%) of cases*	
	Enrolled	*Had gonococcal infection*
Up to class V	180 (32.1)	35 (19.4)
Class VI - class X	219 (39.1)	36 (16.4)
S.S.C. *	23 (4.1)	4 (17.4)
H.S.C. **	60 (10.7)	7 (11.7)
Graduation	44 (7.8)	0 (0)
Post graduation	35 (6.2)	10 (28.6)
Total	561 (100.0)	92 (16.4)

* S.S.C.: Secondary School Certificate, **H.S.C.: Higher Secondary Certificate

χ^2 (chi-square) value= 16.3, at df=5 yielding p value <0.01

4.1.7c: Occupation of the cases

Occupation of the cases showed that the highest enrollment was from the businessmen (269, 26.2%), followed by the labourers (manual workers) (264, 25.8%) and service holders (248, 24.2%). There were some students (71, 6.9%) and jobless/ unemployed (62, 6.1%) individuals. Proportion of each of the occupations among two groups of study population was found almost similar. (Table 4.19)

The rate of gonococcal infection among the male-OPD cases was found the highest among the drivers (19, 48.7%). The differences in the rates of infection among different occupation groups of the male-OPD cases are not significant (chi-square test, p value >0.50). (Table 4.20)

The rate of gonococcal infection among the MSM cases was found the highest among the male sex workers (MSM) (2, 8.0%). Test of significance by chi-square test shows no significant difference in rate of infections among occupations of the MSMs (chi-square test p value >0.10). (Table 4.21)

Table 4.19: Distribution of occupation among two groups of populations

Occupation	*Number (%) of the cases*		
	Total	*Male-OPD, SOMCH*[¶]	*MSM*[§]
Businessmen	269 (26.2)	91 (24.5)	178 (27.2)
Labourer	264 (25.8)	118 (31.8)	146 (22.3)
Service holder	248 (24.2)	75 (20.2)	173 (26.5)
Driver	86 (8.4)	39 (10.5)	47 (7.2)
Student	71 (6.9)	27 (7.3)	44 (6.7)
Jobless	62 (6.1)	21 (5.7)	41 (6.3)
Sex worker	25 (2.4)	0 (0)	25 (3.8)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.20: Relationship between occupation and the rate of gonococcal infection among the male-OPD cases

Occupation of the cases	*No. (%) of the male-OPD, SOMCH*[¶]	
	Enrolled	*Had gonococcal infection*
Labourer	118 (31.8)	44 (37.3)
Businessmen	91 (24.5)	34 (37.4)
Service holders	75 (20.2)	29 (38.7)
Driver	39 (10.5)	19 (48.7)
Student	27 (7.3)	12 (44.4)
Jobless	21 (5.7)	05 (23.8)
Total	371 (100.0)	143 (38.5)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet

χ^2 (chi-square) value= 4.2, at df=6 yielding p value >0. 50

Table 4.21: Relationship between occupation and the gonococcal infection among the MSM cases

Occupation of the cases	*No. (%) of the MSM* [§]	
	Enrolled	*Had gonococcal infection*
Businessmen	178 (27.2)	7 (3.9)
Service holders	173 (26.5)	10 (5.8)
Labourer	146 (22.3)	4 (2.7)
Driver	47 (7.2)	3 (6.4)
Student	44 (6.7)	0 (0)
Jobless	41 (6.3)	2 (4.9)
Male sex worker (MSM)	25 (3.8)	2 (8.0)
Total	654 (100.0)	28 (4.3)

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City

χ^2 (chi-square) value= 7.1, at df= 6 yielding p value >0. 10

4.1.7d: Marital status of the cases

Marital status of the enrolled cases showed that about two-thirds of the total enrollments were unmarried (766, 74.7%). Similarly, majority of the male-OPD (252, 67.9%) and more than two-thirds of the MSM cases were unmarried. (Table 4.22)

The rate of gonococcal infection was found exactly equal (128, 16.7% and 43, 16.7%) between the married and unmarried cases. There was also 1 divorcee case on the list having no gonococcal infection. (Table 4.23)

4.1.7e: Pre-marital sex of the cases

Pre-marital sex among the total 766 unmarried cases showed that almost all had experience of pre-marital sex (741, 96.7%). Distributing cases into two source populations, it was found that 90% among the male-OPD cases had pre-marital sex and by their natural sex practice, all of the MSMs had pre-marital sex. (Table 4.24)

The rate of gonococcal infection was higher among those having no pre-marital sex (20.0%) than the rate among those had pre-marital sex (16.6%) and the difference is not significant (chi-square test p value >0.50). Whereas, considering sex partners among the individuals having pre-marital sex, the rate of gonococcal infection was found significantly higher among the individuals having sex with female CSWs (99, 30.8%) (chi-square test p value <0.001). (Table 4.25)

Table 4.22: Marital status of the two groups of populations (n=1025)

Marital status	*Number (%) of the cases*		
	Total	*Male, OPD*[¶]	*MSM*[§]
Unmarried	766 (74.7)	252 (67.9)	514 (78.6)
Married	258 (25.2)	119 (32.1)	139 (21.3)
Divorcee	1 (0.1)	0 (0)	1 (0.1)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.23: Relationship between marital status and gonococcal infection of the cases (n=1025)

Marital status	*No. (%) of the cases*	
	Enrolled	*Had gonococcal infection*
Unmarried	766 (74.7)	128 (16.7)
Married	258 (25.2)	43 (16.7)
Divorce	01 (0.1)	0 (0)
Total	1025 (100.0)	171 (16.7)

Table 4.24: Pre-marital sex in two groups of populations among the unmarried cases (n=766)

Pre-marital sex	*Number (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Total unmarried cases	766 (74.7)	252 (32.9)	514 (67.1)
No pre-marital sex	25 (3.3)	25 (9.9)	0 (0)
Yes, had pre-marital sex	741 (96.7)	227 (90.1)	514 (100.0)
Yes, with female CSW*	321 (43.3)	202 (89.0)	119 (23.2)
Yes, with MSM	310 (41.8)	3 (1.3)	307 (59.7)
Yes, with others	110 (14.9)	22 (9.7)	88 (17.1)
Not applicable (married)	259 (25.3)	119 (46.0)	140 (54.0)
Total	1025 (100.0)	371 (36.2)	654 (63.8)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

* CSW: commercial sex worker

Table 4.25: Relationship between pre-marital sex practice and gonococcal infection among the unmarried individuals (n=766)

Pre-marital sex	*No. (%) of cases*	
	Enrolled	*Had gonococcal infection*
Total unmarried cases	766 (74.7)	128 (16.7)
No pre-marital sex	25 (3.3)	5 (20.0)
Yes had pre-marital sex	741 (96.7)	123 (16.6)
Yes, with female CSW*	321 (43.3)	99 (30.8)
Yes, MSM[§]	310 (41.8)	12 (3.9)
Yes, with others casually	110 (14.9)	12 (10.9)
Not applicable (married)	259 (25.3)	43 (16.6)
Total	1025 (100.0)	171 (16.7)

*CSW: commercial sex worker

[§]MSM: male individuals having sex with male

Test of significance with status of pre-marital sex: χ^2 (chi-square) value= 0.189, at df =1 yielding p value >0.50

Test of significance with sex partner among cases having pre-marital sex: χ^2 (chi-square) value= 85.9, at df =2 yielding p value <0. 001

4.1.7f: Extra-marital sex of the cases

Among the 258 married individuals, more than two-thirds had extra-marital sex (226, 87.6%) and nearly half of them reported of practicing extra-marital sex with female CSWs (110, 48.7%). In case of male-OPD, almost all individuals had extra-marital sex with female CSWs (85, 95.5%) and in case of the MSMs, majority had extra-marital sex with the males (85, 59.7%). (Table 4.26)

The rate of gonococcal infection was found higher among the cases having extra-marital sex (17.3%) than those having no extra-marital sex (12.5%), but the difference in not significant (chi-square test p value >0.10). Whereas, considering sex partner, the rate of gonococcal infection was found significantly higher among those having extra-marital sex with female CSWs (28/110, 25.5%) (chi-square test p value <0.01). (Table 4.27)

4.1.7g: History of exposure of the cases to female CSWs

History of sexual exposure of the cases with female CSWs (commercial sex workers) showed that majority of the total enrollments had no exposure (594, 57.9%). But distributing the cases into two populations it was found that more than three-fourths of the male-OPD cases (287, 77.4%) had history of exposure with female CSWs, whereas less than one-fourth of the MSM Cases (144, 22.0%) had history of exposure. (Table 4.28)

The rate of gonococcal infection was found higher among the individuals having history of exposure than those having no exposure (127, 29.5% versus 44, 7.4%). The difference in rate of infection among individuals having history of exposure with those having no exposure history is highly significant (chi-square test p value <0.001). (Table 4.29)

Table 4.26: Extra-marital sex in two groups of populations among the married cases (n=258)

Extra-marital sex	*Number (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Total married cases	258 (25.2)	119 (46.1)	139 (53.9)
No extra-marital sex	32 (12.4)	30 (25.2)	2 (1.4)
Yes, had extra-marital sex	226 (87.6)	89 (74.8)	137 (98.6)
- Yes, with female CSW*	110 (48.7)	85 (95.5)	25 (23.2)
- Yes, with MSM	85 (37.6)	0 (0)	85 (59.7)
- Yes, with others	31 (13.7)	4 (4.5)	27 (17.1)
Not applicable (unmarried)	766 (74.7)	252 (32.9)	515 (54.0)
Not applicable (divorce)	1 (0.1)	0 (0)	1 (100.0)
Total	1025 (100.0)	371 (36.2)	654 (63.8)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

*CSW: commercial sex worker

Table 4.27: Relationship between extra-marital sex practice and gonococcal infection among the married individuals (n=258)

Extra-marital sex	*No. (%) of cases*	
	Enrolled	*Had gonococcal infection*
Total Married cases	258 (25.2)	43 (16.7)
No extra-marital sex	32 (12.4)	4 (12.5)
Yes, had extra-marital sex	226 (87.6)	39 (17.3)
- Yes with female CSW*	110 (48.7)	28 (25.5)
- Yes, MSM§	85 (37.6)	6 (7.1)
- Yes with others	31 (13.7)	5 (16.1)
Not applicable (unmarried/ divorcee)	767 (74.8)	128 (16.7)
Total	1025 (100.0)	171 (16.7)

*CSW: commercial sex worker;

§ MSM: male individuals having sex with male attending a medical centre for the MSMs at Sylhet City;

Test of significance with sex partners among the cases with extra-marital sex: χ^2 (chi-square) value= 13.3, at df =2 yielding p value <0.01

Table 4.28: History of exposure to female CSWs of the cases distributed into two groups of populations

*History of exposure to female CSW**	*Number (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Yes	431 (42.1)	287 (77.4)	144 (22.0)
No	594 (57.9)	84 (22.6)	510 (78.0)
Total	1025 (100.0)	371 (100.0)	654 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City;

* CSW: commercial sex worker.

Table 4.29: Relationship between history of exposure to female CSWs and gonococcal infection of the cases

*H/o exposure to female CSW**	*No. (%) of the cases*	
	Enrolled	*Had gonococcal infection*
Yes	431 (42.0)	127 (29.5)
No	594 (58.0)	44 (7.4)
Total	1025 (100.0)	171 (16.7)

*CSW: commercial sex worker;

χ^2 (chi-square) value= 87.4, at df =1 yielding p value <0. 001

4.1.7h: Condom use during sex by the cases

Less than one-tenth of the 962 available cases (9.5%) used condom during sex. The rate of condom use during sexual act was almost similar in the individual groups of study populations (8.5% in male-OPD versus 9.9% in the MSMs). (Table 4.30)

The rate of gonococcal infection was found to be the highest among individuals not using condom during sex (154, 17.7%). Although the differences in rate of infection between individuals used condom and those did not use condom during sex (12.1% and 17.7% respectively) or between those used condom irregularly and did not use condom during sex (13.6% and 17.7% respectively) or between those used condom regularly and used irregularly are not significant (8.0% and 13.6% respectively) (chi-square test p values >0.10 in all the three comparisons). (Table 4.31)

4.1.7i: Sexuality and sexual behaviour of the cases

The type of sexual behaviour showed that the highest number of the enrolled cases were bisexual (337, 33.8%), followed immediately by heterosexuals (332, 33.2%) and MSM only (330, 33.1%). Among the male-OPD, almost all were heterosexual (332, 96.2%) and among the MSMs, almost one half were bisexual (326, 49.8%). (Table 4.32)

The rate of gonococcal infection was found to be the highest among the heterosexuals (134, 40.4%). The differences in rate of infection among the sexual behaviour groups is highly significant (chi-square test p value <0.001). (Table 4.33)

Table 4.30: Condom use during sex by the cases (n=962) in two groups of populations

Condom use	*Number (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
Yes	91 (9.5)	28 (8.5)	63 (9.9)
No	871 (90.5)	300 (91.5)	571 (90.1)
Total	962 (100.0)	328 (100.0)	634 (100.0)

[¶] Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§] MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City.

Table 4.31: Relationship between condom use during sex and gonococcal infection among the cases (n=962)

Condom use	*No. (%) of the cases*	
	Enrolled	*Had gonococcal infection*
Yes, used condoms	91 (9.5)	11 (12.1)
Used regularly (always)	25 (27.5)	2 (8.0)
Used irregularly	66 (72.5)	9 (13.6)
No (never used condoms)	871 (90.5)	154 (17.7)
Total	962 (100.0)	165 (17.2)

Test of significance for condom use and rate of gonococcal infection:

Used regularly vs never: χ^2 (chi-square) value = 2.18, at df =1 yielding p value >0.10

Used condom vs not used: χ^2 (chi-square) value = 1.81, at df =1 yielding p value >0.10

Used irregular vs not used: χ^2 (chi-square) value = 0.69, at df =1 yielding p value >0.10

Used irregularly vs regularly: χ^2 (chi-square) value = 0.96, at df =1 yielding p value >0.10

Table 4.32: Distribution of sexual behaviour in two groups of populations among the cases (n=962)

Condom use	*Number (%) of the cases*		
	Total	*Male-OPD*[¶]	*MSM*[§]
MSM only	330 (33.1)	2 (0.6)	328 (50.2)
MSM-active*	108 (32.7)	2 (100.0)	106 (32.3)
MSM-passive**	45 (13.6)	0 (0)	45 (13.7)
MSM-both	177 (53.7)	0 (0)	177 (54.0)
Heterosexual[¶¶]	332 (33.2)	332 (96.2)	0 (0)
Bisexual[§§]	337 (33.8)	11 (3.2)	326 (49.8)
Total	999 (100.0)	345 (100.0)	654 (100.0)

[¶]Male-OPD, SOMCH cases: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[§]MSM cases: male individuals having sex with male attending the medical centre for the MSMs at Sylhet City;

*MSM-active: MSMs practicing insertive anal sex

**MSM- passive: MSMs practicing receptive anal sex

[¶¶]Heterosexual: male individual having sex with female

[§§]Bisexual: male individual having sex with both male and female

Table 4.33: Relationship between sexual behaviour and gonococcal infection of the cases (n=999)

Sexual behaviour	*No. (%) of the cases*	
	Enrolled	*Had gonococcal infection*
MSM[§]	330 (33.1)	11 (3.3)
MSM-active[§§]	108 (32.7)	4 (3.7)
MSM- passive[¶]	45 (13.6)	1 (2.2)
MSM- both	177 (53.7)	6 (3.4)
Heterosexual[¶¶]	332 (33.2)	134 (40.4)
Bisexual*	337 (33.7)	22 (6.5)
Total	999 (100.0)	167 (16.7)

[§]MSM: male individual having sex with male

[§§]MSM-active: MSMs practicing insertive anal sex

[¶]MSM- passive: MSMs practicing receptive anal sex

[¶¶]Heterosexual: male individual having sex with female

*Bisexual: male individual having sex with both male and female

χ^2 (chi-square) value = 200.9, at df =2 yielding p value <0.001

4.2 CHARACTERIZATION OF THE *N. GONORRHOEAE* ISOLATES

4.2.1: Selection of the *N. gonorrhoeae* isolates for characterization

A total of 495 *N. gonorrhoeae* isolates (54 isolated in Sylhet in 2003 as well as another 441 *N. gonorrhoeae* isolated by the then RTI/STI laboratory of the ICDDR,B in Dhaka) were included for characterization. Thus the *N. gonorrhoeae* included for characterization were isolated during 2003 to 2006 from five different high-risk populations, majority of which (322, 65.1%) were isolated from floating female commercial sex workers (CSWs). (Table 4.34)

4.2.2: Antimicrobial resistance of the *N. gonorrhoeae* isolates

Majority/ almost all of the 495 selected *N. gonorrhoeae* isolates were resistant to penicillin (51.5%), tetracycline (88.1%) and ciprofloxacin (90.9%). Whereas, almost none of the isolates were resistant to ceftriaxone (0.2%), and spectinomycin (1.6%), and none of the tested *N. gonorrhoeae* isolates were resistant to cefixime (0%). (Figure 4.2)

The resistance of the *N. gonorrhoeae* isolates to penicillin, tetracycline and ciprofloxacin showed that the highest number of the organisms were resistant to all three of the antibiotics together (multi-drug resistance, MDR) (230, 46.5%), followed by resistance to any two of them (198, 40.0%). It was further observed that only 13 (2.6%) of the *N. gonorrhoeae* isolates were not resistant (either sensitive or intermediately sensitive) and the remaining 97.3% were resistant to at least any one of the antimicrobial agents tested. (Table 4.35)

The MIC (minimum inhibitory concentration) of the 50% (MIC_{50}), 90% (MIC_{90}) of the *N. gonorrhoeae* isolates for penicillin, tetracycline and ciprofloxacin showed that these values for the antibiotics were very high comparing with the susceptibility breakpoint. (Table 4.36)

4.2.2a: Relationship between antimicrobial resistance and source populations of the N. gonorrhoeae isolates

Comparing the rates of resistance of *N. gonorrhoeae* isolated from different populations, it was found that penicillin resistance was found to be the highest among isolates from male-truckers (57.1%). Similarly, rates of resistance to tetracycline was found to be the highest among isolates from Male-truckers (94.3%) and the rate of ciprofloxacin resistance was found to be the highest among the isolates from the MSMs (100.0%). (Figure 4.3)

Comparing the rates of resistance of *N. gonorrhoeae* isolates between the sites of isolation, it was found that the rate of resistance occurred at the highest rate at Dhaka in all three antibiotics (penicillin-53.7%, tetracycline-90.3% and ciprofloxacin- 91.9%). (Figure 4.4)

Comparing the rates of resistance of the *N. gonorrhoeae* isolates between sex of the source populations it was found that the penicillin (56.5%) and tetracycline (89.5%) resistance were higher among the females, whereas ciprofloxacin resistance (94.2%) was found higher among the male populations. (Figure 4.5)

The relationship between the *N. gonorrhoeae* isolates with resistance to multiple antibiotics and source population of the isolates showed that the highest number of the isolates showing MDR (resistance to all three antibiotics) were isolated from female CSWs-floating (52.2%), whereas the highest percent of the organisms showing resistance to any two of the antibiotics were isolated from the MSMs (71.4%). (Figure 4.6)

Table 4.34: Source of the *N. gonorrhoeae* isolates selected for characterization

Source population[¶] *and site of enrollment*	*Number of N. gonorrhoeae isolates*	*Percent*
Female CSW[§]-floating, Dhaka	322	65.1
Male-OPD, DMCH[¶§], Dhaka	64	12.9
Male-truckers, Dhaka	35	7.1
Male-OPD, SOMCH[§§], Sylhet	46	9.3
MSM[¶¶], Sylhet	8	1.6
Female CSW-brothel based, Gualanda, Faridpur	20	4.0
Total	495	100.0

[¶] Source population: population groups from which the *N. gonorrhoeae* were isolated;

[§] CSW: commercial sex worker;

[¶§] Male-OPD, DMCH: male individuals attending skin and venereal disease outpatients Department of Dhaka Medical College Hospital, Dhaka;

[§§] Male-OPD, SOMCH: male individuals attending skin and venereal disease outpatients Department of Sylhet MAG Osmani Medical College Hospital, Sylhet;

[¶¶] MSM: male individuals having sex with male attending a medical centre for the MSMs at Sylhet City.

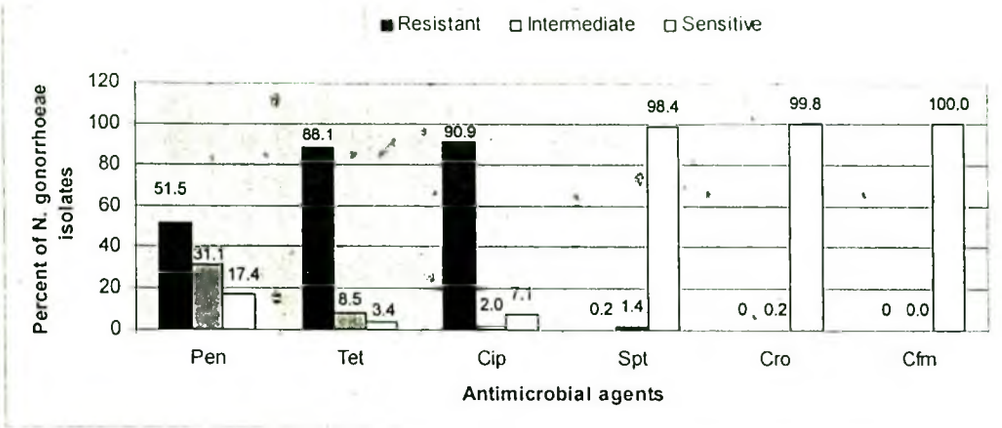


Figure 4.2: Bar diagram showing antimicrobial susceptibility of the *N. gonorrhoeae* isolates (Pen: penicillin, Tet: tetracycline, Cip: ciprofloxacin, Spt: dpectinomycin, Cro: ceftriaxone, Cfm: cefixime)

Table 4.35: The combined resistance of the *N. gonorrhoeae* isolates to three of the antibiotics tested (penicillin, tetracycline and ciprofloxacin)

Type of combined resistance	*Number of N. gonorrhoeae isolates*	*Percent*
Resistance to three antibiotics	230	46.5
Resistance to two antibiotics (MDR[§])	198	40.0
Resistance to one antibiotic	54	10.9
No resistance	13	2.6
Total	495	100.0

[§]MDR: multi-drug resistance

Table 4.36: The MIC parameters of the *N. gonorrhoeae* isolates for penicillin, tetracycline and ciprofloxacin comparing with susceptibility break-point

Antimicrobial Agent	*MIC values in µg/ml for*			
	Susceptible	*MIC_{50}*	*MIC_{90}*	*MIC range*
Penicillin	0.06	2.0	32.0	0.06-64.0
Tetracycline	0.25	16.0	32.0	0.25-128.0
Ciprofloxacin	0.06	8.0	16.0	0.06-16.0

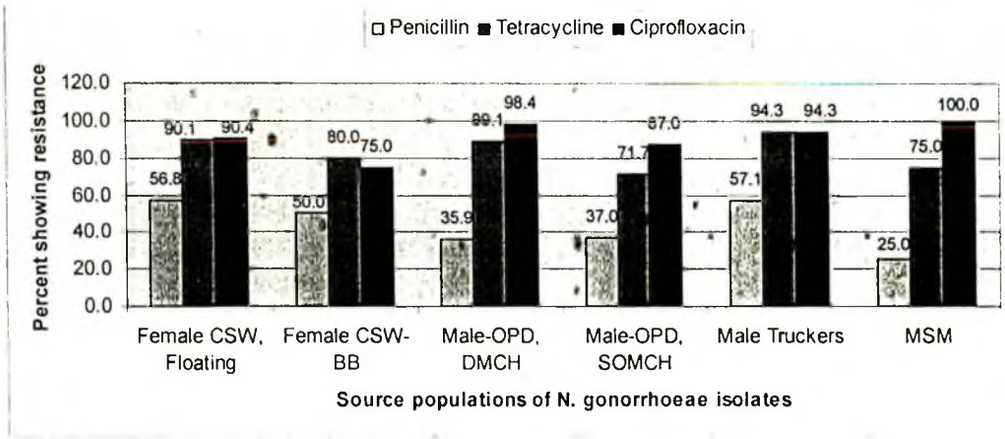


Figure 4.3: Comparison of the rates of resistance of *N. gonorrhoeae* isolates in the different source populations

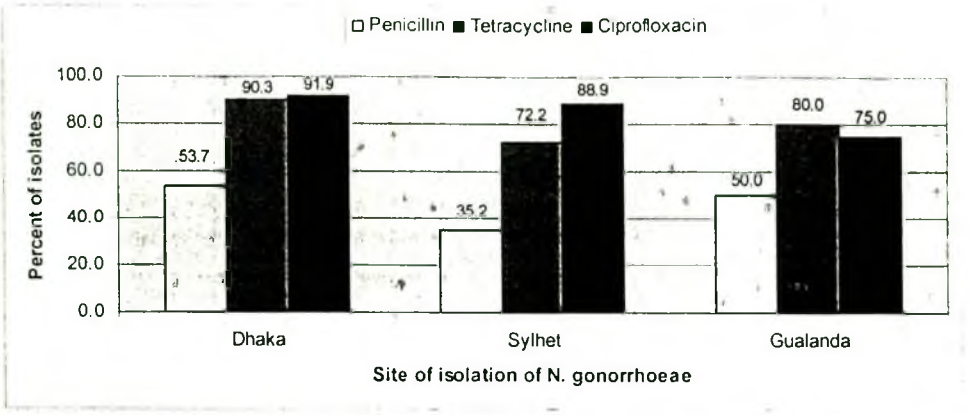


Figure 4.4: Comparison of the rates of resistance of *N. gonorrhoeae* isolates at different sites of isolation

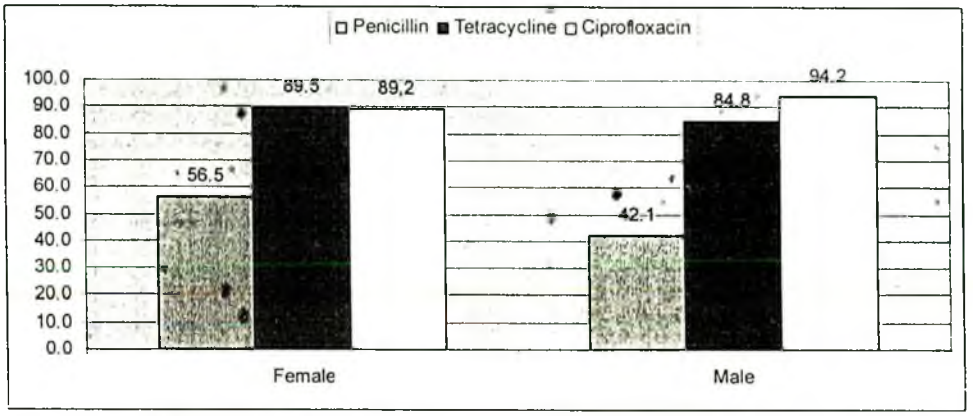


Figure 4.5: Comparison of rates of resistance of *N. gonorrhoeae* isolates in sexes of the source populations

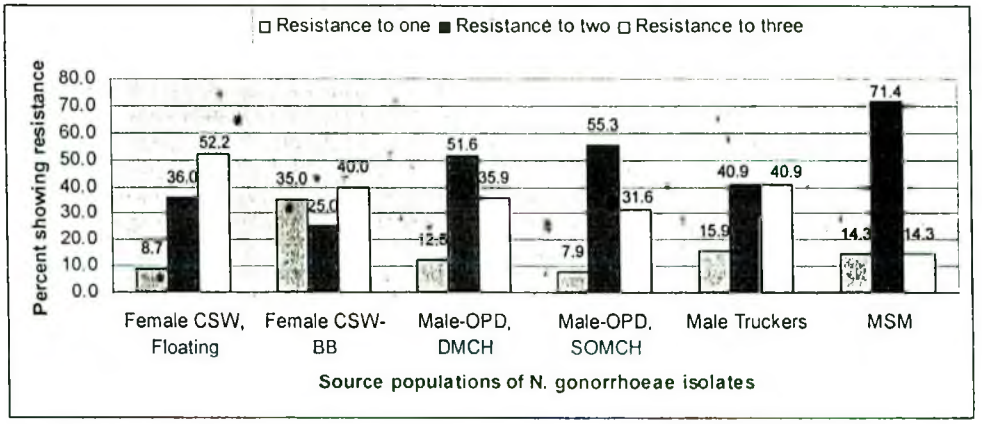


Figure 4.6: Comparison of rates of resistance of *N. gonorrhoeae* isolates to multiple antibiotics and the source populations

4.2.3: Identification of the penicillinase producing *N. gonorrhoeae* (PPNG) strains by paper acidometric method

Among the 495 *N. gonorrhoeae* isolates examined, 178 (36.0%) were found to produce penicillinase (penicillinase producing *N. gonorrhoeae*, PPNG strains) by paper acidometric method. (Table 4.37)

The rate of PPNGs in the first year of study (2003) was 34.5% and it was raised to 36.0% in the last year (2006). The differences in the rate of PPNG isolates in different years during 2003 to 2006 is not significant (chi-square test, p value >0.50). (Table 4.38)

Relationship between penicillin MIC and the identified PPNG stains (by paper acidometric method) showed that the highest rate of penicillinase production occurred among the *N. gonorrhoeae* isolates (PPNG) having penicillin MIC 64.0 μg/ml (100.0%) and the rate of PPNG strains increased gradually with increase in the penicillin MICs. A few of the PPNGs identified by paper acidometric method (6, 3.4%) also found to occur among the isolates having penicillin MIC $\leq$ 2.0 μg/ml which were also tested by PCR method for confirmation (results shown under PPNG plasmid identification by PCR). (Table 4.39)

Table 4.37: Penicillinase production by the *N. gonorrhoeae* isolates as detected by paper acidometric method

Result of penicillinase production	*Frequency*	*Percent*
Positive (PPNG¶)	178	36.0
Negative	317	64.0
Total	495	100.0

¶PPNG: penicillinase producing *N. gonorrhoeae*

Table 4.38: Year-wise distribution of the PPNG strains identified by paper acidometric method

Year wise distribution	*No. (%) of N. gonorrhoeae studied*		
	Total	*PPNG*	*Non-PPNG*
2003	319 (100.0)	110 (34.5)	209 (65.5)
2004	80 (100.0)	30 (37.5)	50 (62.5)
2005	50 (100.0)	18 (36.0)	32 (64.0)
2006	46 (100.0)	20 (43.5)	26 (56.5)
Total	495 (100.0)	178 (36.0)	317 (64.0)

Chi-square= 1.1 at df=3 yielding p value > 0.50

Table 4.39: Relationship between penicillin MIC and penicillinase production (PPNG) identified by paper acidometric method

Penicillin MIC[¶] *(µg/ml)*	*Number of N. gonorrhoeae investigated*	*PPNG*[§] *strains*	
		Number	*Percent*
0.06	84	4	4.8
0.12	13	0	0
0.25	40	0	0
0.50	43	1	2.3
1.00	60	1	2.3
2.00	59	24	40.7
4.00	64	40	62.5
8.00	37	27	73.0
16.00	28	22	78.6
32.00	63	55	87.3
64.00	4	4	100.0
Total	495	178	36.0

[¶]MIC: minimum inhibitory concentration

[§]PPNG: penicillinase producing *N. gonorrhoeae* by paper acidometric method

4.2.4: Identification of PPNG Plasmids by PCR

A total of 169 (34.1% of total 495 *N. gonorrhoeae* isolates and 66.3% of the 255 penicillin-resistant isolates) were found positive for PPNG plasmids by PCR (polymerase chain reaction) method. (Table 4.40)

Almost all of the PPNG plasmids identified were "Africa" type (161, 95.3%) yielding 3.1 kb plasmids. (Figure 4.7) A few (8, 4.7%) among the identified PPNG plasmids showed indefinable double bands (one at 3 kb and other at 1.5 kb), images of which were supposed to be of Africa type plasmids, digested into two pieces due to technical errors (personal communication, Professor Jo-Anne R. Dillon, May 10, 2009, Appendix IX). (Figure 4.8)

Relationship between results of PCR for PPNG plasmids and penicillin MIC showed that all of the *N. gonorrhoeae* strains having PPNG plasmids had penicillin MIC $\geq$2.0 μg/ml and the highest number of *N. gonorrhoeae* strains showing PPNG plasmids (55, 32.5%) had penicillin MIC 32.0 μg/ml. All of the 6 PPNG strains identified by paper acidometric method with penicillin MIC $\leq$2.0 μg/ml were negative for PPNG plasmids by PCR method and were excluded from the list of PPNG. (Table 4.41)

Table 4.40: The PPNG plasmids identification by PCR method

PPNG [§] plasmids by PCR [¶]	*Frequency*	*Percent*
Positive	169	34.1
Negative	92	18.6
Not applicable (penicillin MIC* ≤2.0 µg/ml)	234	47.3
Total	495	100.0

[§]PPNG: penicillinase (plasmid-mediated) producing *Neisseria gonorrhoeae*

[¶]PCR: polymerase chain reaction

*MIC: minimum inhibitory concentration

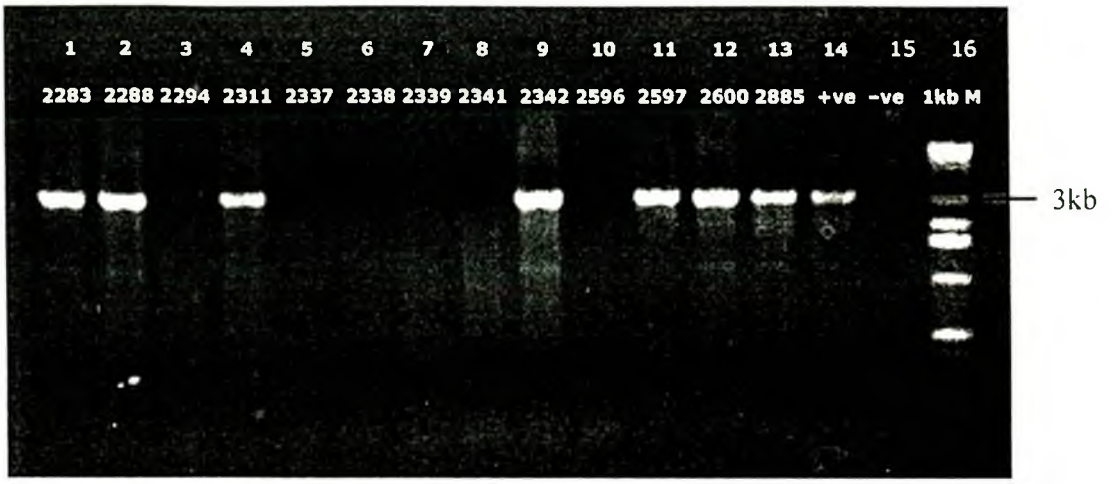


Figure 4.7: Agarose gel image of the PPNG PCR products

Lanes 1, 2, 4, 9, 11, 12, 13 & 14 showing 3.1 kb Africa type plasmids

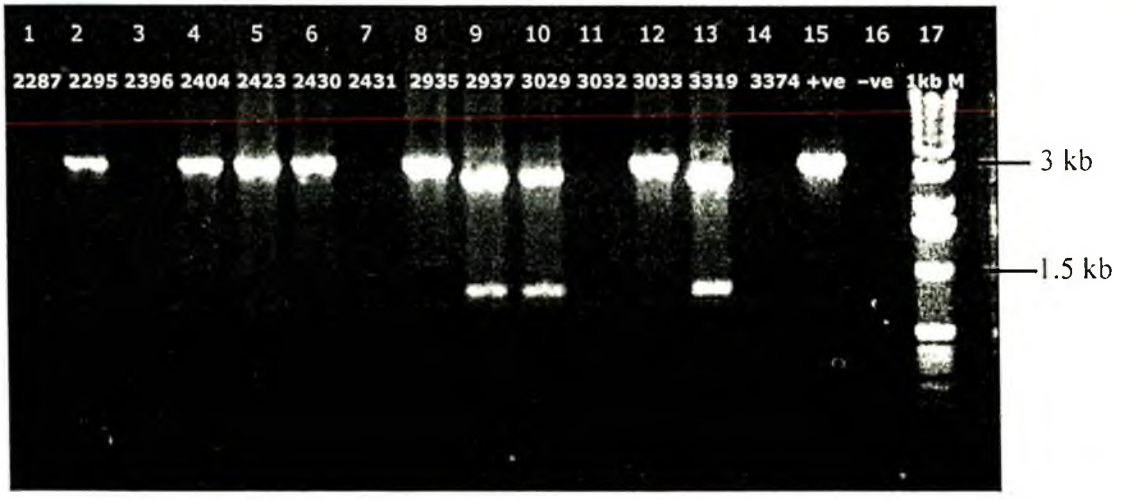


Figure 4.8: Agarose gel image of the indefinable PPNG PCR products

Lanes 9, 10 & 13 showing indefinable double bands

Lanes 2, 4, 5, 6, 8 & 12 showing 3.1 kb Africa type plasmids

Table 4.41: Relationship between penicillin MIC and PPNG plasmids identified by PCR method

Penicillin MIC[¶] *(µg/ml)*	*No. (%) of N. gonorrhoeae isolates*			
	Total	*PCR*[§] *result for PPNG* plasmids*		
		Positive	*Negative*	*NA***
0.06	84 (17.0)	0 (0)	4***(4.3)	80 (34.2)
0.12	13 (2.6)	0 (0)	0 (0)	13 (5.5)
0.25	40 (8.1)	0 (0)	0 (0)	40 (17.1)
0.50	43 (8.7)	0 (0)	1*** (1.1)	42 (18.0)
1.00	60 (12.1)	0 (0)	1*** (1.1)	59 (25.2)
2.00	59 (11.9)	22 (13.0)	37 (40.2)	0 (0)
4.00	64 (12.9)	41 (24.3)	23 (25.0)	0 (0)
8.00	37 (7.5)	27 (16.0)	10 (10.9)	0 (0)
16.00	28 (5.7)	20 (11.8)	8 (8.7)	0 (0)
32.00	63 (12.7)	55 (32.5)	8 (8.7)	0 (0)
64.00	4 (0.8)	4 (2.4)	0 (0)	0 (0)
Total	495 (100.0)	169 (100.0)	92 (100.0)	234 (100.0)

[¶] MIC: minimum inhibitory concentration;

[§] PCR: polymerase chain reaction;

* PPNG: penicillinase producing *Neisseria gonorrhoeae;*

** NA: not applicable, *N. gonorrhoeae* isolates having penicillin MIC ≤ 2.00 µg/ml are not resistant to penicillin and were not tested for PPNG plasmids;

*** Six *N. gonorrhoeae* isolates with penicillin MIC ≤ 2.00 µg/ml were found positive by paper acidometric method (PPNG) and tested by PCR for confirmation of having any PPNG plasmid.

4.2.4a: Relationship between paper acidometric method and PCR for identification of PPNG strains

Among the 178 PPNG strains identified by paper acidometric method, 168 (94.4%) were positive for PPNG plasmids by PCR method. Again, among 317 non-PPNG strains by paper acidometric method, 1 (0.3%) was found positive by PCR method. (Table 4.42)

4.2.4b: Measures of validity of paper acidometric method for identification of PPNG strains

To calculate validity (sensitivity, specificity, positive predictive value, negative predictive value and predictive accuracy of the test) of paper acidometric method for identification of PPNG strains, results of the PCR was considered as the gold standard. Therefore, number of true positive (a) cases was 168, false positive (b) cases was 10, false negative (c) was 1 and true negative (d) was 316 (82+234). Thus, the calculated values of validity of paper acidometric method by sensitivity, specificity, positive predictive value, negative predictive value and predictive accuracy were 99.4%, 96.9%, 94.4%, 99.7% and 97.8% respectively. (Table 4.43)

4.2.4c: Relationship between PPNG plasmid and antimicrobial resistance

Majority of the 255 penicillin-resistant *N. gonorrhoeae* isolates were plasmid-mediated (PPNG) (169, 66.3%). (Figure 4.9) (Table 4.44)

Whereas, majority of the tetracycline-resistant (64.1%) and ciprofloxacin-resistant (63.3%) *N. gonorrhoeae* isolates were not infected by PPNG plasmid. (Table 4.44)

Table 4.42: Relationship between paper acidometric method and PCR for identification of PPNG stains

PPNG by paper acidometric method		*Result of PPNG plasmids by PCR*[§]	
Result	*Number of N. gonorrhoeae isolates*	*Positive*	*Percent*
Positive (PPNG[¶])	178	168	94.4
Negative	317	1	0.3
Total	495	169	34.1

[¶]PPNG: penicillinase producing *Neisseria gonorrhoeae*

[§]PCR: polymerase chain reaction

Table 4.43: The two-by-two table for calculating measures of validity of paper acidometric method (PCR method considered as gold standard) for identification of PPNG strains

PPNG by paper	*Number of N. gonorrhoeae isolates*		
	PCR result for PPNG plasmids		*Total*
	Positive	*Negative*	
Positive	168 (a)	10 (b)	178 (a+b)
Negative	1 (c)	316 (d)	317 (c+d)
Total	169 (a+c)	326 (b+d)	495 (a+b+c+d)

Calculated values of validity measures of paper acidometric method:

Sensitivity (a/a+c): 99.4%

Specificity (d/b+d): 96.9%

Positive predictive value (a/a+b): 94.4%

Negative predictive value (d/c+d): 99.7%

Predictive accuracy (a+d)/(a+b+c+d): 97.8%

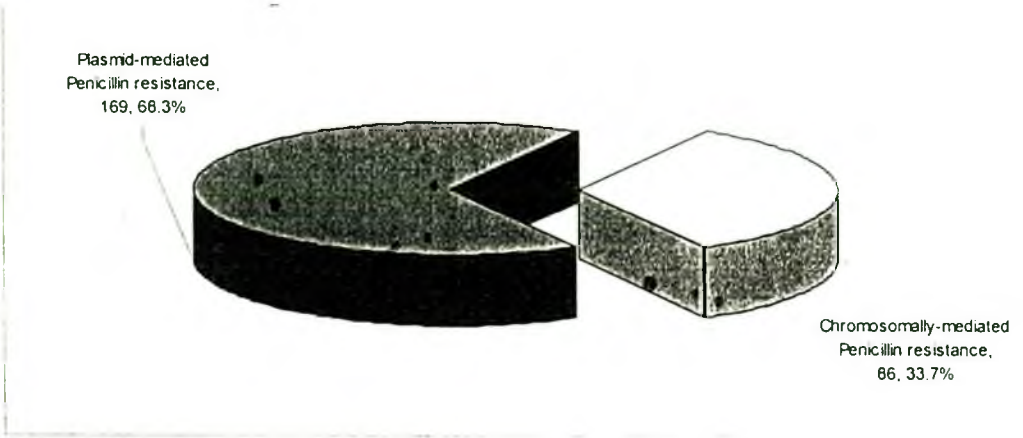


Figure 4.9: Pie diagram showing the types of penicillin resistance found among the *N. gonorrhoeae* isolates (n=255)

Table 4.44: Relationship between PPNG plasmids infection and antimicrobial resistance

PCR result for PPNG¶ plasmid	*No. (%) of N. gonorrhoeae isolates resistant to-*		
	Penicillin	*Tetracycline*	*Ciprofloxacin*
Positive (PPNG)	169 (66.3)	156 (35.9)	165 (36.7)
Negative (Non-PPNG)	86 (33.7)	279 (64.1)	285 (63.3)
Total	255 (100.0)	435 (100.0)	450 (100.0)

¶PPNG: penicillinase (plasmid-mediated) producing *N. gonorrhoeae*

4. 2.5: Identification of TRNG Plasmids by PCR

Among the 495 *N. gonorrhoeae* isolates, 396 (80.0%) strains, having tetracycline MIC ≥ 8.0μg/ml, were investigated for TRNG plasmids by PCR (polymerase chain reaction). A total of 382 (77.2%) of the isolates were found infected with TRNG plasmids. Two types of TRNG plasmids were identified of which almost all (377/382, 98.7 %) were Dutch type (700bp) and a few (5/382, 1.3%) were "American type" (1600 bp). (Table 4.45) (Figures 4.10 and 4.11)

4.2.5a: Relationship between TRNG plasmid and antimicrobial resistance

Observing the rate of plasmid infection among the tetracycline resistant isolates of *N. gonorrhoeae* (n=435), it was found that majority of the tetracycline resistant isolates were plasmid-mediated (382, 88.1%) and the remaining 54 (12.4%) were chromosomally-mediated. (Figure 4.12)

Year-wise rates of TRNG plasmid infection of the *N. gonorrhoeae* showed that the highest rate of plasmid infection occurred in 2004 (69, 86.3%), followed by that in 2006 (39, 84.8%). The rate of TRNG strains increased by 11.4% from 2003 to that in 2006, and the difference in rate is found significant statistically (chi square test p value <0.05). (Table 4.46)

Relationship between tetracycline MIC and the TRNG plasmid infection showed that the highest number (186, 49.3%) of Dutch type plasmids were found among *N. gonorrhoeae* isolates having tetracycline MIC of 32.0 μg/ml, followed by those having tetracycline MIC 16.0 μg/ml (98, 26.0%). Majority of the TRNG plasmids (329/382, 86.1%) are present among the isolates having tetracycline MIC ≥16.0μg/ml, while some 53 (14.1%) of the TRNG plasmids (all Dutch type) were present among the isolates having tetracycline MIC 8.0 μg/ml. (Table 4.47)

Table 4.45: The TRNG plasmids identified by PCR method

Result of TRNG[¶] PCR[§]	*Frequency*	*Percent*
Positive	382	77.2
At 700 bp (Dutch type)	377	98.7
At 1600 bp (American type)	5	1.3
Negative	14	2.8
Not applicable*	99	20.0
Total	495	100.0

[¶]TRNG: tetracycline resistant *N. gonorrhoeae;*

[§] PCR: polymerase chain reaction

* *N. gonorrhoeae* isolates having tetracycline MIC <8µg/ml

Table 4.46: Year-wise distribution of the TRNG strains identified among the *N. gonorrhoeae* isolates

Year wise distribution	*No. (%) of N. gonorrhoeae studied*		
	Total	*TRNG*	*Non-TRNG*
2003	319 (100.0)	234 (73.4)	85 (26.6)
2004	80 (100.0)	69 (86.3)	11 (13.7)
2005	50 (100.0)	40 (80.0)	10 (20.0)
2006	46 (100.0)	39 (84.8)	7 (15.2)
Total	495 (100.0)	382 (77.2)	113 (22.8)

Chi-square= 8.1 at df=3 yielding p value < 0.05

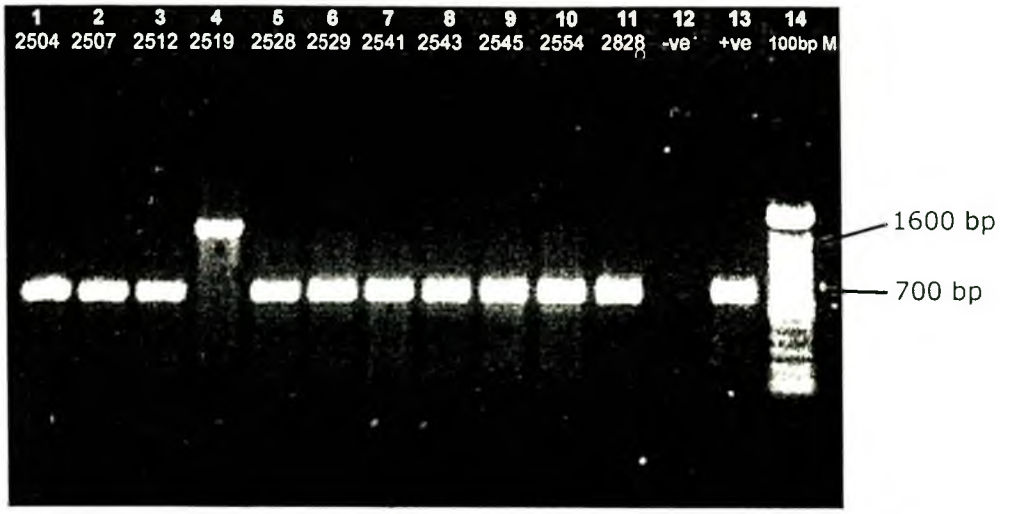


Figure 4.10: Agarose gel image of the TRNG PCR products

Lanes 1,2,3,5,6,7,8,9,10,11: Dutch type TRNG plasmids at 700 bp

Lane 4: American type at 1600 bp

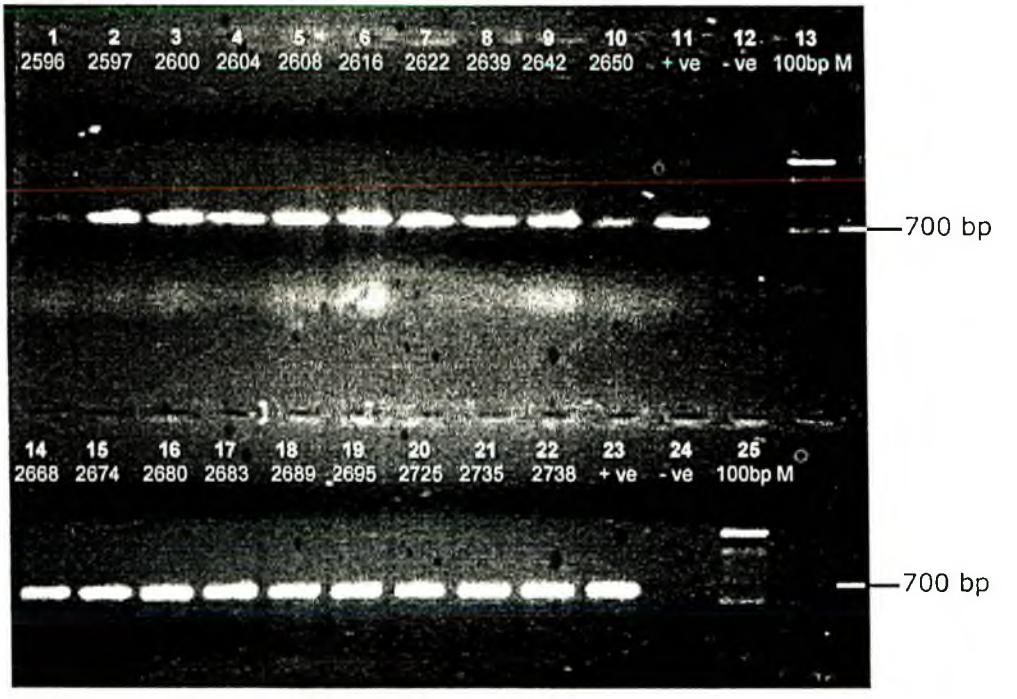


Figure 4.11: Agarose gel image of the TRNG PCR products

Lanes 2, 3, 4, 5, 6, 7, 8, 9,10,14, 15, 16,17,18,19, 20, 21 & 22: Dutch type TRNG plasmids at 700 bp

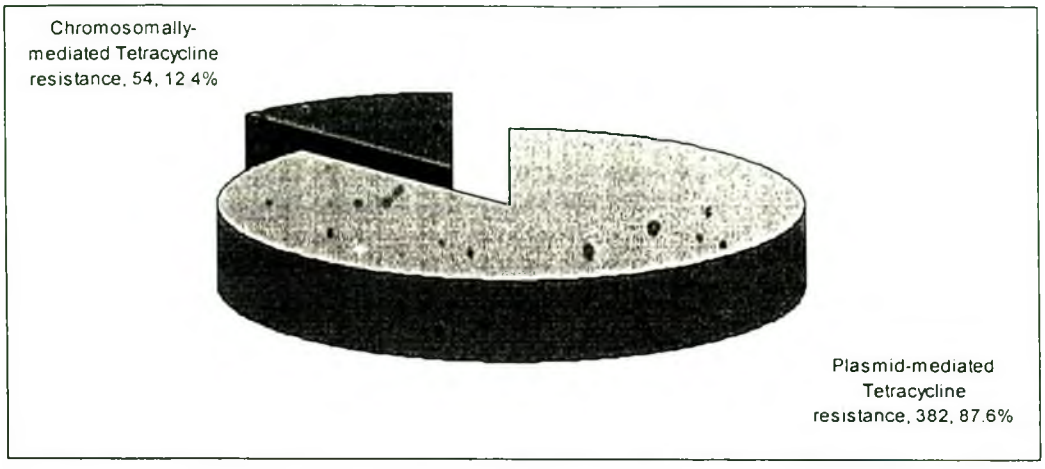


Figure 4.12: Pie diagram showing distribution of the *N. gonorrhoeae* isolates by mechanism of tetracycline resistance (n=436)

Table 4.47: Relationship between tetracycline MIC and the TRNG strains among the *N. gonorrhoeae* isolates

Tetracycline MIC (µg/ml)	*No. (%) of N. gonorrhoeae showing TRNG PCR results*			
	Total	*Positive (TRNG plasmids)*	*Negative*	*Not applicable*[¶]
0.25	19 (3.8)	0 (0)	0 (0)	19 (100.0)
0.50-1.00	40 (8.1)	0 (0)	0 (0)	40 (100.0)
2.00-4.00	40 (8.1)	0 (0)	0 (0)	40 (100.0)
8.00	56 (11.3)	53 (94.6)	3 (5.4)	0 (0)
16.0	106 (21.4)	101 (92.5)	5 (4.7)	0 (0)
32.0	190 (38.4)	186 (49.3)	4 (28.6)	0 (0)
64.0	38 (7.7)	36 (89.5)	2 (5.2)	0 (0)
128.0	6 (1.2)	6 (100.0)	0 (0)	0 (0)
Total	495 (100.0)	382 (76.2)	14 (2.8)	99 (20.0)

[¶]*N. gonorrhoeae* isolates having tetracycline MIC <8.0 µg/ml were not tested for TRNG by PCR

4.2.5b: Relationship between TRNG plasmids and source population

A few of the tetracycline-resistant (Tet-R) *N. gonorrhoeae* isolates carrying TRNG plasmids were the American type (1.1%) and majority others were the Dutch type (86.5%). All of the few American type TRNG plasmids were found to occur among the isolates from floating female CSWs. (Table 4.48)

4.2.5c: Relationship between PPNG and TRNG plasmids

Observing the relationship of the TRNG plasmids and PPNG plasmids content of the *N. gonorrhoeae* isolates, it was found that more than two-thirds (150, 89.3%) of the isolates were found to harbour both PPNG and TRNG plasmids. (Table 4.49)

4.2.5d: Types of N. gonorrhoeae isolates by mechanism of resistance to penicillin and tetracycline

Distributing the *N. gonorrhoeae* isolates into the defined types of plasmid content (criteria defined in Material and Methods section, vide Table 3.6), it was found that the highest (230, 46.5%) number of isolates of the organism was TRNG, followed by PPNG-TRNG (152, 30.7%). (Table 4.50)

The year-wise rate of plasmid infection among the *N. gonorrhoeae* isolates showed that the single plasmid infection (either PPNG for penicillin resistance or TRNG for tetracycline resistance) had a fall at the last year (PPNG from 4.7% in 2003 to 0% in 2006 and TRNG from 45.8% in 2003 to 41.3% in 2006), whereas double infection of the plasmids (both PPNG and TRNG) was on a gradual rise from 27.6% in 2003 to 43.5% in 2006. (Figure 4.13)

Table 4.48: Relationship between TRNG plasmids and source population of the tetracycline-resistant (Tet-R) *N. gonorrhoeae* isolates

Isolate source	*No. (%) of Tet-R** N. gonorrhoeae isolates (n=436)*			
	Total	*Plasmid-mediated*		*No plasmid*
		American	*Dutch*	
Female CSW[¶]-floating	291 (66.7)	5 (1.7)	255 (87.6%)	31 (10.7)
Female CSW- bb[§]	16 (3.7)	0 (0)	14 (87.5)	2 (12.5)
Male-OPD[¶§], DMCH[¶¶]	57 (13.0)	0 (0)	48 (84.2)	9 (15.8)
Male-OPD, SOMCH[§§]	33 (7.6)	0 (0)	25 (75.8)	8 (24.2)
Male truckers	33 (7.6)	0 (0)	30 (90.9)	3 (9.1)
MSM*	6 (1.4)	0 (0)	5 (83.3)	1 (16.7)
Total	436 (100.0)	5 (1.1)	377 (86.5)	54 (12.4)

[¶] CSW: commercial sex worker; [§] bb- brothel-based;

[¶§] OPD: outpatients department;

[¶¶] DMCH: Dhaka Medical College Hospital;

[§§] SOMCH: Sylhet MAG Osmani Medical College Hospital;

* MSM: Male individuals having sex with male;

** Tet-R: tetracycline resistant

Table 4.49: Relationship between PPNG and TRNG strains among *N. gonorrhoeae* isolates

PPNG[¶]-PCR result*	*No. (%) of N. gonorrhoeae isolates*			
	Total	*TRNG[§] PCR results*		
		Positive	*Negative*	*Not applicable*
Positive	168 (100.0)	150 (89.3)	0 (0)	18 (10.7)
Negative	89 (100.0)	69 (77.5)	5 (5.6)	15 (16.9)
Not applicable	238 (100.0)	163 (68.5)	11 (4.6)	64 (26.9)
Total	495 (100.0)	382 (77.2)	16 (3.2)	97 (19.6)

[¶]PPNG- penicillinase (plasmid-mediated) producing *N. gonorrhoeae*

[§]TRNG- tetracycline resistant (plasmid-mediated) *N. gonorrhoeae*

*PCR- polymerase chain reaction

Table 4.50: Cumulative percent of types of *N. gonorrhoeae* isolates by plasmid content

Plasmid-mediated resistance types	*No. of isolates*	*Percent*	*Cumulative percent*
PPNG[¶]	18	3.6	3.6
TRNG[§]	230	46.5	50.1
PPNG-TRNG[¶¶]	152	30.7	80.8
Non-PPNG[§§]	6	1.2	82.0
Non-TRNG[¶§]	36	7.3	89.3
Non-PPNG-non-TRNG*	13	2.6	91.9
Not applicable	40	8.1	100.0
Total	495	100.0	

[¶] PPNG- *N. gonorrhoeae* containing single plasmid for penicillin resistance

[§] TRNG- *N. gonorrhoeae* containing single plasmid for tetracycline resistance

[¶¶] PPNG-TRNG- *N. gonorrhoeae* containing double plasmids for both penicillin and tetracycline resistance

[§§] Non-PPNG- *N. gonorrhoeae* with chromosomally-mediated resistance to penicillin and no resistance to tetracycline

[¶§] Non-TRNG- *N. gonorrhoeae* with chromosomally-mediated resistance to tetracycline and no resistance to penicillin

* Non-PPNG-non-TRNG- *N. gonorrhoeae* with chromosomally-mediated resistance to both penicillin and tetracycline

** Not applicable- *N. gonorrhoeae* with no resistance to penicillin and tetracycline

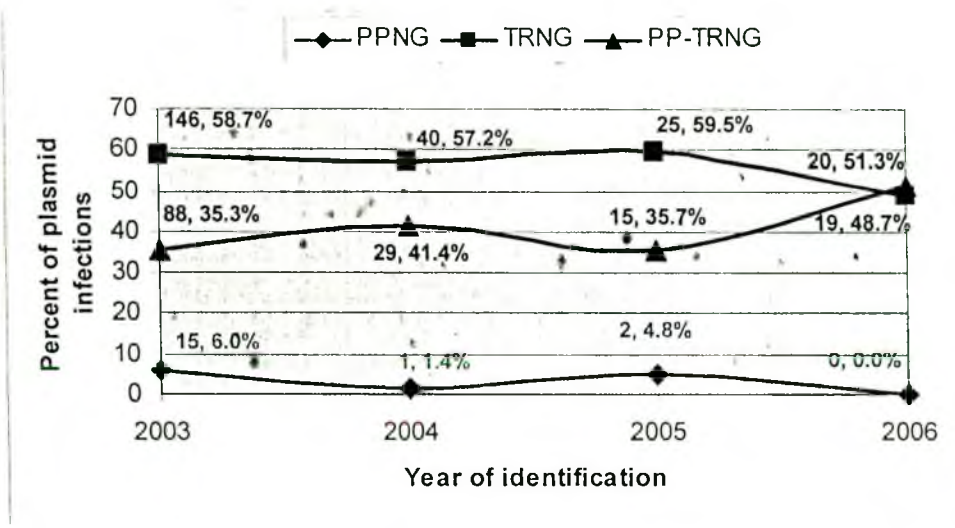


Figure 4.13: Year-wise distribution of the rate of plasmid infection among the *N. gonorrhoeae* isolates (n=495)

4. 2.6: Serotypes and serovars of the *N. gonorrhoeae* isolates

Majority of the investigated *N. gonorrhoeae* isolates (142/ 239, 59.4%) were type B (Figure 4.15) and a total of 41 different serovars were identified. Among the serovars, "Arst" was found to include the highest number of isolates (46/239, 19.2%) in a combined list of the all serotypes, followed by Bropt (22, 9.2%) and Bopt (18. 7.5%). Only 5 among the serovars namely "Arst", "Bropt", "Bopt" "Arost" and "Brop" included nearly 50% (136, 49.3%) of the isolates. (Table 4.51)

Considering serovars of the type A, "Arst" (46/88, 52%) was at top on the list, followed by "Arost" (17, 19%) and "Ast" (12, 14%). (Figure 4.16) On the other hand among the serovars of Type B, "Bropt" (22/142, 15%) was represented by highest number of isolates, followed by "Bopt" (18, 13%), "Brop" (15, 11%). (Figure 4.17)

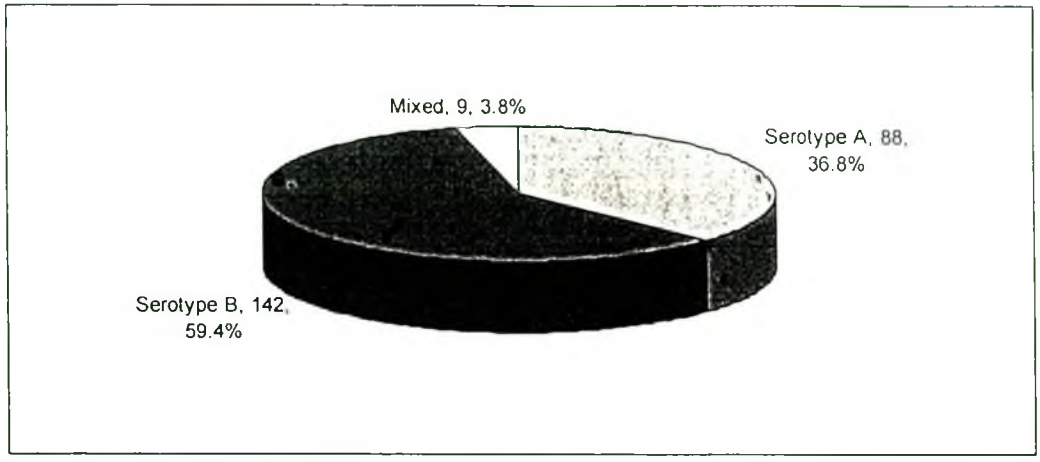


Figure 4.15: Pie diagram showing frequency of serotypes among the *N. gonorrhoeae* isolates (n=239)

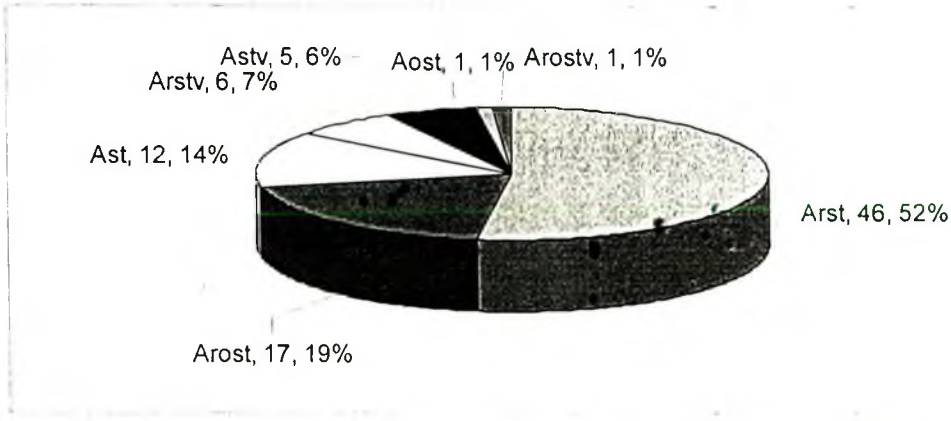


Figure 4.16: Pie diagram showing distribution of type A serovars

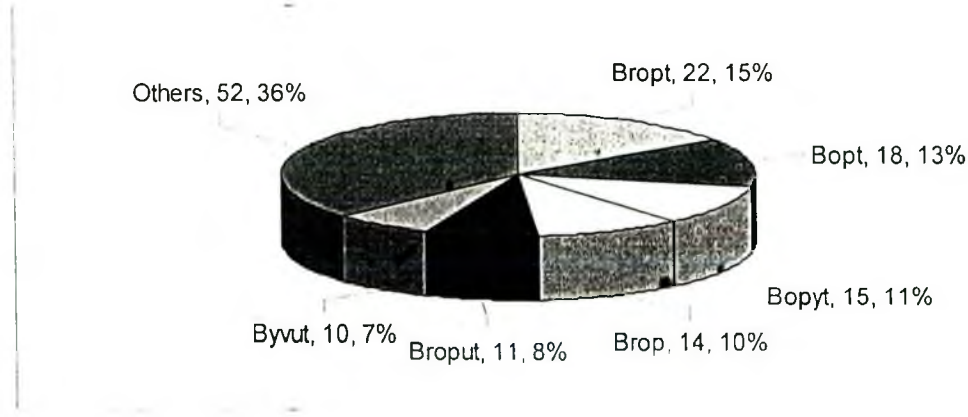


Figure 4.17: Pie diagram showing distribution of type B serovars

Table 4.51: Cumulative frequency distribution of serovars of the *N. gonorrhoeae* isolates

Sl no	*Serovar*	*Frequency*	*Valid Percent*	*Cumulative Percent*
1	Arst	46	19.2	19.2
2	Bropt	22	9.2	28.4
3	Bopt	18	7.5	35.9
4	Arost	17	7.1	43.0
5	Brop	15	6.3	49.3
6	Bopyt	14	5.9	55.2
7	Ast	12	5.0	60.2
8	Broput	11	4.6	64.8
9	Byvut	10	4.2	69.0
10	Bpyvut	8	3.4	72.4
11	Arstv	6	2.6	75.0
12	AvBx	6	2.6	77.6
13	Astv	5	2.1	79.7
14	Bro	4	1.7	81.4
15	Bropys	4	1.7	83.1
16	Brpyt	4	1.7	84.8
17	AstvBx	3	1.3	86.1
18	Bopst	3	1.3	87.4
19	Brpyus	3	1.3	88.7
20	Brpyvu	3	1.3	90.0
21	Bopvt	2	0.8	90.8
22	Bopyst	2	0.8	91.6
23	Bropvu	2	0.8	92.4
24	Bys	2	0.8	93.2
25	Aost	1	0.4	93.6
26	Arostv	1	0.4	94.0
27	Bop	1	0.4	94.4
28	Bopyvs	1	0.4	94.8
29	Bopyvu	1	0.4	95.2
30	Bos	1	0.4	95.6
31	Bot	1	0.4	96.0
32	Bpyst	1	0.4	96.4
33	Bpyt	1	0.4	96.8
34	Bropyt	1	0.4	97.2
35	Bropyv	1	0.4	97.6
36	Brot	1	0.4	98.0
37	Brpt	1	0.4	98.4
38	Bryst	1	0.4	98.8
39	Byst	1	0.4	99.2
40	Byus	1	0.4	99.6
41	Byvutx	1	0.4	100.0
	Total	239	100.0	

4.2.6a: Relationship between serotypes and source populations of the N. gonorrhoeae isolates

Relationship between serotypes of the *N. gonorrhoeae* isolates and the source populations from whom the organisms were isolated showed that the organisms isolated from majority of the populations were serotype B (54.5% isolated from female CSW-floating, 68.8% isolated from male-OPD, Dhaka, 83.3% isolated from male-OPD, Sylhet, 62.5% isolated from Male-Truckers). (Table 4.52)

4.2.6b: Relationship between serovars and source populations of the N. gonorrhoeae isolates

Distributing the serovars of the *N. gonorrhoeae* among source populations from which the organisms were isolated, it was found that among the female CSWs-floating, "Arst" (22.5%) was the highest, followed by "Arost" (8.3%), and "Bopt"/ "Bopyt" (7.1% each). Among the male-OPD, Dhaka, "Broput" (12.5%) was the highest, among the male-OPD, Sylhet, "Bropt" (25.0%) was the highest, and among the male-truckers, "Arst" (18.8%) was the highest. Only two among the serovars ("Arst" and "Bopt") were found in all of the source populations. (Table 4.53)

4.2.6c: Relationship between serotypes and antimicrobial resistance of the N. gonorrhoeae isolates

Comparing the rates of resistance of *N. gonorrhoeae* among different serotypes it was found that the highest rate of penicillin (80.7%) and tetracycline (89.8%) resistance was found among the serotype A, whereas the highest rate of ciprofloxacin resistance was found among the serotype B (100.0%). (Figure 4.18)

Table 4.52: Distribution of the *N. gonorrhoeae* serotypes into source populations

N. gonorrhoeae serotype	*No. (%) of N. gonorrhoeae isolates distributed into source populations*						
	Female CSW-floating [§]	*Female CSW-bb*[¶¶]	*Male-OPD, DMCH*[§§]	*Male-OPD, SOMCH*[¶§]	*Male-truckers**	*MSM***	*Total*
Type A	65 (41.7)	04 (50.0)	09 (28.1)	04 (16.7)	05 (31.3)	01 (33.3)	88 (36.8)
Type B	85 (54.5)	03 (37.5)	22 (68.8)	20 (83.3)	10 (62.5)	02 (66.7)	142 (59.4)
Mixed type	06 (3.8)	01 (12.5)	01 (3.1)	0 (0)	01 (6.2)	0 (0)	09 (3.8)
Total	156 (100.0)	08 (100.0)	32 (100.0)	24 (100.0)	16 (100.0)	03 (100.0)	239 (100.0)

[¶] Source populations: population groups from which the *N. gonorrhoeae* were isolated;

[§] CSW-floating: commercial sex workers engaged in unlawful sex work in the parlours. hotels, parks etc in the Dhaka capital City;

[¶¶]CSW-bb: CSWs engaged in the brothel at Gualanda ghat (the Padma river cruisine) nearby Faripur district City;

[§§]Male-OPD, DMCH: male individuals attending outpatients Department of skin and venereal diseases of Dhaka Medical college Hospital, Dhaka;

[¶§]Male-OPD, SOMCH: male individuals attending outpatients Department of skin and venereal diseases of Sylhet MAG Osmani Medical college Hospital, Sylhet;

* Male truckers: long-distant male truckers attending the temporary medical centre at Tejgaon central truck stand, Dhaka;

**MSM: male individuals having sex with male attending the medical centre for the MSM at Sylhet City.

Table 4.53: Distribution of the *N. gonorrhoeae (Ng)* serovars among source populations

Ng Serotype	*Ng Serovar*	*No. (%) of N. gonorrhoeae isolates distributed into source populations*						
		CSW-floating	*CSW-bb*	*Male-OPD, Dhaka*	*Male-OPD, Sylhet*	*Male-truckers*	*MSM*	*Total*
Type A (n=88)	Arst	35 (22.5)	1 (12.5)	3 (9.4)	3 (12.5)	3 (18.8)	1 (33.3)	46 (19.2)
	Arost	13 (8.3)	1 (12.5)	2 (6.3)	0 (0)	1 (6.3)	0 (0)	17 (7.1)
	Ast	8 (5.1)	2 (25.0)	1 (3.1)	0 (0)	1 (6.3)	0 (0)	12 (5.0)
	Others <10	9 (5.8)	0 (0)	3 (9.4)	1 (4.2)	0 (0)	0 (0)	13 (5.4)
Type B (n=142)	Bropt	10 (6.4)	2 (25.0)	2 (6.3)	6 (25.0)	2 (12.5)	0 (0)	22 (9.2)
	Bopt	11 (7.1)	1 (12.5)	2 (6.3)	1 (4.2)	2 (12.5)	1 (33.3)	18 (7.5)
	Bopyt	11 (7.1)	0 (0)	1 (3.1)	1 (4.2)	2 (12.5)	0 (0)	15 (6.3)
	Brop	10 (6.4)	0 (0)	1 (3.1)	2 (8.3)	1 (6.3)	0 (0)	14 (5.9)
	Broput	5 (3.2)	0 (0)	4 (12.5)	1 (4.1)	1 (6.2)	0 (0)	11 (4.6)
	Byvut	6 (3.8)	0 (0)	3 (9.3)	0 (0)	1 (6.2)	0 (0)	10 (4.2)
	Others <10	32 (20.5)	0 (0)	9 (28.1)	9 (37.5)	1 (6.2)	1 (33.4)	52 (21.8)
Mixed (n=9)	AstvBx	1 (0.6)	0 (0)	1 (3.1)	0 (0)	1 (6.2)	0 (0)	3 (1.3)
	AvBx	5 (3.2)	1 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	6 (2.5)
	Total	156 (100.0)	8 (100.0)	32 (100.0)	24 (100.0)	16 (100.0)	3 (100.0)	239 (100.0)

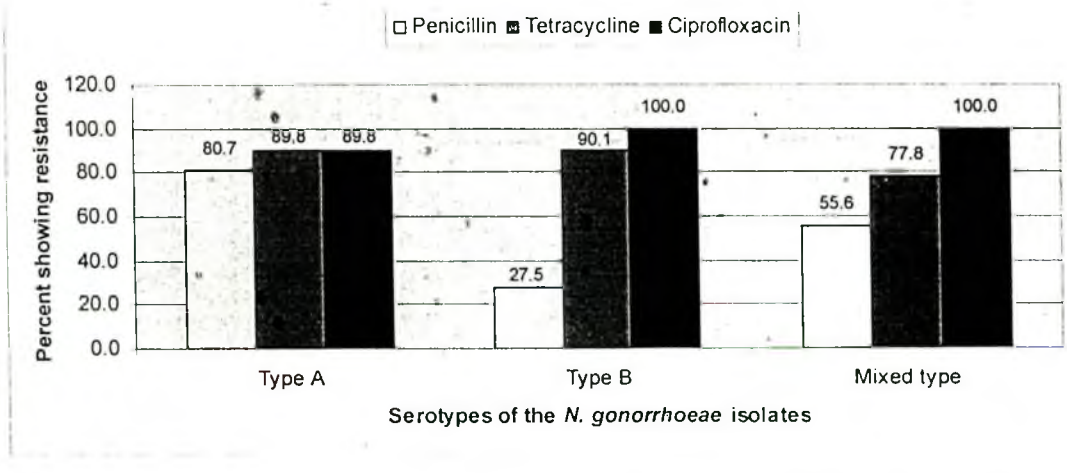


Figure 4.18: Relationship between the serotypes and the rates of resistance of the *N. gonorrhoeae* isolates

4.2.6d: Relationship between serovars and antimicrobial resistance of the N. gonorrhoeae isolates

Distributing the major serovars of the *N. gonorrhoeae* isolates resistant to penicillin, tetracycline and ciprofloxacin, it was found that the highest percent of penicillin-resistant organisms were of serovar "Ast" (83.3%), followed by "Arst" (35.8%) and "Bopt" (27.8%). Among the isolates showing resistance to tetracycline, the highest percent of organisms were of serovars "Ast", "Brop" and "Bopyt" (100.0% each). Among the ciprofloxacin-resistant isolates, the highest percent were of "Bopyt" (100.0%), "Broput" (100.0%) and "Brop" (93.3%). (Table 4.54)

4.2.6e: Relationship between serotypes and plasmid profile of the N. gonorrhoeae isolates

Distributing *N. gonorrhoeae* serotypes into different types of plasmid-mediated resistance of the organism, it was found that majority (5, 55.6%) of the PPNG isolates were type A, more than two-thirds (90, 80.3%) of the TRNG isolates were type B, and more than two-thirds (60, 81.1%) of the PPNG-TRNG isolates were Type A. (Table 4.55)

4.2.6f: Relationship between serovars and plasmid profile of the N. gonorrhoeae isolates

Distributing *N. gonorrhoeae* major serovars into different types of plasmid-mediated resistance of the organism, it was found that majority (5, 55.6%) of the few PPNG isolates were type A, more than two-thirds (90, 80.3%) of the TRNG isolates were type B, and more than two-thirds (60, 81.1%) of the PPNG-TRNG isolates were type A. (Table 4.56)

Table 4.54: Relationship between the major serovars and antimicrobial resistance of the *N. gonorrhoeae* isolates

Serotype	*Major serovars (with ≥10 isolates)*	*No. (%) of N. gonorrhoeae showing resistance to*		
		Penicillin	*Tetracycline*	*Ciprofloxacin*
Type A	Arst (n=46)	41 (35.8)	41 (35.8)	41 (35.8)
	Arost (n=17)	7 (6.1)	13 (76.5)	15 (88.2)
	Ast (n=12)	10 (83.3)	12 (100.0)	10 (83.3)
Type B	Bropt (n=22)	2 (9.1)	18 (81.8)	15 (68.2)
	Bopt (n=18)	5 (27.8)	15 (83.3)	16 (88.9)
	Brop (n=15)	2 (13.3)	15 (100.0)	14 (93.3)
	Bopyt (n=14)	3 (21.4)	14 (100.0)	14 (100.0)
	Broput (n=11)	3 (27.3)	3 (27.3)	11 (100.0)
	Byvut (n=10)	0 (0)	6 (60.0)	9 (90.0)

Table 4.55: Relationship between the serotypes and plasmid-mediated resistance of the *N. gonorrhoeae* isolates

Serotype	*No. (%) of N. gonorrhoeae & plasmid-mediated resistance types*				
	Total	*PPNG*	*TRNG*	*PP-TRNG*	*Non-PPNG-non-TRNG*
Type A	88 (36.8)	5 (55.6)	19 (17.0)	60 (81.1)	4 (9.1)
Type B	142 (59.4)	3 (33.3)	90 (80.3)	11 (14.9)	38 (86.4)
Mixed type	9 (3.8)	1 (11.1)	3 (2.7)	3 (4.0)	2 (4.5)
Total	239 (100.0)	9 (100.0)	112 (100.0)	74 (100.0)	44 (100.0)

Table 4.56: Relationship between the serovars and plasmid-mediated resistance of the *N. gonorrhoeae* isolates

Serotypes	*Serovars*	*No. (%) N. gonorrhoeae & plasmid profile*				
		PPNG	*TRNG*	*PP-TRNG*	*Non-PPNG - non-TRNG*	*Total*
Type A	Arst	4 (44.5)	6 (5.4)	35 (47.3)	1 (2.3)	46 (19.2)
	Arost	1 (11.1)	8 (7.1)	5 (6.8)	3 (6.8)	17 (7.1)
	Ast	0 (0)	4 (3.6)	8 (10.8)	0 (0)	12 (5.0)
Type B	Bropt	0 (0)	12 (10.7)	0 (0)	10 (22.7)	22 (9.2)
	Bopt	0 (0)	10 (8.9)	2 (2.7)	6 (13.6)	18 (7.5)
	Brop	0 (0)	14 (12.5)	0 (0)	1 (2.3)	15 (6.3)
	Bopyt	0 (0)	13 (11.6)	0 (0)	1 (2.3)	14 (5.9)
	Broput	0 (0)	10 (8.9)	1 (1.4)	0 (0)	11 (4.6)
	Byvut	0 (0)	5 (4.5)	0 (0)	5 (11.4)	10 (4.2)

4.2.7 Emergence of new serovars among the *N. gonorrhoeae* isolates

Majority (23/ 41, 56.1%) of the identified serovars had not been reported previously and considered new emergent. Among the 23 new serovars, "Ast" included the highest (12, 20.7%) number of *N. gonorrhoeae* isolates, followed by "Arstv" (6, 10.4%), and 12 of the new serovars included 81.3% of the isolates. The new serovars also found to include a total of 58 (24.3%) of the *N. gonorrhoeae* isolates tested. (Table 4.57)

4.2.7a: Relationship between new serovars and source population of the N. gonorrhoeae isolates

Relationship between the new serovars and source populations of the *N. gonorrhoeae* isolates showed that the highest percent new serovars were found among the isolates from male-OPD, DMCH, followed by those from female CSW-brothel based and male-OPD, SOMCH (each 25.0%). (Table 4.58)

4.2.7b: Relationship between new serovars and sex of the source population of the N. gonorrhoeae isolates

Relationship between the new serovars and sex of the source populations of the *N. gonorrhoeae* isolates showed that higher percent of the organisms of the new serovars were included among the male population. (Table 4.59)

4.2.7c: Relationship between new serovars and site of isolation of the N. gonorrhoeae isolates

Relationship between the new serovars and site of isolation of the *N. gonorrhoeae* isolates showed that the highest rate of new serovars were identified among the organism isolated in Gualanda, Faridpur (25.0%), followed by Dhaka (24.5%) and Sylhet (22.2%). (Table 4.60)

4.2.7d: Relationship between new Serovars and multiple antimicrobial resistance of the N. gonorrhoeae isolates

Relationship between the new serovars and multiple antimicrobial resistance of the *N. gonorrhoeae* isolates showed that the highest rate of new serovars were identified among the organisms having resistance to three antibiotics together (multidrug resistant, MDR) (39.2%). (Table 4.61)

4.2.7e: Relationship between new serovars and plasmid content of the N. gonorrhoeae isolates

Relationship between the new serovars and plasmid content of the *N. gonorrhoeae* isolates showed that the highest rate of new serovars were identified among the organisms possessing both PPNG and TRNG plasmids (36.5%), followed by those possessing only TRNG plasmids (19.6%). (Table 4.62)

Table 4.57: Newly emergent serovars of the *N. gonorrhoeae* isolates investigated

Sl no	*New serovars*	*Number of N. gonorrhoeae isolates*		
		Showing new serovars	*Percent*	*Cumulative percent*
1	Ast	12	20.7	20.7
2	Arstv	6	10.4	31.1
3	Astv	5	8.6	39.7
4	Bropys	4	6.9	46.6
5	Brpyt	4	6.9	53.5
6	AstvBx	3	5.2	58.7
7	Brpyus	3	5.2	63.9
8	Brpyvu	3	5.2	69.1
9	Bopvt	2	3.5	72.6
10	Bropvu	2	3.5	76.1
11	Bopyst	2	3.5	79.6
12	Aost	1	1.7	81.3
13	Arostv	1	1.7	83.0
14	Bop	1	1.7	84.7
15	Bopyvs	1	1.7	86.4
16	Bopyvu	1	1.7	88.1
17	Bot	1	1.7	89.8
18	Bpyst	1	1.7	91.5
19	Bpyt	1	1.7	93.2
20	Bropyv	1	1.7	94.9
21	Bryst	1	1.7	96.6
22	Byst	1	1.7	98.3
23	Byvutx	1	1.7	100.0
	Total	58	100.0	

Table 4.58: Relationship between new serovars and source population of the *N. gonorrhoeae* isolates

Source population[¶]	*Number of N. gonorrhoeae*		*Percent of N. gonorrhoeae in new serovars*
	Investigated	*Showing new serovars*	
Female CSW-floating[§]	156	38	24.4
Female CSW-brothel based[¶¶]	8	2	25.0
Male-OPD, DMCH[§§]	32	10	31.3
Male-OPD, SOMCH[¶§]	24	6	25.0
Male truckers*	16	2	12.5
MSM**	3	0	0
Total	239	58	24.3

[¶] Source population: population group from which the *N. gonorrhoeae* were isolated;

[§] CSW-floating: commercial sex workers engaged in unlawful sex work in the parlours, hotels, parks etc in the Dhaka capital City;

[¶¶]CSW- brothel-based: engaged in commercial sex work in a brothel at Gualanda ghat (the Padma river cruisine) nearby Faripur district City;

[§§]Male-OPD, DMCH: male individuals attending outpatients Department of skin and venereal diseases of Dhaka Medical college Hospital, Dhaka;

[¶§]Male-OPD, SOMCH: male individuals attending outpatients Department of skin and venereal diseases of Sylhet MAG Osmani Medical college Hospital, Sylhet;

* Male truckers: long-distant male truckers attending the temporary medical centre at Tejgaon central truck stand, Dhaka;

**MSM: male individuals having sex with male attending the medical centre for the MSM at Sylhet City.

Table 4.59: Relationship between new serovars and sex of the source population of the *N. gonorrhoeae* isolates

Sex of the source population	*N. gonorrhoeae isolates*		
	Number investigated	*Number showing new serovars*	*Percent showing new serovars*
Male	84	21	25.0
Female	155	37	23.9
Total	239	58	24.3

Table 4.60: Relationship between new serovars and site of isolation of the *N. gonorrhoeae* isolates

Site of isolation of N. gonorrhoeae	*N. gonorrhoeae isolates*		
	Number investigated	*Number showing new serovars*	*Percent showing new serovars*
Dhaka	204	50	24.5
Sylhet	27	6	22.2
Gualanda, Faridpur	8	2	25.0
Total	239	58	24.3

Table 4.61: Relationship between new serovars and multiple antimicrobial resistance of the *N. gonorrhoeae* isolates

Resistance of N. gonorrhoeae to multiple antimicrobial agents	*N. gonorrhoeae isolates*		
	Number investigated	*Number showing new serovars*	*Percent showing new serovars*
Resistance to 1 antibiotic	26	4	15.4
Resistance to 2 antibiotics	107	14	13.1
Resistance to 3 antibiotics (MDR)	102	40	39.2
Not applicable	4	0	0
Total	239	58	24.3

Table 4.62: Relationship between new serovars and plasmid content of the *N. gonorrhoeae* isolates

Type of plasmid	*N. gonorrhoeae isolates*		
	Number investigated	*Number showing new serovars*	*Percent showing new serovars*
PPNG (single plasmid for penicillinase)	9	1	11.1
TRNG (single plasmid for tetracycline resistance)	112	22	19.6
PPNG-TRNG (double plasmids)	74	27	36.5
Non-PPNG-non-TRNG (no plasmids)	36	6	16.7
Total	239	58	24.3

4.2.8: Genotypes of the *N. gonorrhoeae* by NG-MAST

Alleles of the *por* gene were identified in 8 of the *N. gonorrhoeae* isolates, of which majority were known (6/8, 75.0%), whereas alleles of the *tbpB* gene were identified in 12 of the *N. gonorrhoeae* isolates of which majority were found new (8/12, 66.7%). The genotype (sequence type, ST) with combination of the two gene alleles were identified in 7 of the *N. gonorrhoeae* isolates and all of them were found new. (Table 4.63)

The edited and trimmed DNA sequence data (for NG-MAST) of the gene loci for each of the new allele were compared in an alignment with the closest known matched alleles and it was found that the base substitutions were different in each of the new *por* or *tbpB* gene alleles. (Figures 4.19, 4.20, 4.21, 4.22, 4.23, 4.24, 4.25, 4.26, 4.27)

Relationship between the genotype and plasmid infection of the *N. gonorrhoeae* isolates showed that 3 (42.9%) among the isolates were infected by PPNG-TRNG double plasmids, whereas 3 (42.9%) were infected by Dutch type TRNG plasmids and remaining 1 (14.2%) had no plasmid infection.

Table 4.63: Gene alleles and genotypes (ST, sequence type) of the *N. gonorrhoeae*

N. gonorrhoeae strain ID[¶]	*Genes and allele numbers of the N. gonorrhoeae isolates*				*Genotype (sequence type)*
	por		*tbpB*		
	Primary	*Final*	*Primary*	*Final*	
MD[§]-2132	251	251 (known)	11 (86%)[¶§]	741 (NEW)	ST- 3512 (NEW)
MD[§]-2159	49	49 (known)	136	136 (known)	ST- 3507 (NEW)
MD[§]-2232	1061 (91%)[¶§]	2119 (NEW)	646 (75%)[¶§]	784 (NEW)	ST- 3508 (NEW)
MD[§]-2281	160	160 (known)	628 (82%)[¶§]	785 (NEW)	ST- 3509 (NEW)
MD[§]-2287	251	251 (known)	720 (94%)[¶§]	786 (NEW)	ST- 3510 (NEW)
MD[§]-2338	581 (99%)[¶§]	2258 (NEW)	82	82 (known)	ST- 3738 (NEW)
MD[§]-2423	251	251 (known)	ND*	ND*	ND*
MD[§]-2443	ND*	ND*	72	72 (known)	ND*
MD[§]-2596	184	184 (known)	637 (86%)[¶§]	787 (NEW)	ST- 3511 (NEW)
MD[§]-2642	ND*	ND*	747 (91%)[¶§]	788 (NEW)	ND*
MD[§]-2683	ND*	ND*	648 (89%)[¶§]	789 (NEW)	ND*
MD[§]-2725	ND*	ND*	81	81 (known)	ND*
MD[§]-2797	ND*	ND*	300 (77%)[¶§]	790 (NEW)	ND*

[¶]ID- Identification number;

[§]MD-Abbreviated case identity for female commercial sex works (floating)

[¶§] Closest match allele number, data shown in Appendix IV; percent indicates the rate of similarity of the DNA sequence of the *N. gonorrhoeae* isolate test allele with the closest matched known allele;

*ND- not done

***tbpB* gene locus (new allele 741) of *N. gonorrhoeae* isolate MD-2132**

(DNA sequence data edited and trimmed for NG-MAST)

```
122-CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGG-181
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGG

182-AAATCAAAGATCTCGACAACTTCAGCAATGCCGCCCAACTGGTTGTCGACGGCATTATGA-241
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     AAATCAAAGATCTCGACAACTTCAGCAATGCCGCCCAACTGGTTGTCGACGGCATTATGA

242-TTCCGCTCCTGCCCAATGATTCCGGAAGCGGGGGCAGTCAGGCAGATAAAGGTAAAAACG-301
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     TTCCGCTCCTGCCCAATGATTCCGGAAGCGGGGGCAGTCAGGCAGATAAAGGTAAAAACG

302-GTGGAACAGACTTTACCTACACAACAACCTACACGCCGGAAAGCGATAAAGAAGACACCC-361
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     GTGGAACAGACTTTACCTACACAACAACCTACACGCCGGAAAGCGATAAAGAAGACACCC

362-AAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCACAAGTG-421
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     AAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCACAAGTG

422-GCAAAACAAAAACCCATCTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAAT-481
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     GCAAAACAAAAACCCATTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAAT

482-TACGGGTTGCTGACGCGCAAAACCGCCGGCA-511
     |||||||||||||||||||||||||||||
     ACGGGTTGCTGACGCGCAAAACCGCCGGCA
```

Bases in black fonts (CGTCTGAA) : *tbpB* gene locus of MD-2132
Bases in black shade (CGTCTGAA): *tbpB* allele 11 (closest match)
Bases highlighted (CTATG): dissimilar with the closest match

Conserved region (CGTCTGAA): at base 122

Dissimilarity/ Substitutions: 439, 440, 441, 442, 443- 445, 446, 447- 449- 451, 452- 454, 455, 456, 458, 459- 461, 462, 463- 465- 467, 468, 469- 471- 473, 474, 475, 476, 477, 478- 481, 482, 483, 484 - 487- 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499- 503- 505, 506- 508- 511 = 52

Similarity= 338/390, 86.6%

Figure 4.19: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2132

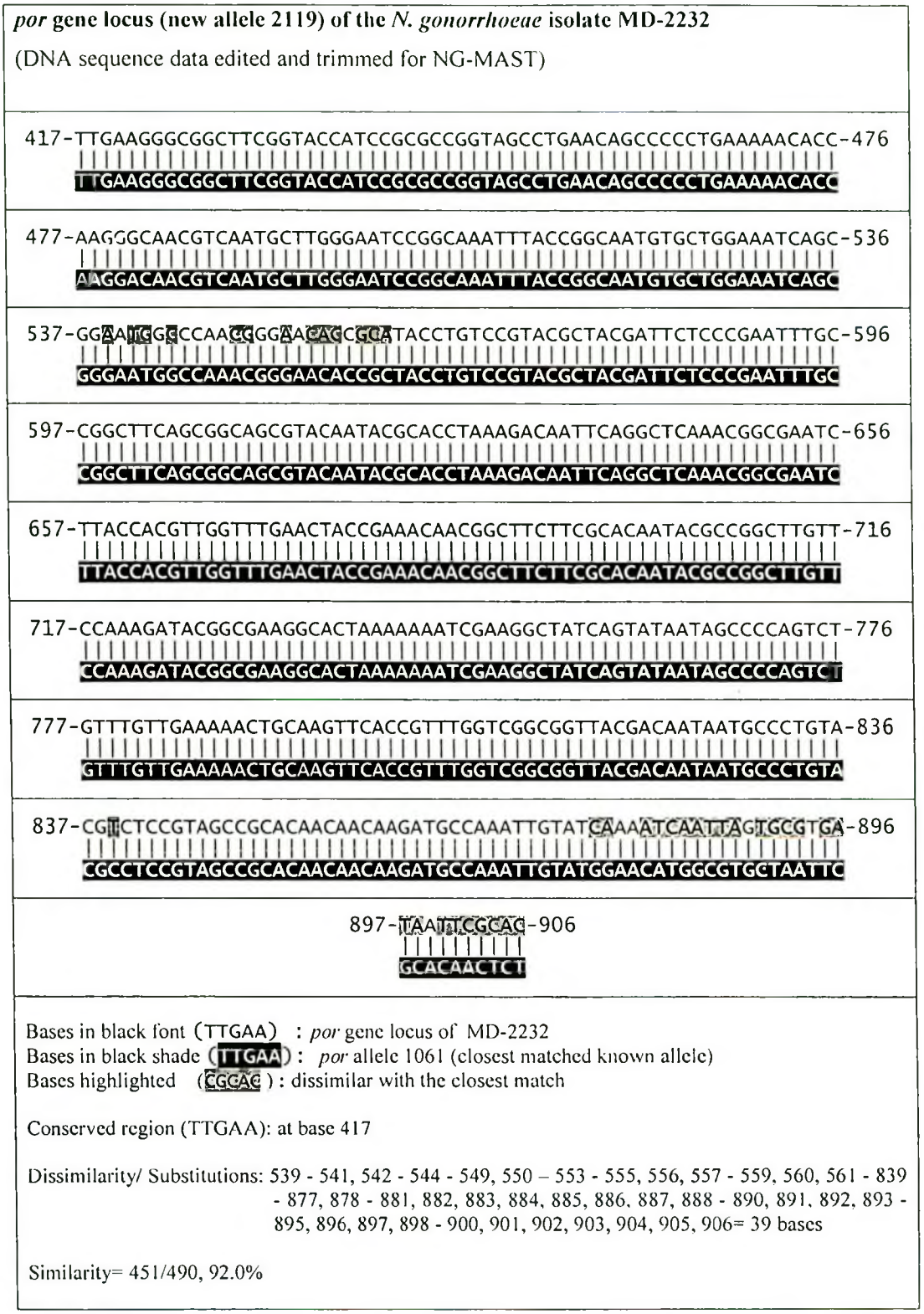


Figure 4.20: Alignment with the closest match of the edited and trimmed *por* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2232

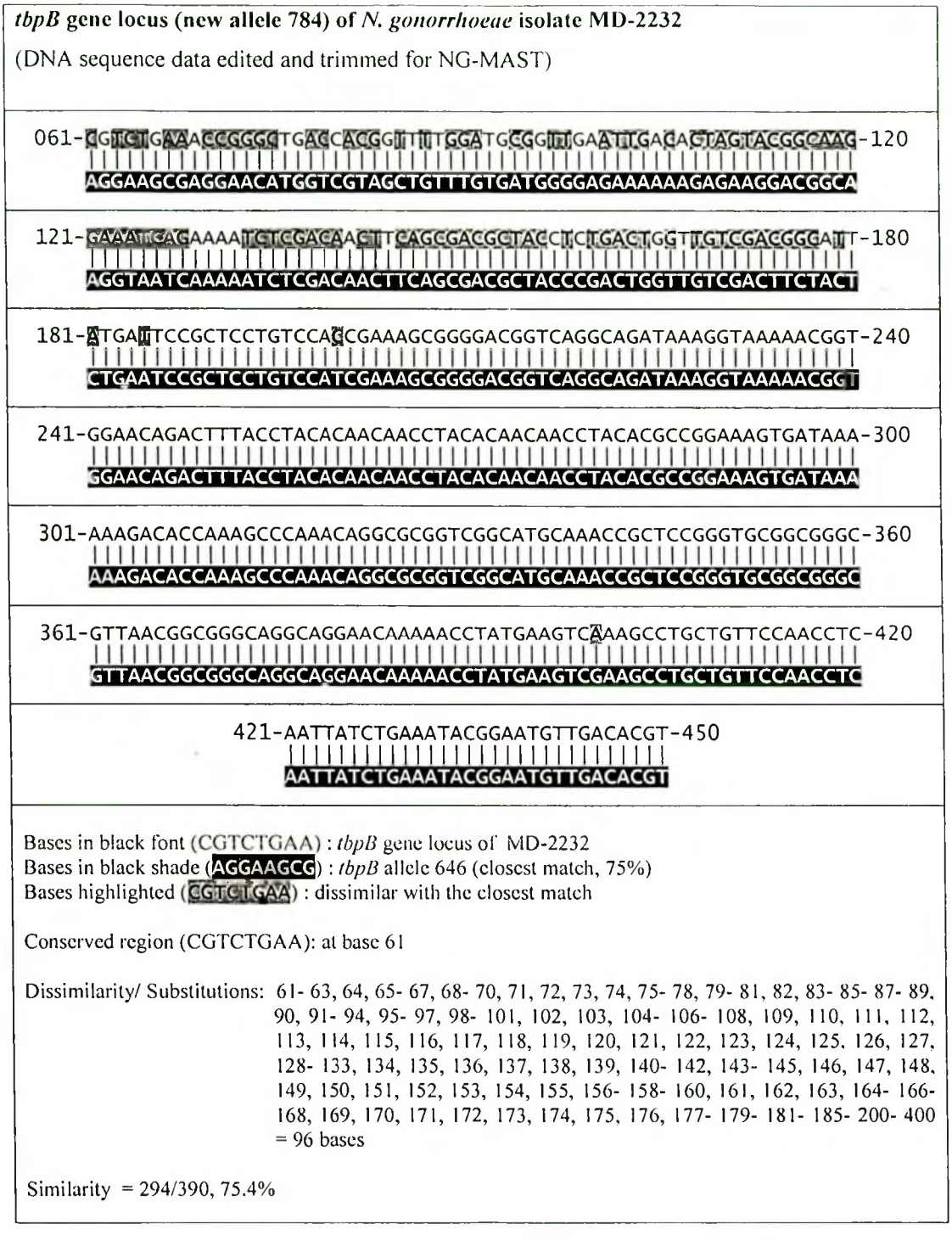


Figure 4.21: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of the *N. gonorrhoeae* isolate MD-2232

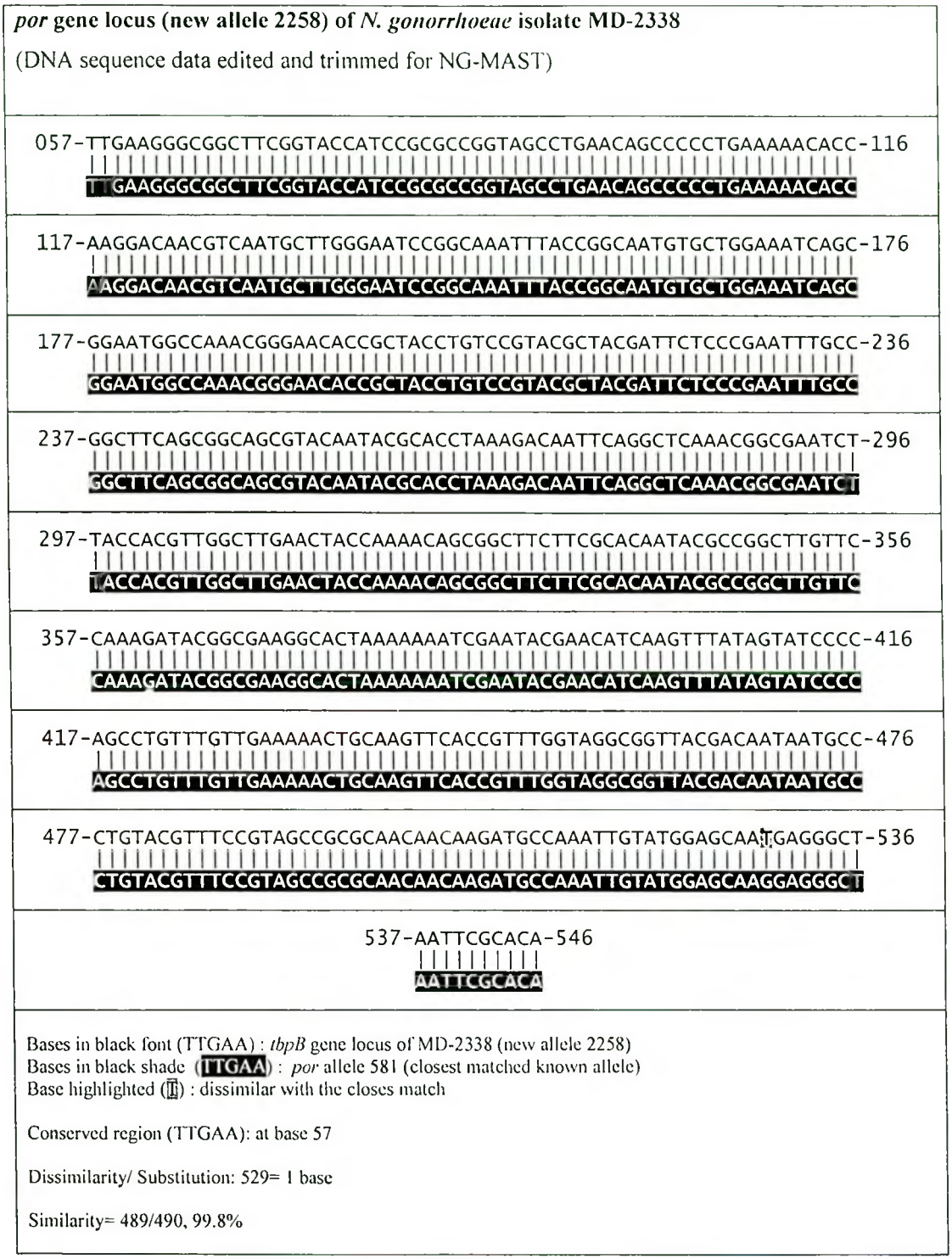
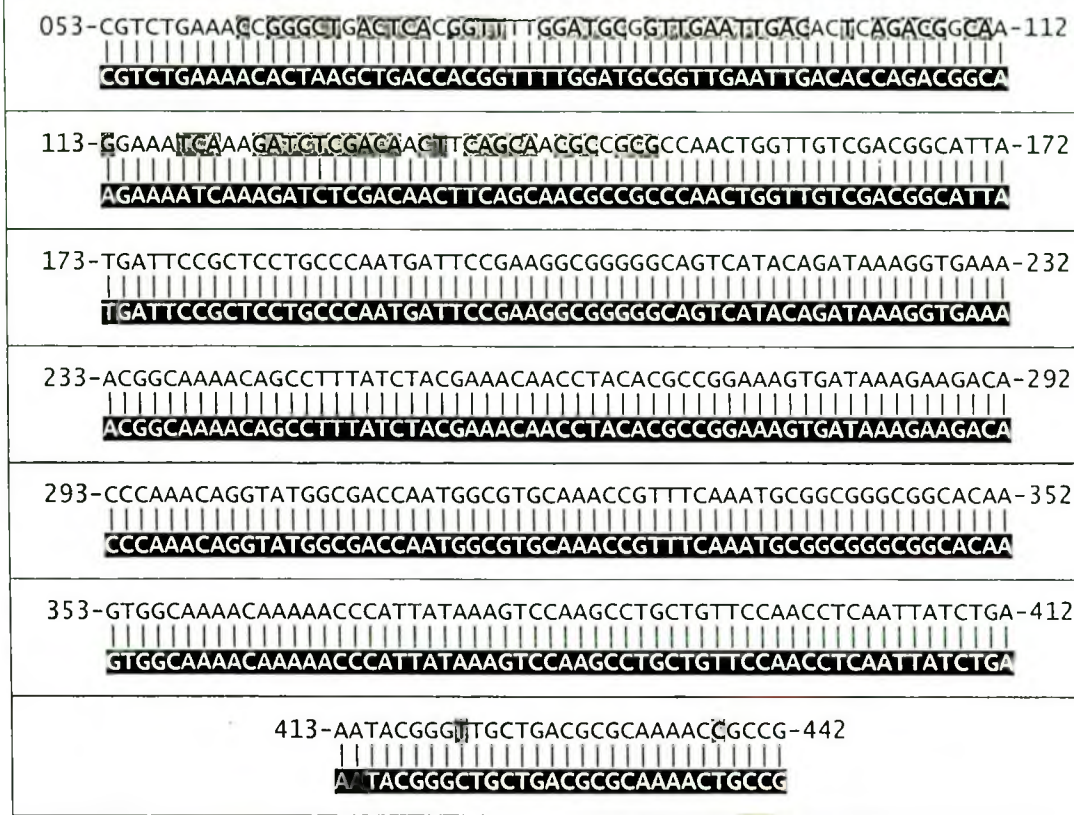


Figure 4.22: Alignment with the closest match of the edited and trimmed *por* gene locus of *N. gonorrhoeae* isolate MD-2338

tbpB gene locus (new allele 785) of *N. gonorrhoeae* isolate MD-2281

(DNA sequence data edited and trimmed for NG-MAST)



Bases in black fonts (CGTCTGAA) : *tbpB* gene locus of MD-2281
Bases in black shade (CGTCTGAA): *tbpB* allele 628 (closest match)
Bases highlighted (GGGCT) : dissimilar with the closest match

Conserved region (CGTCTGAA): at base 53

Dissimilarity/ Substitutions: 62- 64, 65, 66, 67, 68- 70, 71, 72, 73, 74- 76, 77, 78, 79- 82, 83, 84, 85, 86, 87- 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99- 102- 104, 105, 106, 107, 108- 110, 111- 113- 118, 119, 120- 123, 124, 125, 126, 127, 128, 129, 130, 131, 132- 134, 135- 137, 138, 139, 140, 141- 143, 144, 145- 147, 148, 149- 422- 439 =70 bases

Similarity = 320/390, 82.1%

Figure 4.23: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2281

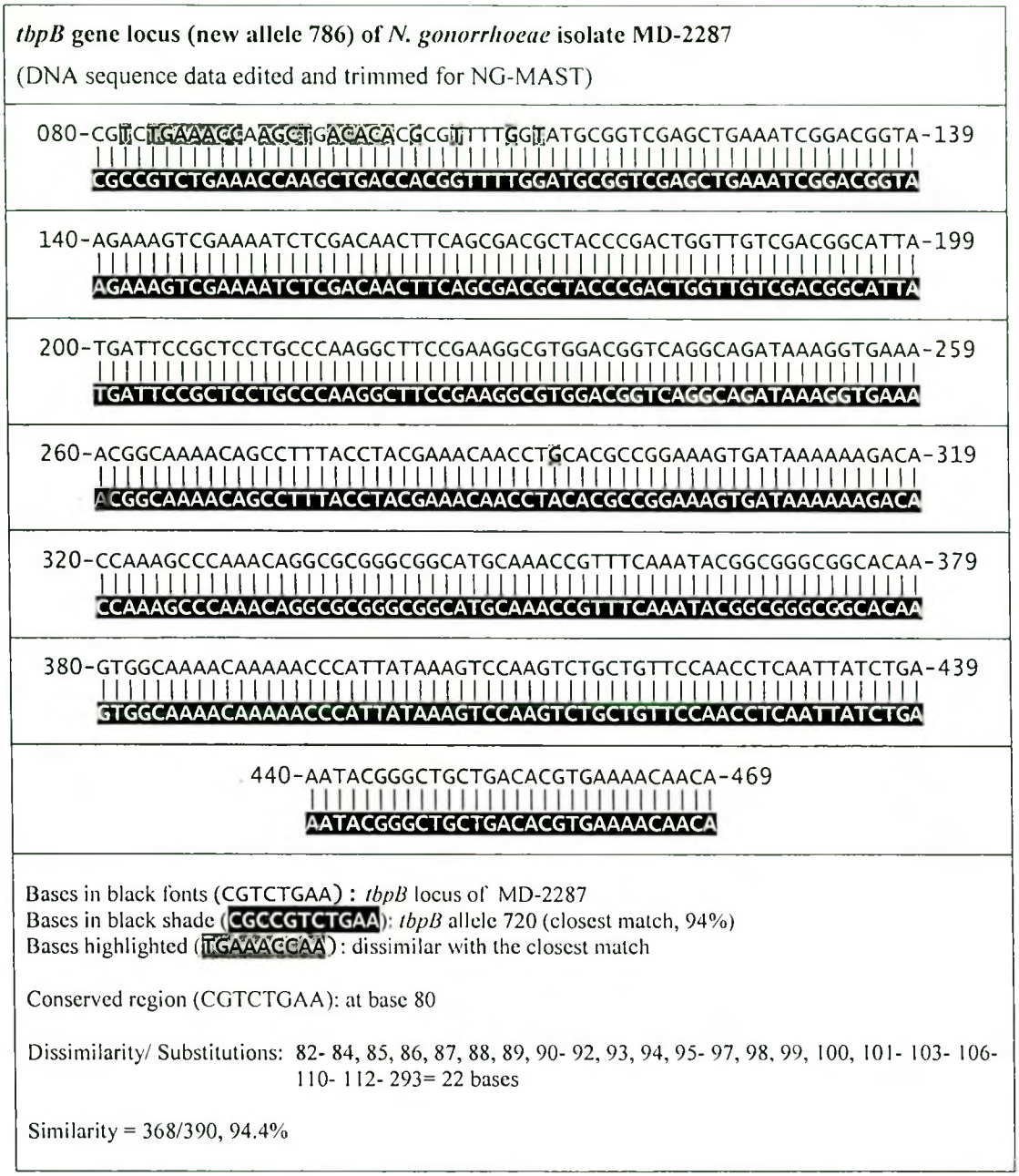


Figure 4.24: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2287

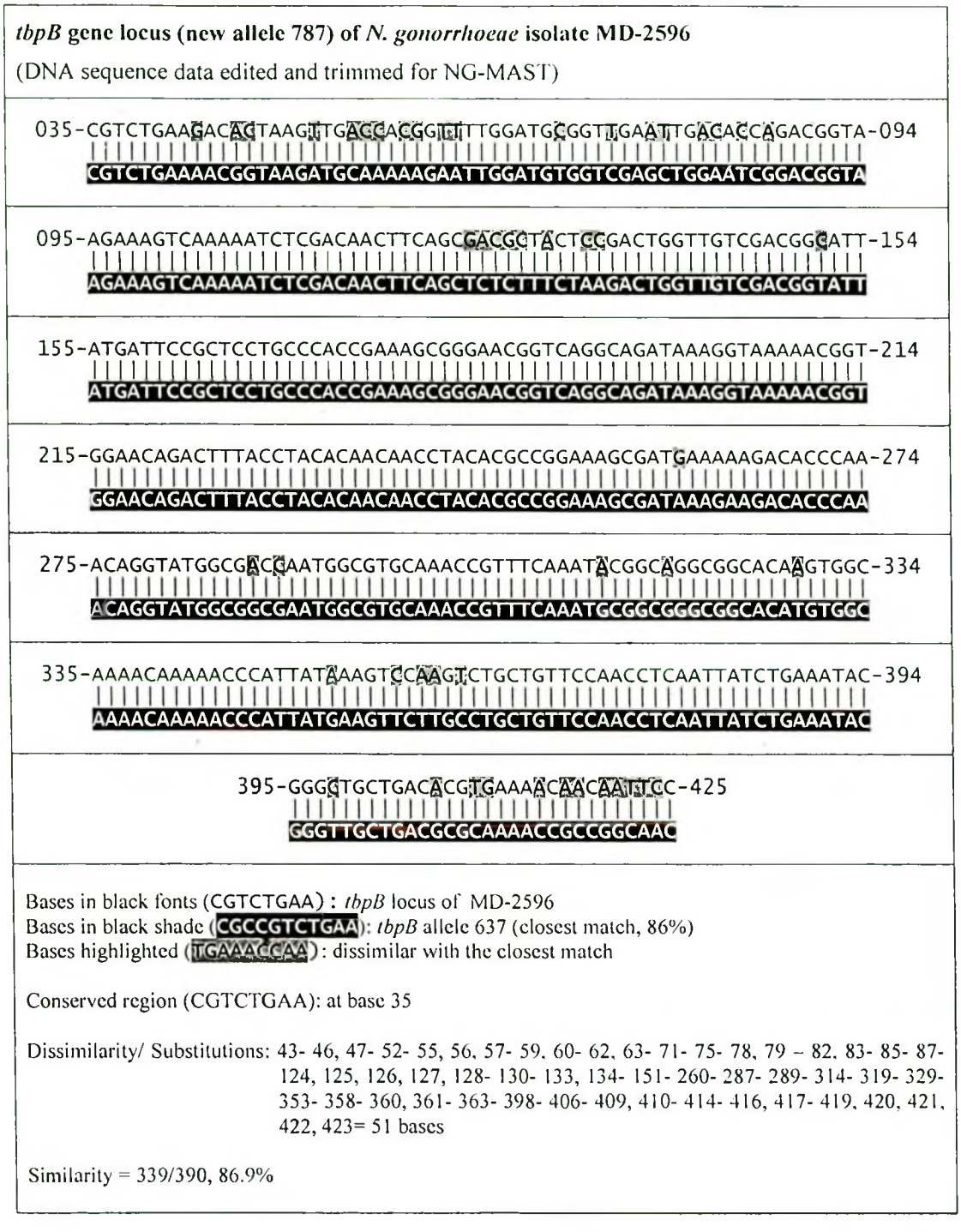


Figure 4.25: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2596

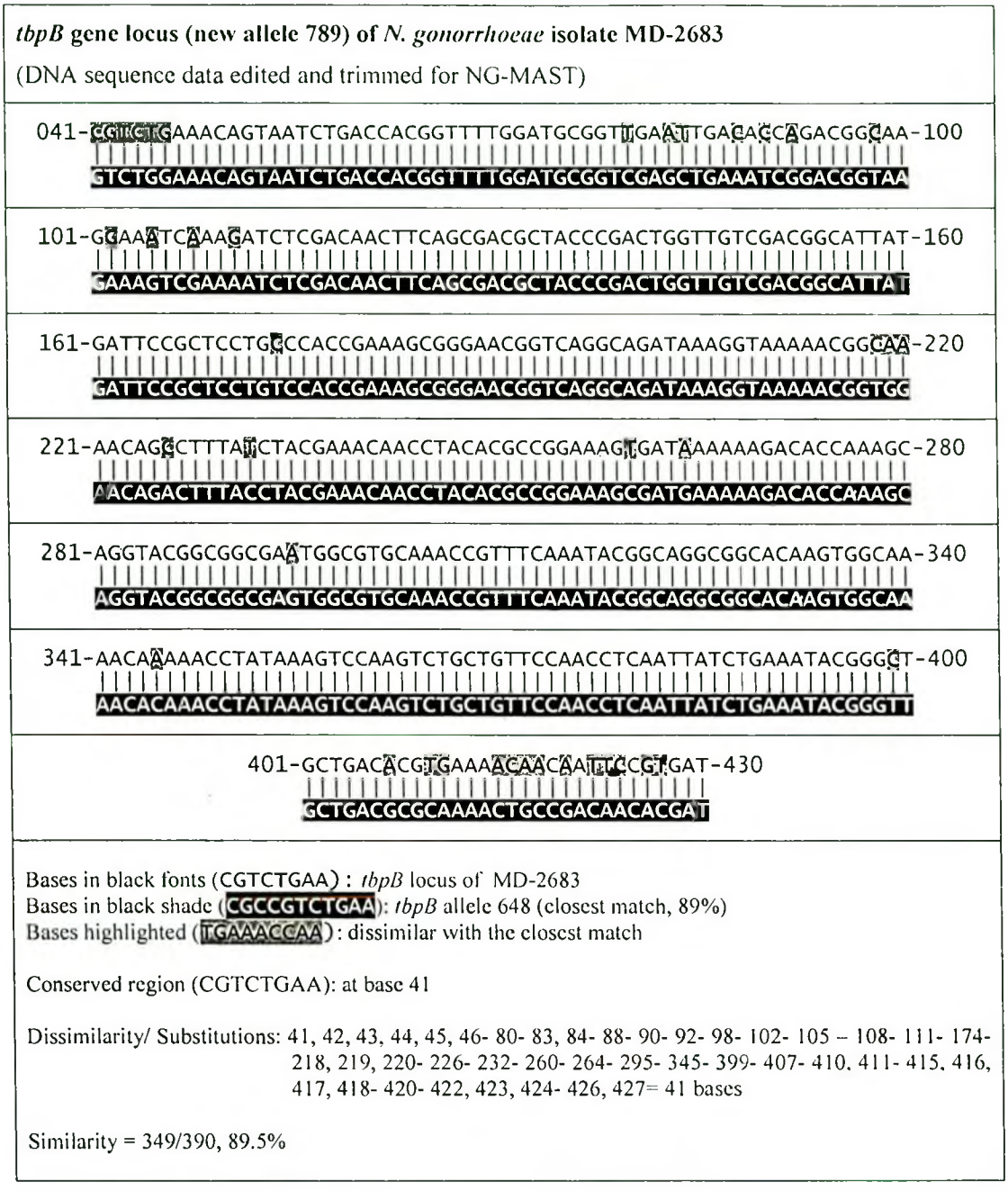


Figure 4.26: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2683

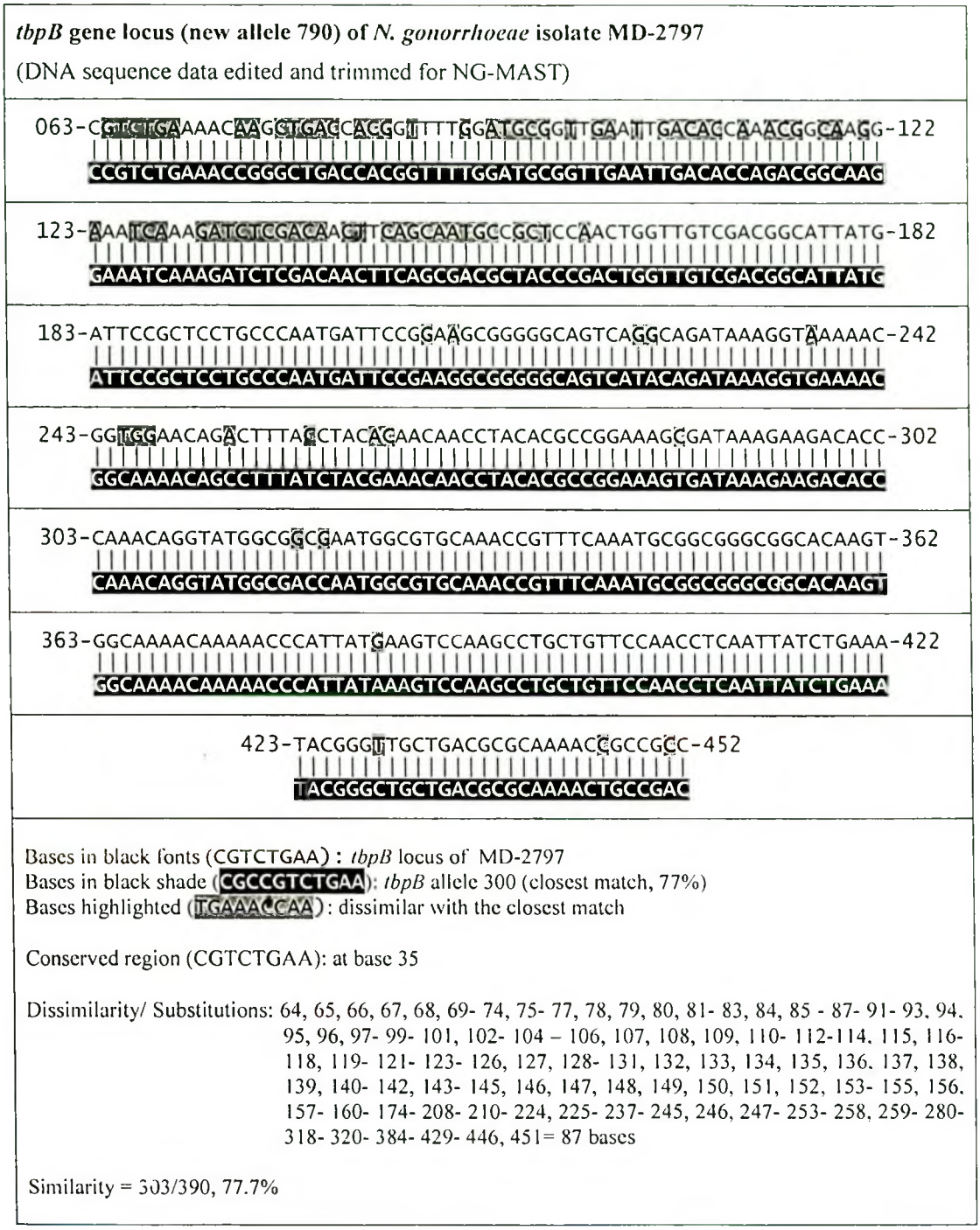


Figure 4.27: Alignment with the closest match of the edited and trimmed *tbpB* gene locus (new allele) of *N. gonorrhoeae* isolate MD-2797

Chapter 5: Discussion and Conclusion

5.1: Discussion

5.1.1: Gonococcal infection and sociodemographic characteristics of the cases

A total of 1025 male individuals in Sylhet were included in the present study to find out sociodemographic characteristics related to gonococcal infection. The enrolled cases were in two groups including 371 (36.2%) individuals attending skin and venereal diseases male-outpatients Department (OPD) of Sylhet MAG Osmani Medical College Hospital (SOMCH) (Male-OPD, SOMCH cases) and 654 (63.8%) male individuals having sex for male (MSMs) attending medical centre for the MSMs (MSM cases). The Male-OPD cases were symptomatic, enrolled purposively when presented with complaints suggestive of gonococcal infection, whereas, all of the MSM cases, attending during the study period, with or without any symptom, were enrolled with a view to identify the asymptomatic cases of gonorrhoea, if any.

Sylhet is the northeastern divisional City of Bangladesh and no reported study with gonococcal infection and susceptibility pattern of the *N. gonorrhoeae* isolates was found in this region. Whereas, male gonococcal infections with frequent therapeutic failures with oral ciprofloxacin were identified in the skin and venereal diseases OPD of SOMCH. Apart from this, a large number of MSMs had been provided with screening and management of sexually transmitted diseases (STDs) by a non-government organization (Bandhu Social Welfare Society, BSWS) in the City. That is why the present study was designed to find out the gonococcal infection and related sociodemographic characteristics of the two groups of male populations in Sylhet.

The overall rate of gonococcal infection among the enrolled cases was 16.7% (Table 4.2) with significantly higher rate among the Male-OPD cases (38.5%) than the MSMs (4.3%) (chi-square test p value <0.001). (Table 4.2) Three major complaints presented by the cases were dysuria (35.4%), non-purulent urethral discharge (30.6%) and purulent/ mucopurulent urethral discharge (15.0%). (Table 4.7) The rate of gonococcal infection was significantly higher among the cases with purulent/ mucopurulent urethral discharge (92.2% with purulent/ mucopurulent urethral discharge, versus 7.3% and 1.4% with non-purulent urethral discharge and dysuria respectively, chi-square test p value <0.001). (Table 4.8)

There was no significant relationship of the gonococcal infection in the present study with age groups, literacy, marital status and occupation of the enrollments. However, the rate was significantly higher among the individuals having post-graduation level of education (Table 4.18) showing no evident reason for this significantly high rate. More than two-thirds (226, 87.6%) of the married individuals had experience of extra-marital sex indicating having multiple sex partners. But yet there was no significant difference in acquiring infection by these polygamous persons enrolled into the present study. The rate of gonococcal infection was significantly higher among the individuals having history of sexual exposure with female CSWs (chi-square test p value <0.001) (Table 4.27) as well as among the heterosexuals (chi-square test p value <0.001) (Table 4.33).

In Bangladesh, on reviewing the previous reports as well as the results of the current study it appears that the rates of gonococcal infection, particularly among the male individuals, is declining (52.7% in 1988 to 16.7% in 2003). The first available report of male gonococcal infection in Bangladesh was very high (52.7%) in 1988 among the attendees at skin and venereal diseases (skin VD) outpatient department (OPD) of Sir Salimullah Medical College Hospital, Mitford, Dhaka (Chowdhury and Amin, 1988). This was followed by another

study reporting 31.0% gonococcal infection (Islam and Rahman, 1992) among the attendees at Skin VD OPD of Chittagong Medical College Hospital and Central Skin and Social Hygiene Centre, Agrabad, Chittagong. In Sylhet, the first available report among sexually active men in 2002 found a further declining rate of gonococcal infection (20.4%) (Alam *et al.*, 2002). The rate of gonococcal infection among the female CSWs in Bangladesh was also found declining (42% in 1999 to 20% in 2003) among street-based (floating) commercial sex workers in Dhaka (ICDDR,B, 2003a).

In the neighbouring India, the rate of gonococcal infection among the men in STD clinics was found to range from 8.5% to 25.9% (Sharma and Khandpur, 2004). In Thailand, a recent study among 824 HIV-seropositive patients (highly vulnerable), the rate of gonococcal infection was only 1.3% (Srifeungfung *et al.*, 2009).

In Canada, the reported gonorrhoea rate in 2006 was very low (0.03%, 33.1 per 100,000 population) (PHAC, 2008). In the USA, the recent national gonorrhoea case rate was 0.12% (120.9 cases per 100,000 population) (Workowski *et al.*, 2008). However, there was yet a higher-than-USA incidence of gonorrhoea in London, UK among the MSM (1.8%, 1834 per 100,000 population) and the heterosexuals of black ethnicity (0.39%, 392 per 100,000 population) (Risley *et al.*, 2007). A further previous report in the UK, among male attendees at a genitourinary medicine (GUM) clinic having evidence of urethritis, gonococcal infection was found among 29.7% of the cases (Turner *et al*, 1995). In Australia, the rate of gonococcal infection was found 11.7% among the men in remote aboriginal communities and in the Alice Springs Gaol (Skov *et al.*, 1997). The rate of infection was found higher among the heterosexuals of male gonorrhoea cases enrolled in "A Sygros" hospital, Athens, Greece (Kyriakis *et al.*, 2002).

The year-wise rate of gonococcal infection among the cases in the present study in Sylhet showed that the rate declined in the last year of study in 2003 (from 21.6% in 2002 to 14.1% in 2003). (Table 4.3) This decline may be due to an effect of provision of free treatment among the positive cases with cap. cefixime, which was also found highly effective against the *N. gonorrhoeae* isolates (98.7% sensitive) (Figure 4.2), indicating that the surveillance of antimicrobial susceptibility of the *N. gonorrhoeae* circulating in a community can help in reducing the rate of gonococcal infection effectively. An effective treatment was thus proposed as the only one element in the successful control of gonorrhoea (Bignell, 1994) and the effective treatment regimen has been prepared with the updated antibiogram of the *N. gonorrhoeae* isolates by the CDC regularly (CDC, 1985a; CDC, 1989; CDC, 1993; CDC, 2006).

A total of 228 (22.2%) among the cases (n=1025) in the present study had urethritis and majority of the urethritis cases was found due to gonococcal infection (170, 74.6%). (Table 4.10) Therefore, the remaining cases (58, 25.4%) were due to non-gonococcal aetiology (non-gonococcal urethritis, NGU). The rate of gonococcal urethritis in the present study also corresponds with a similar report in India showing 83.9% (52/62) gonococcal infection among the urethritis cases in men (Khaki *et al.*, 2007). However, the rate appears very high in comparison to that in the developed countries. In the UK, as mentioned earlier, the GUM clinic attendees were found to have gonococcal urethritis among 29.7% of the cases and remaining 70.3% cases were supposed to have NGU (Turner *et al.*, 1995).

Only a few of the available individuals (91/ 962, 9.5%) reported to use condom anytime during sexual act (Table 4.30) and only about one-fourth (25, 27.5%) of them used condom regularly (Table 4.31). However, there was no significant difference in the rates of gonococcal infection among the individuals with no condom use and those using condom during sexual act, indicating that a few among the cases acquired infection in spite of reported condom use regularly.

Two explanations follow this failure: (i) inappropriate use of condoms that may allow organisms to reach the urethra for infection; and (ii) answering falsely to the interviewer to get positive response of healthcare. Another similar report of the extremely low level of condom use (9%) was found in a behavioural baseline study in Bangladesh by the Family Health International (FHI) in 2001 (cited in Nessa *et al.,* 2004). In a similar report of study among 881 female CSWs, the prevalence of gonococcal infection among the women who reported 100% condom use was 8%, versus 23% among the women who reported <100% condom use (P <.001) (Klausner *et al.,* 1999). There has been sufficient evidence to show that condoms, when used correctly and consistently, are effective in protection against the transmission of all STIs including gonococcal infection (Holmes *et al.,* 2004). On the other hand, inconsistent condom use was identified as one of the risk factors of acquiring gonorrhoea (CDC, 2006). Similarly, very low rate of using condom during sex is an indicator of unsafe sexual practice among majority of the individuals which endangers acquiring of other STIs, especially HIV infection and appropriate counseling and education of safer sex often found very effective in reducing the infections. In Thailand, explicit messages to men about the risks of STIs from unprotected commercial sex found to have resulted in higher reported condom use, lower reported numbers of sex worker visits and lower infection rates measuring a 95% drop in common curable STIs during the 1990s following introduction of the 100% condom use programme (CUP) (Rojanapithayacom, 2006). Therefore, correct and consistent condom use has been advocated by the WHO, promoting the CUP to ensure a policy of "*No Condom- No Sex*" (WHO, 2007; WHO, 2008).

5.1.2: Antimicrobial resistance and other characteristics of the *N. gonorrhoeae* isolates

A total of 495 *N. gonorrhoeae*, isolated during 2003-2006, were investigated for antimicrobial resistance and other characteristics. These isolates included 54 from Sylhet and another 441 from Dhaka and Faridpur from six different high-risk populations: (i) male-OPD, SOMCH (Sylhet, n=46), (ii) MSM (Sylhet, n=8), (iii) female CSWs-floating (Dhaka, n=322), (iv) female CSWs-brothel-based (Gualanda, Faridpur, n=20), (v) male-OPD, DMCH (Dhaka, n=64) and (vi) long-distant truckers (Dhaka, n=35). (Table 3.3) Thus the *N. gonorrhoeae* isolates investigated covered almost all individuals with high-risk sexual behaviour from three different areas of Bangladesh including Sylhet, Dhaka and Faridpur. These three localities include a large area of sexual network and so on to the majority of the "core-transmitters" in the country, because: (i) Sylhet is the northeastern divisional City and was representing a large area of the country providing with the symptomatic male-OPD cases who were attending the Sylhet MAG Osmani Medical College Hospital (the largest tertiary hospital in the region) as well as the homosexuals, attending an exclusive medical centre for the MSMs in Sylhet; (ii) Dhaka, on the other hand, being the capital of Bangladesh, represented almost all areas of the country, included the symptomatic male-OPD cases attending Dhaka Medical College Hospital (the largest hospital in the City), the long-distant male truckers (supposed to have multiple sex partners throughout the country) as well as the floating (hotel- or street-based) CSWs (supposed to have clients visiting from outside as well as single dormitory-living individuals inside Dhaka City); and (iii) Gualanda, Faridpur in addition, being a river port inhabiting a large brothel, was representing the cases of the brothel-based CSWs. Therefore, the *N. gonorrhoeae* investigated was representing almost all of the strains circulating in Bangladesh.

The *N. gonorrhoeae* isolates were resistant in high rates to three of the antimicrobial agents, namely penicillin (51.5%), tetracycline (88.1%) and ciprofloxacin (90.9%). (Figure 4.2)

Observing the previous reports in Bangladesh, it is found that the rates of resistance of the *N. gonorrhoeae* isolates to penicillin is currently declining (51.5% in the present study) following a sharp rise from 8.5% in 1989 (Miah *et al.,* 1989) to 68.0% in 2001 (Rahman *et al.,* 2001). To tetracycline, the rates are increasing steadily from 60.6% in 1999 (Bhuiyan *et al.,* 1999) to 72% in 2001 (Rahman *et al.,* 2001) and 87.9% in the present study. To ciprofloxacin, the rate was abruptly increasing from 11.7% in 1999 (Bhuiyan *et al.,* 1999) to 96% in 2001 (Rahman *et al.,* 2001).

The high rates of resistance (>50%) of *N. gonorrhoeae* isolates to penicillin and tetracycline were also reported from the other developing/ underdeveloped countries: the Asian countries like Indonesia (Donegan *et al.,* 2006), China, Hong Kong, the Philippines, Vietnam and Singapore (Tapsall, 2001) as well as the Indian states (WHO, 2002), the African countries including Zaire (van Dyck *et al.,* 1992) and Northern Tanzania (West *et al.*, 1995), as well as the Caribbean island, Cuba (Sosa *et al.,* 2003). The high rates of resistance of the *N. gonorrhoeae* isolates (>50%) to ciprofloxacin were reported from other Asian countries including India (Sethi *et al.,* 2006), China (Zheng *et al.,* 2003; Wang *et al.*, 2006; Tiejun *et al.,* 2009), Japan (Tanaka *et al.,* 2004) as well as from the developed countries including USA (Wang *et al.,* 2007) and Australia (Tapsall, 2009). Observing the widespread high-level resistance of the *N. gonorrhoeae* isolates in the US, the CDC announced that fluoroquinolones were no longer recommended for treatment of gonococcal infections and related conditions like pelvic inflammatory disease (PID) or epididymitis, in any population in the US (CDC, 2007c).

A high percent of the *N. gonorrhoeae* isolates in the present study (230, 46.5%) showed resistance to three of the antibiotics together (penicillin, tetracycline and ciprofloxacin) (MDR, multidrug resistant) (Table 4.35). The first report of the MDR *N. gonorrhoeae* in the USA was published in 2003 in which 4 urogenital isolates demonstrated resistance to penicillin, tetracycline and ciprofloxacin with reduced susceptibility to cefixime (Wang *et al.*, 2003). In another study in Greece, 15.1% of the isolates of *N. gonorrhoeae* during 1991 to 1998 exhibited resistance to penicillin, tetracycline, erythromycin and chloramphenicol (Mavroidi *et al.*, 2001). The resistant subtypes of the organism in the developed countries are supposed to be imported and eventually introduced into the country's sexual network to achieve sustained endemic transmission (Tapsall, 2009).

Penicillinase production of all of the 495 *N. gonorrhoeae* isolates was tested by paper acidometric method by which a total 178 (36.0%) of the isolates were found positive (PPNG, penicillinase producing *N. gonorrhoeae* strains) (Table 4.37). Observing the penicillin MIC results among the PPNG strains, it was found that almost all (172/178, 96.6%) of the PPNG-positive isolates had penicillin MICs $\geq$2.0 μg/ml, whereas, a few (6, 3.4%) showed positivity having penicillin MICs $\leq$ 2.0μg/ml, (Table 4.39) which were not resistant by standardized susceptibility criteria (CDC, 2003). However, penicillinase (beta-lactamase) enzymes are known to hydrolyse beta-lactam antibiotics (like penicillins) and *N. gonorrhoeae* strains showing beta-lactamase production might not have penicillin MIC $\leq$2.0 μg/ml. These isolates of the *N. gonorrhoeae* were, therefore, subjected to identification of the plasmids by PCR to identify the epidemic PPNG plasmids and all of these 6 isolates were found negative by the PCR method. However, the PCR method identified 169 (34.1%) of the *N.* gonorrhoeae isolates to produce epidemic plasmids and these strains were considered to produce PPNG plasmids actually. (Table 4.40)

The first available report on PPNG in Bangladesh in 1989 found 9.6% PPNG of the *N. gonorrhoeae* isolated from acute urethritis cases attending dermatovenereology departments of different hospitals at Dhaka City (Miah and Rahman, 1989). This was followed within a decade increasing to more than 20.0% among the *N. gonorrhoeae* isolates from CSWs (Bhuiyan *et al.*, 1999; Alam *et al.*, 2002). Comparing with these previous reports, it appears that the PPNG strains increased remarkably during the present study period (36.0% in the current study). Among the 255 penicillin-resistant *N. gonorrhoeae* isolates in the present study, majority (169, 66.3%) were plasmid-mediated and the remaining 87 (34.1%) were chromosomally-mediated. (Figure 4.9) Since the first identification in 1976, PPNG has spread all over the world. Until the mid-1990s the prevalence of PPNG remained low (<10%) in the European countries, the United States, and Australia, but in many countries in Asia and Africa more than 50% of the *N gonorrhoeae* isolates were found to be PPNG (Lind, 1997). In Indonesia, a high rate of PPNG plasmid infection (72.5%) was reported among 160 *N. gonorrhoeae* isolates (Su *et al.*, 2003). Although, in India, in a report in 2006, 22% of the *N. gonorrhoeae* isolates were found to produce beta-lactamase enzymes (Sethi *et al.*, 2006). In another Indian report in 2008, only 10% isolates were PPNG (Shilpee *et al.*, 2008). However, in Thailand, the *N. gonorrhoeae* isolates from HIV-seropositive patients, a very high rate of PPNG strains (102/122, 83.6%) were identified (Srifeungfung *et al.*, 2009).

Observing the plasmid-mediated tetracycline resistance of the *N. gonorrhoeae* isolates, it was found that more than two-thirds (382, 87.6%) of the tetracycline-resistant isolates were plasmid-mediated (Figure 4.12) and the highest rate (186, 49.3%) of plasmid-mediated resistant isolates had tetracycline MIC 32.0 µg/ml (Table 4.47). Distributing the *N. gonorrhoeae* isolates into different types of plasmid-mediated resistance, it was found that the highest number of isolates (230, 46.5%) possessed TRNG plasmids, followed by those possessing both PPNG and TRNG plasmids (PPNG-TRNG)

(152, 30.7%). (Table 4.50) *N. gonorrhoeae* with high-level plasmid-mediated tetracycline resistance (TRNG) was first reported in the United States in 1985 (CDC, 1985b) and since then a rise in the prevalence of TRNG and of PPNG/TRNG have been observed all over the world (Su *et al.*, 2003).

A total of 239 *N. gonorrhoeae* isolates were subjected to serotype and serovar determination by Phadebact monoclonal antibody. Majority (142, 59.4%) of the isolates were type B (protein IB). (Figure 4.15) Among the penicillin-resistant isolates, majority (71, 61.7%) were type A, whereas among the tetracycline-resistant and ciprofloxacin-resistant isolates, majority was type B (128, 59.8% and 129, 59.5% respectively). (Figure 4.18)

A total of 41 different serovars were identified, of which "Arst" (46/239, 19.2%) included the highest number of isolates, followed by "Bropt" (22, 9.2%) and "Bopt" (18. 7.5%). (Table 4.51) Only 5 among the serovars included nearly 50% (136, 49.3%) of the *N. gonorrhoeae* isolates tested. Almost similarly, majority of the isolates were included in 7 of the serovars in a study in Stockholm (Ruden, 1994). Among the 41 serovars, 23 (56.1%) were not reported previously and considered new emergent. The new emergent serovars included a total of 58 (24.3%) of the *N. gonorrhoeae* isolates tested. Among the new emergent, the predominant serovars were: "Ast" (12, 20.7%), "Arstv" (6, 10.4%), "Astv" (5, 8.6%), "Bropys" (4, 6.9%) and "Brpyt" (4, 6.9%) and majority of the *N. gonorrhoeae* isolates in new serovars (40, 39.2%) were multidrug resistant (MDR) (Table 4.61). One study among the homosexuals in Edinburg, UK, over a 5-year period with 175 Gonococcal infections found 32 new serovars of which "Bropyt" (26.3%) and "Brpyust" (16.6%) were predominating (Young *et al.*, 1991). Only 3 *N. gonorrhoeae* isolates from homosexuals were investigated for serovars in the present study and the serovars of the isolates were "Arst", "Bropyt" and "Byvut".

Genotyping of few selected *N. gonorrhoeae* was carried out by NG-MAST using sequence data of two most variable genes *por* and *tbpB* of *N. gonorrhoeae*. All of the *N. gonorrhoeae* strains genotyped were isolated from floating female CSWs and were multidrug resistant (MDR) to penicillin, tetracycline and ciprofloxacin. Although emergence of new sequence types (genotypes) is due to mutations in the two chromosomal genes, majority genotyped isolates (6, 85.7%) showed plasmid infection. This is supposed to be a coincidence and the emergence of new genotypes indicates interstrain transformation and recombination (Norlander *et al.*, 1979).

5.1.3: Molecular epidemiology of gonococcal infection in Bangladesh

The PPNG plasmids were identified by PCR method using primers developed aligning primary DNA sequences of the epidemic plasmids (Asia, Africa and Toronto types) of the PPNG strains (Dillon and Yeung, 1989; Dillon *et al.*, 1999). Only the Africa type (3.1 MDa) of epidemic plasmid was identified in the present study. The other study on PPNG plasmid type in Bangladesh also reported all PPNGs being Africa type (Bhuiyan *et al.*, 1999). All studies in Bangladesh showing only Africa type PPNG plasmid suggests that the strains are all indigenous, disseminating the penicillinase gene via conjugation (Biswas *et al.*, 1980). However, conjugal plasmids were not identified which projects another limitation in the present study and the postulation regarding conjugal transfer of PPNG plasmids can not be confirmed.

The Africa type plasmid was first isolated in the UK in 1976 (Percival *et al.*, 1976; Phillips, 1976), is supposed to be a 2.1 kb deletion derivative of the Asia type plasmid (Yeung *et al.*, 1986), was first identified in the USA in 1976 and named according to the epidemiological origin in the Africa (Ashford *et al.*, 1976; Perine *et al.*, 1977). The Africa type PPNG plasmids, predominantly infecting the *N. gonorrhoeae* isolates, have been reported from the African nations like Rwanda and Jamaica (70.0% each) (Bogaerts *et al.*, 1986; Dillon *et*

al., 1987), from the European countries like Munich, Germany (70.0%) (Abeck *et al.*, 1988) and London, UK (66.1%) (Ison & Easmon, 1991), from the Caribbean countries (67.8%) (Dillon *et al.*, 2001) as well as from Southeast Asian countries like Bangkok, Thailand (96.2%) (Knapp *et al.*, 1997b), Mumbai, India (71.4%) (Divekar *et al.*, 1999) and Bangladesh (100.0%) (Bhuiyan *et al.*, 1999). On the contrary, the Asia type PPNG plasmids (the ancestral of Africa type), predominantly infecting the *N. gonorrhoeae* isolates have been reported from the Far East (100.0%) (Ng *et al.*, 1982), Zurich, Switzerland (96.7%) (Eichmann & Pifferetti, 1984), Taiwan (82.0-95.0%) (Chu *et al.*, 1992) and Bandung, Indonesia (100.0%) (Djajakusumah *et al.*, 1998). Therefore, by the identified PPNG plasmid type, the infecting strains of *N. gonorrhoeae* is supposed to have close relationship with those from African or related countries. Two important factors appear contributory: (i) The isolates from the African countries also found to have high-level of resistance to common antimicrobial agents, similar to the isolates in Bangladesh; (ii) If they would be deletion derivatives of the Asia type, there might be predominantly Asia type plasmids among the *N. gonorrhoeae* isolates in Bangladesh along with the Africa type. However, as the type of PPNG plasmids had not been identified earlier, the origin of Africa type in Bangladesh remains questioned and mostly supposed to be indigenous.

In a recent study in the neighbouring India, all PPNG strains were found to harbour 3.2 MDa Africa type plasmids (Chaudhry *et al.*, 2007). However, another report in 1999 found 71.4% Africa type (3.2 MDa) and other 28.6% Asia type (4.4 MDa) PPNG plasmids in India (Divekar *et al.*, 1999). On the contrary, there was another earlier report showing all PPNG strains in India to carry 4.4 MDa Asia type plasmids (Bhalla *et al.*, 1998), indicating that at least two epidemic PPNG plasmid types are circulating in the neighbouring India.

The majority of the tetracycline-resistant *N. gonorrhoeae* isolates (382/ 436, 87.6%) in the present study was plasmid-mediated (Figure 4.12) and almost all

these TRNG plasmids (377/382, 98.7%) were Dutch type yielding an amplified product of 700 bp (Table 4.45). High-level tetracycline resistance with MICs ≥8.0μg/ml in gonococci is mediated by a *tet-M* determinant carried on a 25.2 MDa conjugative plasmid (Morse *et al.*, 1986). The restriction endonuclease map of the 25.2 MDa conjugative plasmid from a TRNG strain imported from United States has been found to differ from the map derived from a strain isolated in the Netherlands (Gascoyne *et al.*, 1991). These two types of *tet-M* carrying conjugative plasmids were designated "American" and "Dutch type" respectively (Morse *et al.*, 1986). All TRNG strains isolated from *N. gonorrhoeae* in the Southeast Asia were found to carry the Dutch *tet-M* gene (Turner *et al.*, 1999). The Dutch type tet-M may have originated in the Far East, possibly in Indonesia and the American type may have originated in the equatorial regions of the African continent. International travel has been found to distribute both types of the TRNG plasmids to other parts of the world and heterogeneity of auxotype/ serovar class also indicated the existence of interstrain distribution of the *tet-M* gene (Turner *et al.*, 1999).

In addition, molecular typing by NG-MAST was done for few *N. gonorrhoeae* isolates. Due to limited availability of the resources and standardization, only few *N. gonorrhoeae* strains isolated from floating female CSWs and showing multidrug resistance (MDR) were selected for NG-MAST using the sequence-based allele numbers of the *por* and *tbpB* gene of the organism. Surprisingly, all sequence types identified were new (STs 3507, 3508, 3509, 3510, 3511, 3512 and 3738), although majority of the *por* alleles were known (75.0%, 6/8) and 37.5% (3/8) of the *por* genes were found to contain similar allele (*por* allele number 251). These examples of emergence of new alleles or sequence types (genotypes) demonstrate interstrain transformation and recombination of the *N. gonorrhoeae* isolates (Norlander *et al.*, 1979; Graves *et al.*, 1982).

All these molecular characteristics of the *N. gonorrhoeae* isolates in the present study indicate that the isolates in Bangladesh have been originated from those

having high-level antimicrobial resistance and the MDR isolates were predominantly occurring among the female CSWs with widespread interstrain transformation to emerge as new genotypes.

5.2: Conclusions

Although declining from the previously reported rates of gonococcal infection in Bangladesh, the current rate in the present study was very high in comparison to that in the developed countries like Canada, USA and UK as well as the developing nations like sub-Saharan Africa and the Caribbean countries. Purulent / mucopurulent urethral discharge was found associated with majority of the cases of gonococcal infection and this presentation of the infection carries the highest risk of complications like fibrosis and stricture of urethra to sterility. The sexual exposure with female commercial sex workers and heterosexual type of sexual orientation were found to be significantly associated with gonococcal infection of the cases with very low and inconsistent condom use. So, promotion of safer sex, including consistent condom use is very urgent for the control measures of gonococcal infection.

The characterized *N. gonorrhoeae* isolates from the high-risk populations showed high-rate of resistance to penicillin, tetracycline and ciprofloxacin. The rate of plasmid-mediated resistance to penicillin and tetracycline was found very high in comparison to the other reports throughout the world, indicating no hope for the antibiotics of becoming widely sensitive for inclusion in the treatment regimen. Many new serovars have been found to emerge among the isolates investigated of which the highest percent demonstrated multidrug resistance (MDR). The emergence of new genotypes with different types of genetic changes (mutations) among the MDR strains *N. gonorrhoeae* isolated from the floating female CSWs also indicate the widespread transformation and recombination among the circulating endogenous strains of the organism. Additionally, as there is no routine antimicrobial surveillance of the *N.*

gonorrhoeae isolates in Bangladesh, no current/ updated antibiogram is available to follow in the treatment of the gonococcal infections and eventually antibiotics used to treat the infection without sensitivity result are accelerating the survival and widespread dissemination of the resistant isolates. Therefore, before becoming widely resistant to the current highly sensitive antibiotics by interstrain transformation and recombination as well as by survival and dissemination of the resistant strains by selective antibiotic pressure, the circulating *N. gonorrhoeae* in Bangladesh should be treated with careful and appropriate treatment modality.

Limitations of the present study

Limitations of the present study identified are listed below:

(1) Sociodemographic characteristics were identified among the male cases only. Female cases of gonococcal infection from the CSWs are supposed to be potential reservoirs and sources of unknowingly transmission by the asymptomatic cases.

(2) Some important sociodemographic data were not recorded properly like number of sexual partners, site of sexual exposure with the CSWs, reason(s) of not using condoms during sex and oral sex (fellatio), if any.

(3) Many cases of frank urethral discharge showing Gram-negative intracellular diplococci failed to yield growth by culture of the specimens due to technical errors (probably due to uncontrolled raised temperatures of the incubator used).

(4) Not all isolates in Sylhet were included for characterization. Moreover, many of the *N. gonorrhoeae* isolates in Sylhet could not be grown again after transfer in Dhaka probably due to limitation in the transfer protocol.

(5) Auxotyping is another important phenotype that could not be included for a further discrimination tool.

(6) Serotyping and serovar determination was not done to all isolates included for characterization.

(7) For plasmid profile, PCR protocols using primers to detect epidemic plasmids for PPNG or TRNG were used, which can not detect conjugative as well as the cryptic plasmids. Conjugative plasmids have an impact to postulate the transmission of the resistant genes via conjugation.

(8) Few among the *N. gonorrhoeae* isolates showed unidentified products of PPNG plasmids by PCR which could not be re-examined following suggestions available from Professor Jo Anne R Dillon, who developed the PCR protocol for identifying PPNG plasmids.

(8) Genotyping could not be done for all isolates of *N. gonorrhoeae* and no sociodemographic data of all of the source population of the genotyped isolates were available.

Recommendations

(1) Consistent surveillance of antimicrobial susceptibility of the *N. gonorrhoeae* isolates from the high-risk populations throughout Bangladesh in a central, well-organized reference centre. This can be achieved by making a network of laboratories at divisional Cities of the country which will carry out the data collection, isolation and antimicrobial susceptibility tests of the *N. gonorrhoeae* isolates conveying all the data to the central reference laboratory;

(2) Regular update of treatment regimen for gonococcal infection whenever rate of resistance exceeds >5% for a recommended antimicrobial agent;

(3) A national Gonococcal infection control guideline can be designed by identifying sociodemographic characteristics of all high-risk population groups including the female commercial sex workers;

(4) Contact tracing by identifying the sexual network of the commercial sex workers and their clients for effective treatment of all infected cases;

(5) Control measures in an integrated approach for all sexually transmitted infections (STIs);

(6) Promotion and implementation of safer sex practice including consistent and right use of condoms during sex which may ultimately help in reducing other STIs as well including the HIV infection;

(7) Genotyping of some representative isolates to find out genetically related strains of *N. gonorrhoeae* circulating in the community.

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Chapter 7: Appendices

Appendix I: Preparation of Reagents

Appendix I.1: Oxidase reagent

Ingredients (to make 100 ml stock solution):

Tetramethyl-*p*-phenylene-diamine hydrochloride... 1 gm

Ethanol, 95% ..... 100 ml

Working solution: Stock solution.....1 ml

Distilled water ..... 2 ml

Procedure:

i. The oxidase reagent was weighed and dissolved in 95% ethanol in a brown reagent bottle (250 ml) and labeled properly with reagent name and date of preparation;

ii. The working solution was prepared by adding two volumes of distilled water to one volume stock solution;

iii. The working solution should be freshly prepared before use.

iv. *Storage:* The stock solution was stored at 4^0C in the brown bottle protecting from direct light exposure.

Appendix I.2: Saponin solution, 10%

Ingredients (to make 100 ml):

Saponin powder ..... 10 gm

Distilled water ..... 100 ml

Procedure:

i. The saponin powder was weighed and mixed with the measured volume of distilled water. The reagent was labeled properly with name of the reagent and date of preparation;

ii. *Sterilization*: autoclaving;

iii. *Storage:* The saponin solution was kept on a suitable reagent rack at room temperature maintaining all aseptic procedure.

Appendix I.3: Carbohydrate solutions

Ingredients (to make 10 ml each of 20% w/v solution of the sugars):

Carbohydrate powders (glucose, maltose, lactose and sucrose) .... 2 gm

Distilled water ..... 10 ml

Procedure:

i. The reagent grade carbohydrate powders were weighed, dissolved in distilled water in four properly labeled screw-capped test tubes/ falcon tubes and mixed thoroughly to dissolve the sugars completely;

ii. *Sterilization:* The sugar solutions were sterilized by filtration;

iii. *Storage:* The sugar solutions were stored at -20^{0}C in screw-capped test tubes/ falcon tubes.

Appendix I.4: Preparation of Buffered Salt Indicator Solution (BSS)

Ingredients (to prepare 100 ml)

K_2HPO_4 .....0.04 gm

KH_2PO_4 .....0.01 gm

KCl ..... 0.8 gm

Phenol red ..... 0.06 gm

ddH_2O ..... up to 100 ml

Procedure

i. All the reagents were weighed and transferred in a conical flask;

ii. 50 ml ddH_2O was added into the flask, mixed well and transferred to a measuring cylinder;

iii. Distilled water was added into the cylinder till 100 ml;

iv. The solution was transferred to the properly labeled reagent bottle (250 ml);

v. *Sterilization:* autoclaving;

vi. *Storage:* at room temperature.

Appendix I.5: Preparation of Phosphate Buffered Solution (PBS)

(1) Master stock solution: 10x PBS pH 7.2/ 6.5 (to prepare 100 ml)

NaCl..... 8.9 gm

NaH_2PO_4.....0.34 gm

Na_2HPO_4.....1.2 gm

dd H_2O.....up to 100 ml

(2) Working solution: 1x PBS (to prepare 100 ml):

10x PBS.....10 ml

dd H_2O..... 90 ml

Procedure (10x PBS, pH 7.2/ 6.5):

i. Ingredients (NaCl-8.9 gm, NaH_2PO_4-0.34 gm, Na_2HPO_4-1.2 gm) were weighed in appropriate electronic balances and kept in a 250 ml beaker.

ii. 95 ml ddH_2O was measured in a 250 ml measuring cylinder and added to the beaker containing the salts and mixed adequately by a magnetic stirrer.

iv. pH of the solution was then read by a pH meter and adjusted if required to a pH of 7.2/ 6.5.

v. The solution was transferred to the measuring cylinder, ddH_2O was added to make volume 100 ml.

vi. The solution was then transferred to a 250 ml reagent bottle with proper labeling and made ready for autoclaving.

vii. *Sterilization:* Autoclaving at 121^{0}C for 15 minutes.

viii. *Storage:* at room temperature for next use.

Appendix I.6: Preparation of Antibiotic solutions for Agar dilution AST

To prepare Penicillin stock solution (5 ml of 5120µg/ml)

Penicillin G powder (reagent grade with 100% purity) ……..25.6 mg

0.1 M Phosphate buffer solution (PBS) at pH 6.5……..up to 5 ml

To prepare Tetracycline stock solution (5 ml of 5120µg/ml)

Tetracycline powder (reagent grade with 100% purity) ……..25.6 mg

ddH_2O……..up to 5 ml

To prepare Ciprofloxacin stock solution (5 ml of 5120µg/ml)

Ciprofloxacin powder (reagent grade with 100% purity) ……..25.6 mg

ddH_2O……..up to 5 ml

To prepare Spectinomycin stock solution (5 ml of 5120µg/ml)

Spectinomycin powder (reagent grade with 100% purity) ……..25.6 mg

ddH_2O……..up to 5 ml

To prepare Ceftriaxone stock solution (5 ml of 160µg/ml)

Ceftriaxone powder (reagent grade with 100% purity) .....0.8 mg

0.1 M Phosphate buffer solution (PBS), pH 6.5.....up to 5 ml

To prepare Cefixime stock solution (5 ml of 160µg/ml)

Cefixime powder (reagent grade with 100% purity) .....0.8 mg

ddH_2O.....up to 5 ml

Procedure:

i. Stock solutions of the antibiotics were prepared with sterile 0.1M PBS at pH 6.5 (Penicillin, Ceftriaxone and Cefixime) or deionized distilled water (Tetracycline, Ciprofloxacin and Spectinomycin).

ii. Working solutions of different required concentrations were prepared from the stock solution following the table below.

iii. 2 ml of the working solution at required concentration was mixed with 18 ml GC agar medium with 1% Isovitalex (Appendix II.4) to give final concentrations of the antibiotic from 128.0 µg/ml to 0.002 µg/ ml to prepare the antibiotic media.

iv. *Storage:* The stock antibiotic solution was stored at -20^{0}C for next use.

Sl no	Stock soln conc (µg/ml)	Stock volume (ml)	Sterile ddH_2O added (ml)	Working soln Intermediate Conc (µg/ml)	Working soln added to media (ml)	Amount of media (ml) at 1:10 dilution	Final conc in media (µg/ml)
1	5120	2	6	1280	2	18	128.0
2	1280	2	2	640	2	18	64.0
3	1280	1	3	320	2	18	32.0
4	1280	1	7	160	2	18	16.0
5	160	2	2	80	2	18	8.0
6	160	1	3	40	2	18	4.0
7	160	1	7	20	2	18	2.0
8	20	2	2	10	2	18	1.0
9	20	1	3	5	2	18	0.5
10	20	1	7	2.5	2	18	0.25
11	2.5	2	2	1.25	2	18	0.125
12	2.5	1	3	0.6	2	18	0.06
13	2.5	1	7	0.3	2	18	0.03
14	0.3	2	2	0.16	2	18	0.016
15	0.3	1	3	0.08	2	18	0.008
16	0.3	1	7	0.04	2	18	0.004
17	0.04	2	2	0.02	2	18	0.002

Appendix I.7: Preparation of McFarland turbidity standards

McFarland turbidity standards were prepared by mixing two reagents: (1) 1% Ba chloride solution, and (2) 1% Sulfuric acid solution. The required standard of McFarland tube was prepared adding appropriate amount of the solutions as mentioned below:

(1) Preparation of 1% Ba-chloride solution:

Ingredients (to prepare 10 ml):

Ba-chloride powder.....0.1gm

ddH_2O.....10 ml

Procedure:

i. The Ba-chloride powder 0.1gm was weighed and taken into a clean 50ml reagent bottle
ii. 10 ml ddH_2O was measured in a cylinder, and carefully added to the reagent bottle
iii. The mixture in the bottle was mixed gentle shaking
iv. The 1% Ba-chloride solution was then ready for use and kept on reagent racks with proper labeling (1% Ba chloride and date of preparation)

(2) Preparation of 1% Sulfuric acid solution:

Ingredients (to prepare100 ml):

Concentrated Sulfuric acid .....1 ml
ddH_2O ..... up to 100 ml

Procedure:

i. 90 ml ddH_2O was measured in a cylinder and poured into a properly labeled (1% Sulfuric acid with date of preparation) 200 ml reagent bottle
ii. 1 ml of concentrated Sulfuric acid was measured in a glass pipette suctioned by a Pi-pump very carefully to avoid highly inflammable acid burn
iii. The acid was then added to the bottle containing water drop by drop and mixed by gentle shaking of the bottle during addition

(3) Protocol for preparation of different Mc Farland turbidity standards

Mc Farland standard	*Amount of solutions mixed*		*Corresponding bacterial CFU/ml ($x10^x$)*
	1% Barium chloride	*1% Sulfuric acid*	
0.5	0.05 ml	9.95 ml	1
1	0.1 ml	9.9 ml	3
2	0.2 ml	9.8 ml	6
3	0.3 ml	9.7 ml	9
4	0.4 ml	9.6 ml	12
5	0.5 ml	9.5 ml	15
6	0.6 ml	9.4 ml	18
7	0.7 ml	9.3 ml	21
8	0.8 ml	9.2 ml	24
9	0.9 ml	9.1 ml	27
10	1.0 ml	9.0 ml	30

Appendix I.8: Preparation of Penicillin Solution for Beta-lactamase test (van Dyck *et al*, 1989)

Ingredients (to prepare 10 ml):

Penicillin powder (reagent grade, buffer free), 5%.....5.0 gm

Bromocresol purple, 0.2%..... 0.2 gm

0.05M Phosphate buffer solution, pH 8.0..... up to 100 ml

Procedure:

i. 5.0 gm Buffer free crystalline Penicillin powder (Upjohn, USA) was measured and kept in a 50 ml beaker;

ii. For preparation of 0.05 M Phosphate buffer at pH 8.0, stock solutions were prepared with 0.68 gm KH_2PO_4 in 10 ml and 0.89 gm $Na_2HPO_4.2H_2O$ in 100 ml ddH_2O. Working solution was then prepared by addition of 5.5 ml of KH_2PO_4 solution in 94.5 ml $Na_2HPO_4.2H_2O$ solution;

iii. 0.2 gm Bromocresol purple (Oxoid, UK) was measured and added to the beaker and mixed on a magnetic stirrer;

iv. pH of the solution was adjusted to 8.0 by adding NaOH/ HCl if necessary;

v. *Sterilization:* The solution was then filtered by disposable filters and made aliquots at 1.5 ml volume into eppendorf tubes labeled properly;

vi. *Preservation:* The eppendorfs were preserved at -20 degrees Celsius till next use.

Appendix I.9: Preparation and dispensing of PPNG-PCR master mix

Composition of the PPNG-PCR master mix:

ddH_2O for PCR	- 16.8 µl,
10x PCR buffer	- 2.5 µl,
$MgCl_2$	- 0.75 µl,
dNTPs	- 2.0 µl,
primers: JDA, JDB	- 0.15 µl each,
Taq DNA polymerase	- 0.15 µl,
DNA template	- 2.5 µl

Procedure:

i. All of the ingredients were taken in ice in a box;

ii. The ingredients were mixed within a PCR hood in a large (1.5 ml) eppendorf keeping in ice and dispensed into the PCR tubes labeled properly;

iii. The DNA template was added from the properly thawed DNA preparations keeping in ice;

iv. The PCR tubes after addition of DNA template were placed into the PCR amplifier machine immediately for run in the targeted program.

Appendix I.10: Preparation and dispensing of TRNG-PCR master mix

Composition of TRNG-PCR master mix:

ddH_2O for PCR	- 19.3 µl,
10x PCR buffer	- 2.5 µl,
$MgCl_2$	- 1.0 µl,
dNTPs	- 1.0 µl,
primers: RM4, G1	- 0.05 µl each,
Taq DNA polymerase	- 0.1 µl,
DNA template	- 1.0 µl

Procedure:

i. All of the ingredients were taken in ice in a box

ii. The ingredients were mixed within a PCR hood in a large (1.5 ml) eppendorf keeping in ice and dispensed into the PCR tubes labeled properly.

iii. The DNA template was added into the corresponding PCR tube from the properly thawed DNA preparations keeping in ice.

iv. The PCR tubes after addition of DNA template were placed into the PCR amplifier machine immediately for run in the targeted program.

Appendix I.11: Preparation of 3M Na-Acetate solution (pH 4.6)

Ingredients (to prepare 50 ml):

Na acetate ($CH_3COONa.3H_2O$, trihydrate).....20.412 gm

Acetic acid (to pH) .....~ 6 ml

H_2O.....up to 50 ml

Procedure:

i. 20.312 gm of Na acetate was weighed carefully and transferred to a 50 ml reagent bottle

ii. 40 ml H_2O was added to the bottle to dissolve the Na acetate

iii. Glacial acetic acid ~6 ml was added to adjust the pH of the solution to 4.6 and the volume was adjusted to 50 ml by H_2O if required

v. *Sterilization:* by filtration

vi. *Storage:* at room temperature in small aliquots

Appendix II: Preparation of media

Appendix II.1: Preparation of Modified Thayer Martin (MTM) Medium

Ingredients (to prepare 500 ml):

GC Agar Base.....	18.0 gm
Defibrinated, saponin-lysed, sheep blood..... ...	50 ml
Growth supplement (Isovitalex) solution.....	5 ml
Antibiotic supplement (VCNT) solution.....	5 ml
Deionized distilled water (ddH_2O).....	Up to 500 ml

Procedure:

i. GC Agar Base (Oxoid, UK) was measured and transferred to a 1 liter conical flask

ii. The deionization plant (Nanopure Infinity, Barnstead, USA) was switched on and 450 ml ddH_2O was measured in a 500 ml cylinder, added to the GC agar base, and mixed gently to dissolve the base.

iii. The conical flask containing GC agar base solution was closed with a suitable cotton plug stopper and autoclaved properly. The solution after autoclaving was kept in a water-bath fixing at a temperature of 55 degrees Celsius till dispensing.

iv. The sheep blood, defibrinated with glass beads, was transferred to a sterile glass bottle, and lysed adding a 0.5% sterile saponin solution (2.5 ml of a 10% sterile saponin solution) with all aseptic precautions.

v. The Isovitalex powder (BBL, USA) and the diluent (BBL, USA) were kept at room temperature and 10 ml diluent was added to the powder, and 5 ml of Isovitalex solution was drawn in a sterile disposable syringe.

vi. The VCNT (BBL, USA/ Oxoid, UK) powder was kept to room temperature,10 ml of sterile ddH_2O was added to dissolve, and 5 ml of the VCNT solution was drawn in a sterile disposable syringe.

vii. The autoclaved GC agar base solution made at 55 degrees Celsius was brought in a laminar air flow hood (BH 2000 Series, CLYDE APAC, USA, Model no. BHA 180) and 50 ml saponin-lysed defibrinated sheep blood, 5 ml Isovitalex solution, and 5 ml VCNT

solution were added to GC agar solution quickly and all ingredients mixed gently avoiding formation of froths.

viii. The MTM medium was thus prepared and dispensed into disposable plastic Petri-plates labeled properly in an amount of about 20 ml making a thickness of about 4 mm.

ix. After solidification of the MTM medium plates were then preserved in a refrigerator within plastic bags for next use.

Appendix II.2: Preparation of GC Agar Medium for subculture and agar dilution AST

Ingredients (to prepare 500 ml):

GC Agar Base.....	18.0 gm
Isovitalex supplement solution.....	5 ml
Deionized distilled water (ddH_2O).....	Up to 500 ml

Procedure:

i. GC Agar Base (Oxoid, UK) was measured and transferred to a 1 litre conical flask.

ii. An amount of 450 ml ddH_2O was measured in a 500 ml cylinder, added to the GC agar base and mixed gently to dissolve the base.

iii. The conical flask containing GC agar base solution was closed with a suitable cotton plug stopper and autoclaved properly. The solution after autoclaving was kept in a water-bath fixing at a temperature of 55 degrees Celsius till dispensing.

v. The Isovitalex powder (BBL, USA) and the diluent (BBL, USA) were kept at room temperature for 30 minutes and 10 ml diluent coming close to room temperature was added to the powder.

vii. The autoclaved GC agar base solution made at 55 degrees Celsius was brought in a laminar air flow hood (Appendix IV.2) and the Isovitalex solution- 5 ml was added to GC agar solution quickly and the ingredients mixed gently avoiding formation of froths.

viii. For subculture, an amount of about 20 ml GC agar medium with Isovitalex was dispensed into disposable plastic Petri-plates labeled properly making a thickness of about 4 mm.

ix. For agar dilution AST to determine MICs of Penicillin, Tetracycline, Ciprofloxacin, Spectinomycin, Ceftriaxone and Cefixime, different concentrations of the antibiotic solutions were prepared (Appendices I.6). An amount of 18 ml of the GC agar medium with Isovitalex and 2 ml of the respective antibiotic solution at different concentrations was mixed and dispensed into disposable plastic petri-plates labeled properly.

x. *Storage:* After solidification, the MTM medium plates (both for subculture and agar dilution AST) were preserved in a refrigerator within plastic bags for next use with 4 weeks.

Appendix II.3: Preparation of 20% Glycerol broth

Ingredients (to prepare 100 ml):

Tryptic Soy Broth.....	3.0 gm
Yeast Extract.....	0.6 gm
Glycerol.....	20 ml
ddH_2O.....	Up to 100 ml

Procedure:

i. Tryptic Soy Broth (Oxoid, UK) and Yeast Extract (Oxoid, UK) were measured and kept into a 500 ml beaker.

ii. 70 ml of ddH_2O was added and mixed using a magnetic stirrer (Appendix II.7)

iii. 20 ml Glycerol (Oxoid, UK) was added to the beaker and allowed to mix properly.

iv. The properly mixed solution was taken in a 100 ml measuring cylinder and ddH_2O was added to make the volume 100 ml. The solution was transferred again into the beaker and mixed properly.

v. The 20% Glycerol broth was thus prepared and dispensed at a volume of 1.5 ml in 2 ml vials.

vi. *Sterilization:* The storage medium vials were then autoclaved properly in a 9 x 9 nalgene box.

vii. *Storage:* The medium vials were then preserved at -20 degrees Celsius till next use.

Appendix III: Tests procedures

Appendix III.1: Superoxol test

Requirement

30% Hydrogen peroxide

Test tube

Wire loop

Burner

Test procedure

i. Three-fourth of the test tube is filled with the hydrogen peroxide;

ii. After flaming and cooling the wire loop, a distinct colony of the test organism was picked up and dissolve into the hydrogen peroxide in the test tube;

iii. Test result was observed by rapidly developing bubbles coming out over the surface of the reagent;

iv. Positive result was indicated by the rapid development of bubbles, whereas negative result was indicated by no bubbles.

Appendix III.2: Oxidase test

Requirements

Working oxidase reagent (Appendix I.5)

Blotting paper

Wire loop

Burner

Test procedure

i. A piece of blotting paper was fitted into a petriplate with proper labeling and soaked with the working oxidase reagent;

ii. A single colony of the test organism is picked up by the properly flamed and cooled wire loop and rubbed over the reagent soaked in blotting paper;

iii. Test result was observed by change in colour of the rubbed area of the blotting paper;

iv. Positive result was indicated by immediate change in colour of the colony-rubbed blotting paper to purple and negative result showed no colour change.

Appendix III.3: Sugar fermentation test

Requirements

Sugar solution, Test tubes

Buffered salt indicator solution (BSS) (Appendix I.4)

Fresh pure subculture of the test organism

Wire loop, Burner

Test procedure

i. Fresh subcultures of the test organism were prepared on GC agar medium with growth supplement (Appendix II.2);

ii. An amount of 0.05 ml of the 20% sterile glucose, maltose, sucrose and lactose solutions were taken into the test tubes labeled properly;

iii. An amount of 0.1 ml PBS was added to each of the test tubes containing the sugars as well as a fifth tube without sugar serving as control;

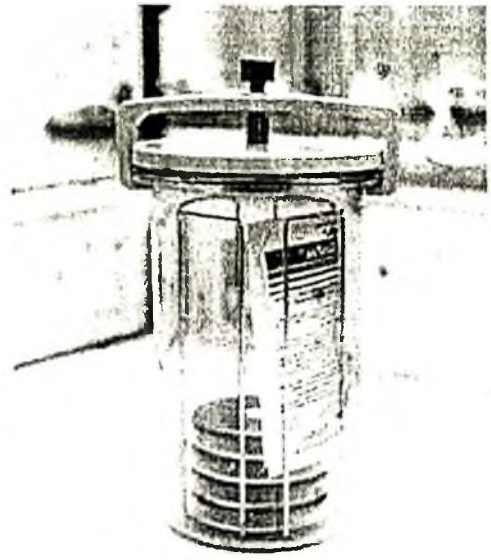
iv. Two loopfuls in a 3mm wire loop of the test organism from the fresh subculture were emulsified into 0.3 ml -0.4 ml sterile PBS and transferred to each of the five test tubes;

v. The suspensions were mixed well and incubated in a waterbath at 37^0C for 4 hours;

vi. After proper incubation, a colour change from red to yellow was considered as positive result and no change as negative.

Appendix IV: Unique Instruments used

Appendix IV.1: CO_2 extinction jar

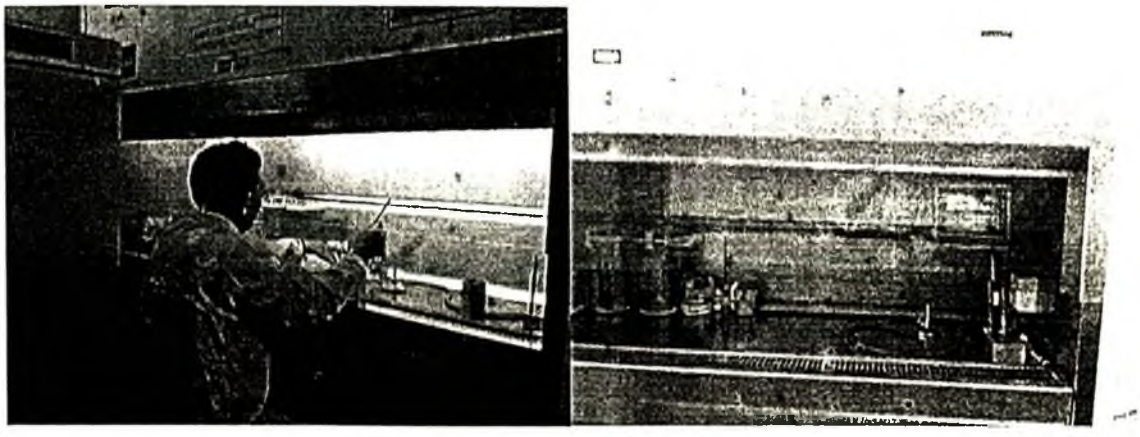


CO_2 extinction (Anaerobic) Jar (unvented)
(BD GasPak™ 100 Anaerobic Unvented Jar)

Manufacturer: Becton Dickinson, 1 Becton Drive, Franklin Lakes, NJ USA 07417, 201.847.6800

Description: BD GasPak™ 100 Anaerobic Jars offer waterless, catalyst-free environment in producing anaerobic, microaerophilic, or CO_2-enriched environments. Anaerobic as well as CO_2 Indicators are available to provide reliable methods for assuring ideal anaerobic and CO_2 enriched environments are achieved. The complete GasPak™ 100 System consists of polycarbonate jar, lid with "O" ring gasket, improved clamp/ thumbscrew assembly and catalyst reaction chamber, two catalyst charges, one rack and one tube holder. Also available as a vented system, which is complete with vent installed, rubber tube and tubing clamp. The Jar also can be used with white, non-scented burning candles to produce 3-5% CO_2 instead of using gas packs.

Appendix IV.2: Label -2 safety hood (Laminar Air Flow)



Photograph of Laminar Air Flow
Left: Dr. Ashraf working in a Laminar Air Flow at then RTI/STI lab, ICDDR,B, Right: Inside view of the Laminar Air Flow

Name of the instrument: Laminar Air Flow (type II safety hood)

Model: BH 2000 series, BHA 180

Manufacturer: CLYDE-APAC, Australia

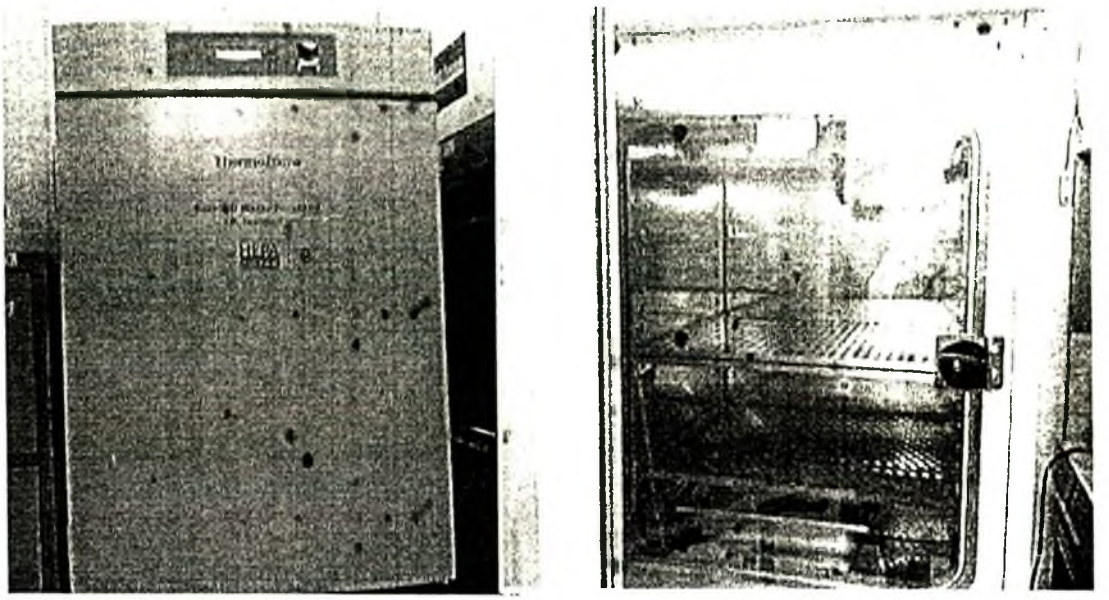
Description of the instrument:

BH2000 Series cabinets are part-recirculating laminar air flow enclosures with high efficiency particulate air (HEPA) filtration of exhaust air and an air barrier at the work opening. The HEPA-filtered vertical laminar air flow, which is recirculated in the work zone, creates an ultra-clean work environment for product protection. An air barrier between the operator and the work zone is maintained by a flow of room air into a full width grille in the work opening.

The barrier air mixes with the laminar flow air in a sump underneath the work surface, and is exhausted to the room via a HEPA filter. All positive pressure zones and filter seals are surrounded by negative pressure zones, so as to contain potentially hazardous aerosols.

Standard cabinets have exhaust discharge on the right hand side. Optional left hand side or top exhaust is available. Cabinets are produced with a work zone width of 90cm, 120cm or 180cm and may be installed on standard laboratory benches or on optional floor stands. Embodying advanced system-monitoring technology, enhanced ergonomics and styling, BH2000 Series cabinets set a new standard in safety cabinet design and performance.

Appendix IV.3: CO_2 Incubator



Photograph of CO_2 Incubator
Left: Front view, Right: Inside view

Part no. 3111, REL #5

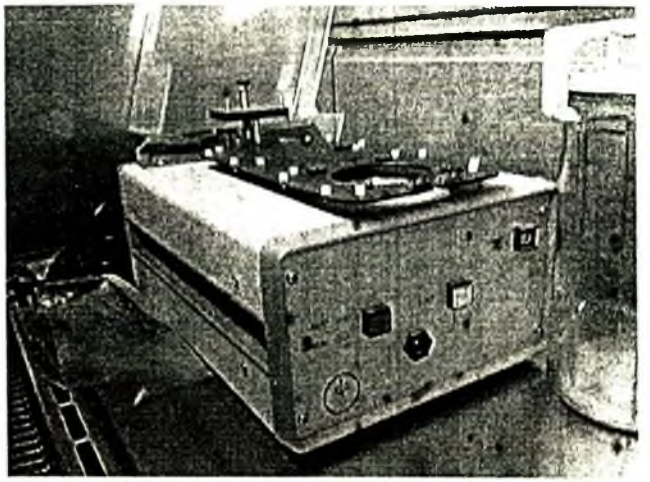
Manufacturer: Thermo Forma, Ohio, USA

Description of the instrument:

Thermo Scientific forma series II water jacketed CO_2 incubator combine precise CO_2 control with sensors, unsurpassed temperature stability, and superior parameter recovery characteristics with innovative continuous contamination control technology.

Maximal thermal stability and quick recovery are assured with unique triple wall construction providing superior protection against temperature loss in the event of unexpected power cutoff. The incubator minimizes risk of airborne contaminants entering the incubator upon door openings with a validatable in-chamber HEPA air filtration system.

Appendix IV.4: Multipoint inoculator



Name of the instrument: Multipoint inoculator

Model: NA

Manufacturer: NA

Description of the instrument:

The Multipoint Inoculator is a precision unit for the multiple inoculation of petri dishes, replica and microtitration plates. It is ideally suited for critical assay work where rapid, repetitive multipoint inoculation is required with a high degree of precision. Any format of head may be used from 5 to 100 pins. The inoculation pins are available to dispense 0.3µl and 1.0µl volumes.

Appendix IV.5: PCR machine

Photograph of the PCR amplifier (GeneAmp®) in running condition

Name of the instrument: PCR amplifier

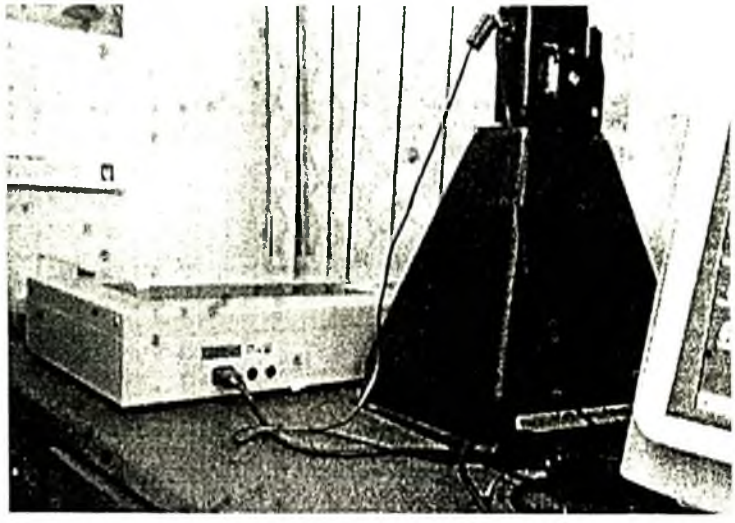
Model: Gene Amp PCR system 9700, Part no. N 8050200

Manufacturer: Applied Biosystems, Foster City CA 94404, USA

Description of the instrument:

GeneAmp® PCR System 9700 is specifically designed for the amplification of nucleic acids. The System consists of a base module and one of many interchangeable sample block modules. The 96-well GeneAmp® PCR System 9700 is designed for use with 0.2 ml reaction tubes or 96-well reaction plates for all of your routine PCR applications.

Appendix IV.6: Gel Documentation unit



Photograph of Gel documentation unit comprising of Kodak digital camera and transilluminator

Name of the instrument: Gel documentation unit

Model: Electrophoresis, Documentation and Analysis system 120

Manufacturer: Kodak Digital Science, Japan

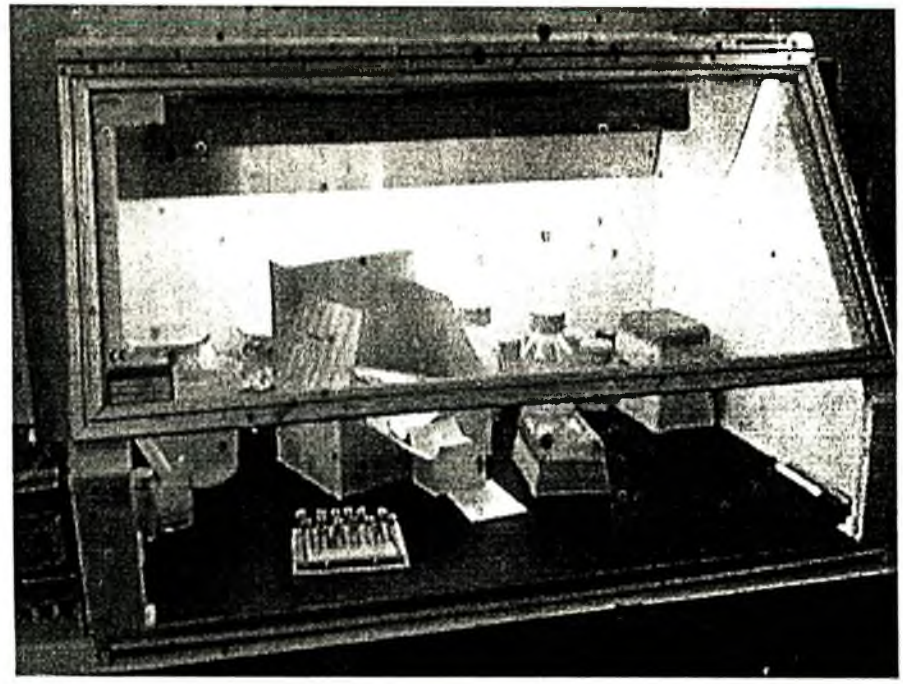
Description of the instruments:

The gel documentation unit comprises principally of two instruments: (i) The flat bed Transilluminator and (ii) The Kodak positive camera.

Appendix IV.7: DNA Calculator

Photograph of DNA Calculator
Left: The Instrument, Right: Dr. Ashraf working with DNA Calculator

Appendix IV.8: PCR Hood



Photograph of a PCR hood at Molecular Laboratory of CLS, LSD, ICDDR,B

Appendix V: Ethical permission letter copy

The Ethical clearance by the Ethical Review Committee, ICDDR,B was issued to Dr. Motiur Rahman, who was then the supervisor of the Ph.D. synopsis and provided the logistic supports for the Ph.D. through his protocol # 2000-036 (Epidemiology and etiology of sexually transmitted infections and antimicrobial susceptibility surveillance of *Neisseria gonorrhoeae* in Bangladesh). Dr. Md. Ashraful Alam was the Co-Investigator of this protocol and later allowed to work at the ICDDR,B as Elective Ph.D. Fellow (Identification Card image on next page).

ICDDR,B
CENTRE FOR HEALTH AND POPULATION RESEARCH

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH
Mail : ICDDR,B, GPO Box 128, Dhaka-1000, Bangladesh
Phone : 871751-60, Telex : 675612 ICDD BJ
Fax : 880-2 883116, 836052, 871568, 871686, Cable : Cholera Dhaka

MEMORANDUM

17 January 2001

To Dr. Motiur Rahman
Laboratory Sciences Division

17/01/2001

From Professor Mahmudur Rahman
Chairman, Ethical Review Committee (ERC)

Sub Protocol # 2000-036

This has reference to your memo of 7th January 2001 with a modified copy of your protocol # 2000-036 entitled "Epidemiology and etiology of sexually transmitted infections and antimicrobial susceptibility of surveillance of *N. gonorrhoea* in Bangladesh". The protocol is hereby approved upon your satisfactory addressing of the issues raised by the Committee in its meeting held on 29th November 2000.

Thanking you and wishing you success in running the said study

cc: Acting Associate Director
Laboratory Sciences Division

Principal Investigator (Last, First, middle [illegible]

KEY PERSONNEL (Do not duplicate personnel listed in Participating Organizations)

Name	Professional Discipline	Specific Project
A. Laboratory Sciences Division:		
1. Mostur Rahman	MBBS, PhD	Molecular Biologist
2. Joseph Bogaerts	MBBS, MPH, PhD	Molecular Biologist
B. Health and Population Extension Division:		
1. Dr. M. A Quaiyum	MBBS, [illegible]	[illegible]
C. Concern Bangladesh (Dhaka)		
1. Dr. Shahnewaz Alam Khan	MBBS, MPH	Head, Health and Nutrition Programme, Concern Bangladesh
D. Salvation Army (Jessore)		
1. Mr. Victor [illegible]		[illegible]
E. Paricharja (Dhaka)		
1. Dr. Parvez Sabrin Chowdhury	MBBS, DGO	[illegible]
F. Momota (Chittagong)		
1. Dr. Saiful Islam	MBBS	[illegible]
G. M. A. G. Osmani Medical College, (Sylhet)		
1. Dr. Ashraful Alam	MBBS, M. Phil Assistant Prof Dept. of Pathology	Camp coordinator

Name: Mr. Ashraful Alam

Title: Elective Fellow

Appendix VI: Patient's Consent form in Bangla (modified from the ICDDR,B protocol # 2000-036)

গবেষণায় অংশগ্রহণকারীর Serial no. | | | | | |

গবেষণায় অংশগ্রহণকারীর Reg. no. | | | | | |

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, মহাখালী, ঢাকা-১২১২ ও
মাইক্রোবায়োলজী বিভাগ, সিলেট এমএজি ওসমানী মেডিকেল কলেজ, সিলেট

স্বেচ্ছা সম্মতি পত্র (পুরুষ)

আন্তর্জাতিক উদরাময় গরেষণা কেন্দ্র-এর সহায়তায় আমি বর্তমানে "গনোরিয়া রোগের ব্যাপ্তি এবং এই রোগের জীবানুর এন্টিবায়োটিক স্পর্শকাতরতা" শিরোনামে একটি পি,এইচ,ডি গবেষণা পরিচালনা করছি। এই গবেষণা সিলেট অঞ্চলে গনোরিয়া সংক্রমণের ব্যাপকতা এবং এর জীবাণুর এন্টিবায়োটিক সংবেদনশীলতা সম্পর্কে জানতে সহায়তা করবে এবং এই গবেষণার ফলাফল গনোরিয়া সংক্রমণ প্রতিরোধে সহায়তা করবে। আপনি এই গবেষণায় অংশগ্রহণ করে আমাকে সহায়তা করতে পারেন। আপনি যদি আমাদের এই গবেষণায় অংশগ্রহণ করতে চান তাহলেঃ

১। আপনার স্বাস্থ্য সম্পর্কে কিছু প্রশ্ন করা হবে এবং একজন ট্রেনিংপ্রাপ্ত পুরুষ চিকিৎসক আপনার শারীরিক পরীক্ষা করবেন যার মধ্যে আপনার লিঙ্গ পরীক্ষাও অন্তর্ভুক্ত থাকবে।

২। একটি সোয়াব কাঠির সাহায্যে আপনার লিঙ্গ থেকে সোয়াব সংগ্রহ করা হবে। এতে আপনি সামান্য ব্যাথা অনুভব করবেন, তবে আপনার কোন ক্ষতির সম্ভাবনা নেই।

৩। ইতিমধ্যে আপনার লিঙ্গ হতে নেওয়া সোয়াব হতে গরোরিয়া রোগ নির্ণয়ের জন্য পরীক্ষা করা হবে।

৪। উপরোক্ত পরীক্ষায় যদি আপনার গনোরিয়া রোগ নির্ণয় হয় তবে আপনাকে এই রোগের চিকিৎসা প্রদান করা হবে।

৫। আমাদের এই গবেষণা হতে আপনি সরাসরি উপকৃত হবেন, কারণ আমরা আপনার রোগের কারণ নির্ণয় করে আপনাকে বিনামূল্যে চিকিৎসা প্রদান করছি। এই ব্যবস্থা আপনার রোগটি অন্যদের মধ্যে ছড়াতে প্রতিরোধ করবে। উপরন্তু, এই গবেষণার ফল সমাজকে ভীষণভাবে উপকৃত করবে।

৬। এই গবেষণায় অংশগ্রহণ করলে আপনার শারীরিক ঝুঁকির সম্ভাবনা নগণ্য।

৭। আপনি যদি এই গবেষণায় অংশগ্রহণ নাও করেন তাহলেও এই হাসপাতালের প্রচলিত ব্যবস্থা অনুযায়ী আপনি চিকিৎসা পাবেন। অন্যদিকে অংশগ্রহণ করেও যে কোন সময় কোনরূপ ক্ষতিপূরণ ছাড়াই আপনি আপনার সম্মতি প্রত্যাহার করতে পারবেন। এমনকি এই হাসপাতাল হতে প্রাপ্ত আপনার পরবর্তী চিকিৎসা ব্যবস্থারও কোনরূপ পরিবর্তন হবে না।

৮। ল্যাবরেটরী পরীক্ষার ফলাফল ও আপনার যাবতীয় তথ্য সম্পর্কে গোপনীয়তা রক্ষা করা হবে। এই গবেষণায় অংশগ্রহণকারী, গবেষকগন এবং মানবিক অধিকার রক্ষায় ইথিক্যাল কমিটি ছাড়া এই সব তথ্য অন্য কেউ জানতে পারবে না।

৯। আমরা যে কোন সময় আপনার যে কোন প্রশ্নের উত্তর দিতে বাধ্য থাকবো।

আপনি যদি এই গবেষণায় অংশগ্রহণ করতে ইচ্ছুক হন, তাহলে অনুগ্রহ করে নিচে প্রদত্ত স্থানে স্বাক্ষর বা টিপসহি দিন। আপনার সহযোগিতার জন্য ধন্যবাদ।

গবেষণায় অংশগ্রহণকারীর স্বাক্ষর/টিপসহি
তারিখঃ

পরীক্ষকের স্বাক্ষর
তারিখঃ

Appendix VII: Patient Data collection Questionnaire (modified from the ICDDR,B protocol # 2000-036)

পি,এইচ,ডি গবেষণার জন্য তথ্য সংগ্রহ প্রশ্নমালা

গবেষণার বিষয়: অংশ-১। গনোরিয়া রোগের ব্যাপ্তি ও এই রোগের জীবানুর এন্টিবায়োটিক স্পর্শকাতরতা

গবেষক: ডা: মো: আশরাফুল আলম, সহকারী অধ্যাপক (চলতি দায়িত্ব), মাইক্রোবায়োলজী বিভাগ, সিলেট এম,এ,জি ওসমানী মেডিকেল কলেজ, সিলেট

সহযোগিতায়: আই,সি,ডি,ডি,আর,বি, মহাখালী, ঢাকা

ক্রমিক নং ☐☐☐☐☐ তারিখঃ ☐☐☐☐☐

রেজিষ্ট্রেশন নং ☐☐☐☐☐

১। রোগীর নাম ঃ

২। বয়স (বছর)ঃ ☐☐

৩। ঠিকানা ঃ

৪। ধর্ম ঃ (ইসলাম-১, হিন্দু-২, বৌদ্ধ-৩, খ্রীষ্টান-৪, অন্যান্য-৯) ☐

৫। শিক্ষা ঃ (শিক্ষিত-১, অশিক্ষিত-২) ☐

শিক্ষিত হলে কত বছর শিক্ষালাভ করেছেন/ কোন ক্লাশ পর্যন্ত পড়েছেন ? ☐☐

৬। পেশা ঃ (যৌণ কর্মী-১, দিন মজুর-২, ড্রাইভার-৩, চাকুরী-৪, ছাত্র-৫, ব্যবসা-৬, বেকার-৯) ☐

৭। বৈবাহিক অবস্থা ঃ (অবিবাহিত-১, বিবাহিত-২, তালাকপ্রাপ্ত/ বিপত্নিক-৩) ☐

৮। কত বছর বয়সে আপনার প্রথম যৌণ মিলনের অভিজ্ঞতা হয়েছিল? ☐☐

এবং তখন আপনার যৌণ সঙ্গী কে ছিলেন? (স্ত্রী-১, বান্ধবী-২, যৌণ কর্মী-৩, অন্যান্য-৯) ☐

৯। আপনার কি কোন নিয়মিত যৌণ সঙ্গী আছে (বিবাহিতদের ক্ষেত্রে স্ত্রী ছাড়া) (হ্যাঁ-১, না-২) ☐

হ্যঁ হলে, আপনার যৌণ সঙ্গী কে? (সমকামী -১, মহিলা যৌণ কর্মী-২, অন্যান্য-৯) ☐

সমকামী যৌণ সঙ্গী হলে আপনার অংশগ্রহণ কি রকম? (সক্রিয়-১, অক্রিয়-২, উভয়-৩) ☐

মহিলা যৌণ কর্মী হলে কোথায় আপনাদের মিলন হয়? (বাসায়-১, হোটেলে-২, পতিতালয়ে-৩, অন্যান্য-৯) ☐

সর্বশেষ কতদিন আগে মহিলা যৌণ কর্মীর সাথে আপনার মিলন হয়? (একদিন আগে-১, এক সপ্তাহ আগে-২, এক মাস আগে-৩, ছয় মাস আগে-৪, এক বছর বা অধিক আগে-৫) ☐

১০। এ সব যৌণ মিলনের সময় আপনি কি কনডম ব্যবহার করেছেন? (হ্যঁ-১, না-২) ☐

উত্তর হ্যাঁ হলে কি হারে ব্যবহার করেছেন? (নিয়মিত-১, অনিয়মিত-২) ☐

১১। রোগ লক্ষণ ও চিকিৎসা:

(ক) প্রশ্রাবে জ্বালা-পোড়া আছে কিনা? (হ্যাঁ-১, না-২) ☐

উত্তর হ্যাঁ হলে, কতদিন যাবৎ প্রশ্রাবে জ্বালা-পোড়া? (দিন/ মাস/ বছর) ☐☐

(খ) প্রশ্রাবের রাস্তায় কোন নিঃসরণ (তরল/ পুঁজ) আছে? (হ্যাঁ-১, না-২) ☐

উত্তর হ্যাঁ হলে, কতদিন যাবৎ প্রশ্রাবে জ্বালা-পোড়া? (দিন/ মাস/ বছর)

(গ) এই সব সমস্যার জন্য আপনি কোন চিকিৎসা নিয়েছেন কি? (হ্যাঁ-১, না-১)

হ্যাঁ হলে কার কাছে চিকিৎসা নিয়েছিলেন? (এমবিবিএস ডাক্তার-১, ঔষুধের দোকানদার-২, হোমিওপ্যাথি ডাক্তার-৩, আয়ুর্বেদী ডাক্তার-৪, অন্যান্য-৯)

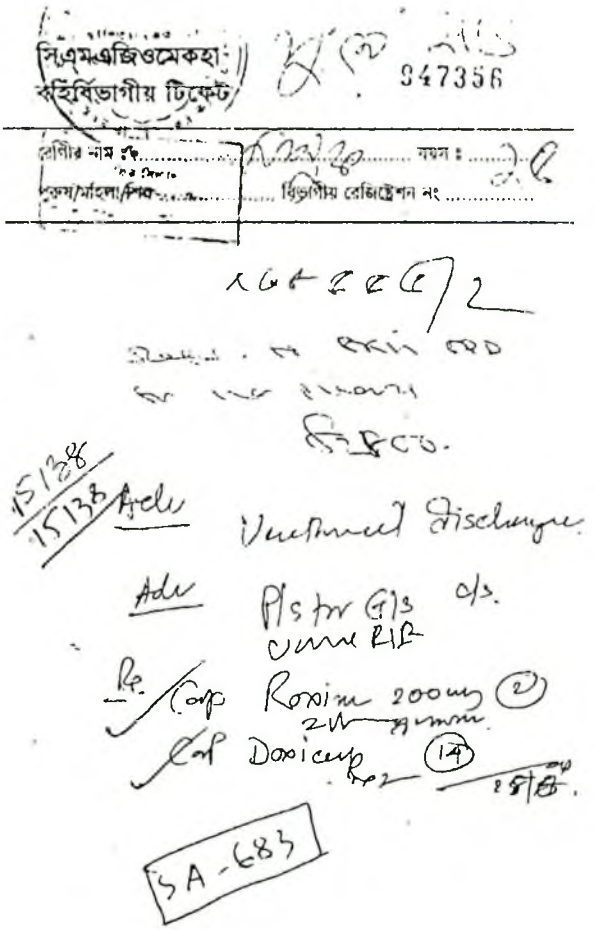
সম্পূর্ণ চিকিৎসা নিয়েছিলেন কিনা? (হ্যাঁ-১, না-২)

(ঘ) অতীতে কখনো এই রকম সমস্যায় ভুগেছেন কিনা? (হ্যাঁ-১, না-২)

উত্তর হ্যাঁ হলে, কত দিন আগে? (মাস/ বছর)

উত্তর হ্যাঁ হলে, চিকিৎসা নিয়েছিলেন কিনা? (হ্যাঁ-১, না-২)

Appendix VIII: SOMCH OPD prescription (scan copy)



Appendix IX: E-mail copy of Prof. Jo Anne R Dillon for indefinable PPNG plasmids (red-font-writings are the comments from Prof. J.A.R. Dillon)

YAHOO! MAIL Classic

Re: Seeking help for PPNG pcr product Thursday, May 7, 2009 10:05 AM

From: "Jo-Anne Dillon" <j.dillon@usask.ca>
To: "Ashraf Alam" <ashalam61@yahoo.com>
Message contains attachments
1 File (62KB)

DOC Alam -PCR products for PPNG plasmids -comments.doc

Dear Dr Alam:

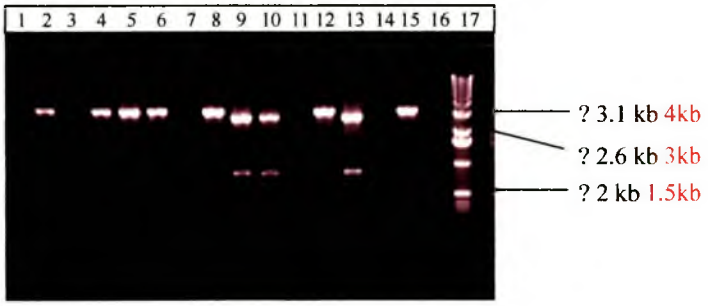
Please see attached. It seems that you had some problems with digestion as only single bands should be observed. You need better mw markers and control DNA as well.

Thank you for contacting us.

Yours sincerely,

Jo-Anne Dillon

Ashraf Alam wrote:
> Dear Mam
> Please find in attachment a gel doc from a PCR for PPNG with unusual bands following your protocol (Mol Cell Probes 1999; 13: 89-92). Would you please spare a little of your valuable times to identify the product?

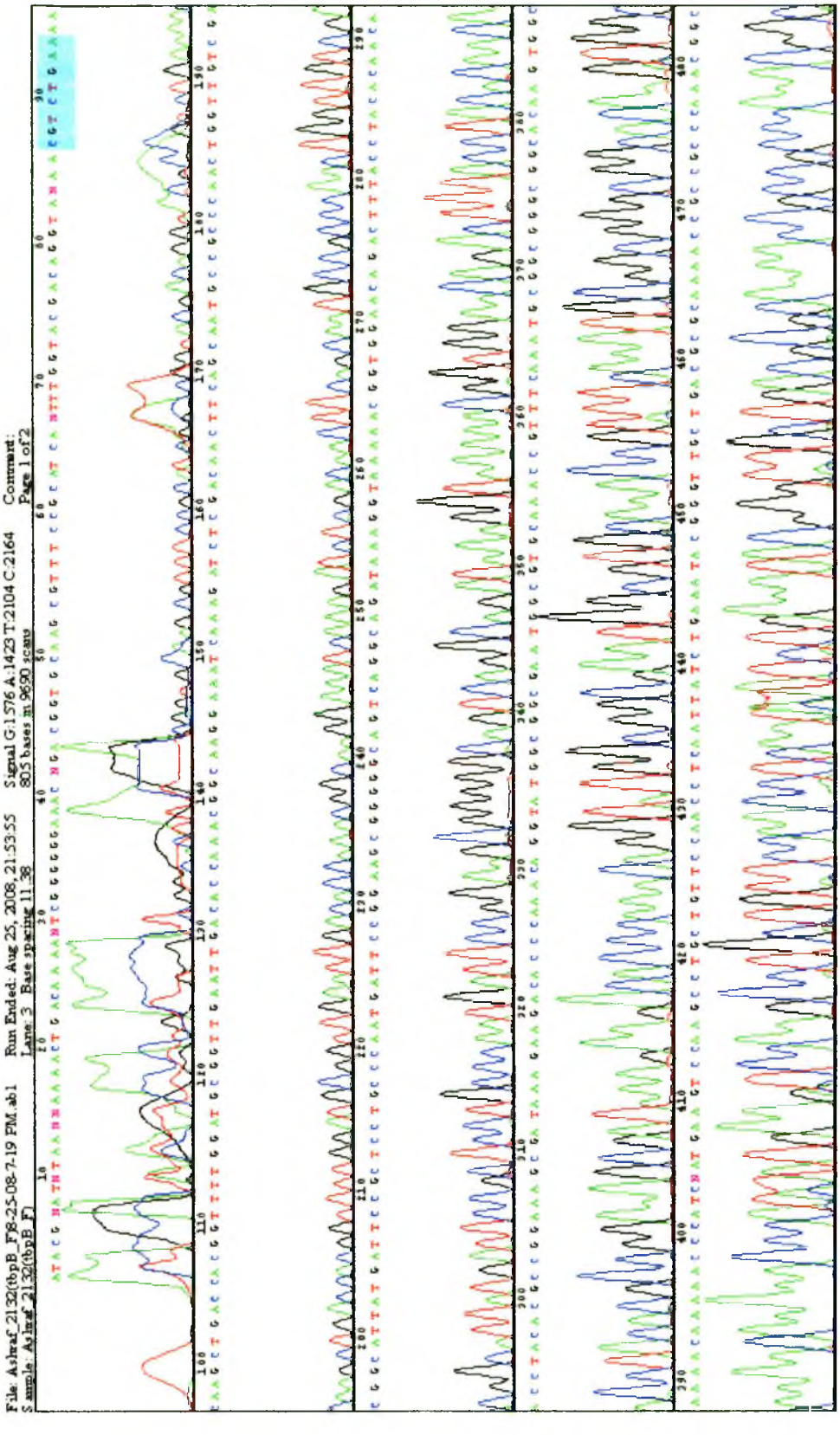


Dr. Md. Ashraful Alam*,MBBS,MPhil
> Officer on Special duty, DGHS, Dhaka, Bangladesh
> (Assistant Professor of Microbiology) Deputed to NIPSOM, Mohakhali, Dhaka &
> PhD Fellow, Laboratory Sciences Division, ICDDR,B, Dhaka
>

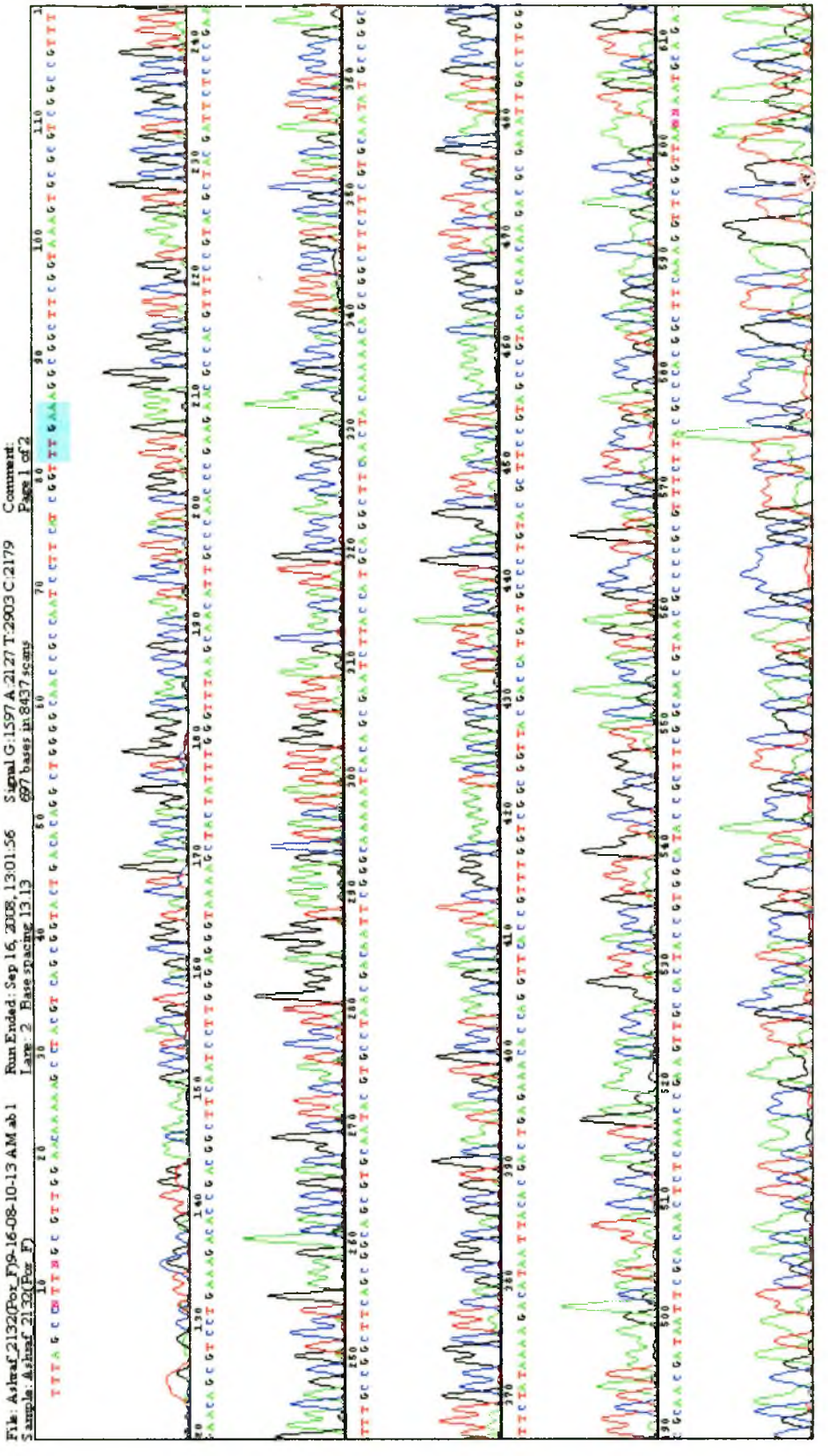
The biggest band could be Africa-type (~3.1kb). Need better differentiation for the markers with higher molecular weights. Don't have an explanation for the double bands and the small fragments (~1.3kb); it seems to me that the PCR products were digested into two pieces. I suggested that plasmids pJD4/pJD5/pJD7 be as positive controls.

Appendix X: Chromatogram of the bases sequence

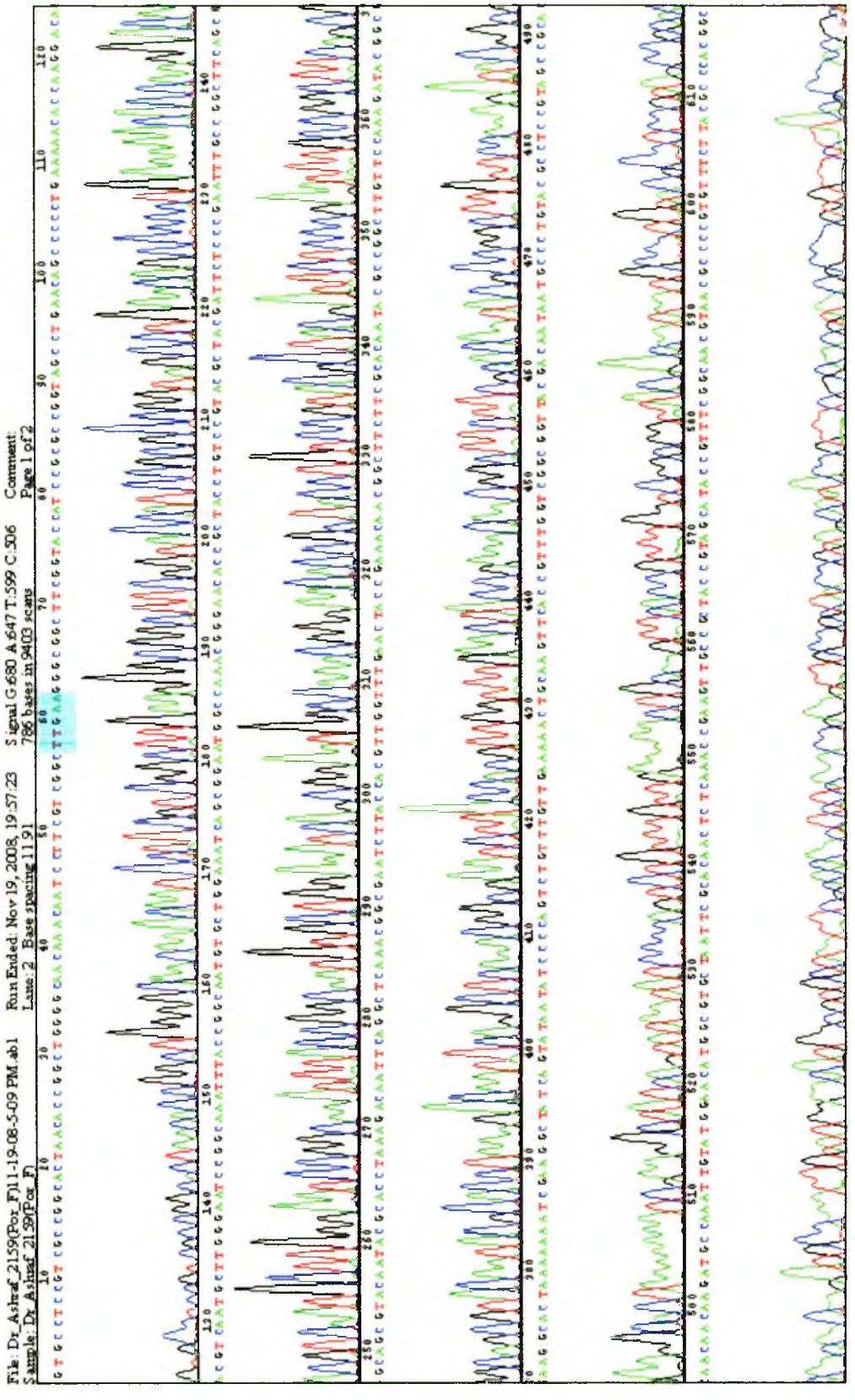
Appendix X.1: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2132 (conserved region, blue highlighted: TTGAA at base-86)



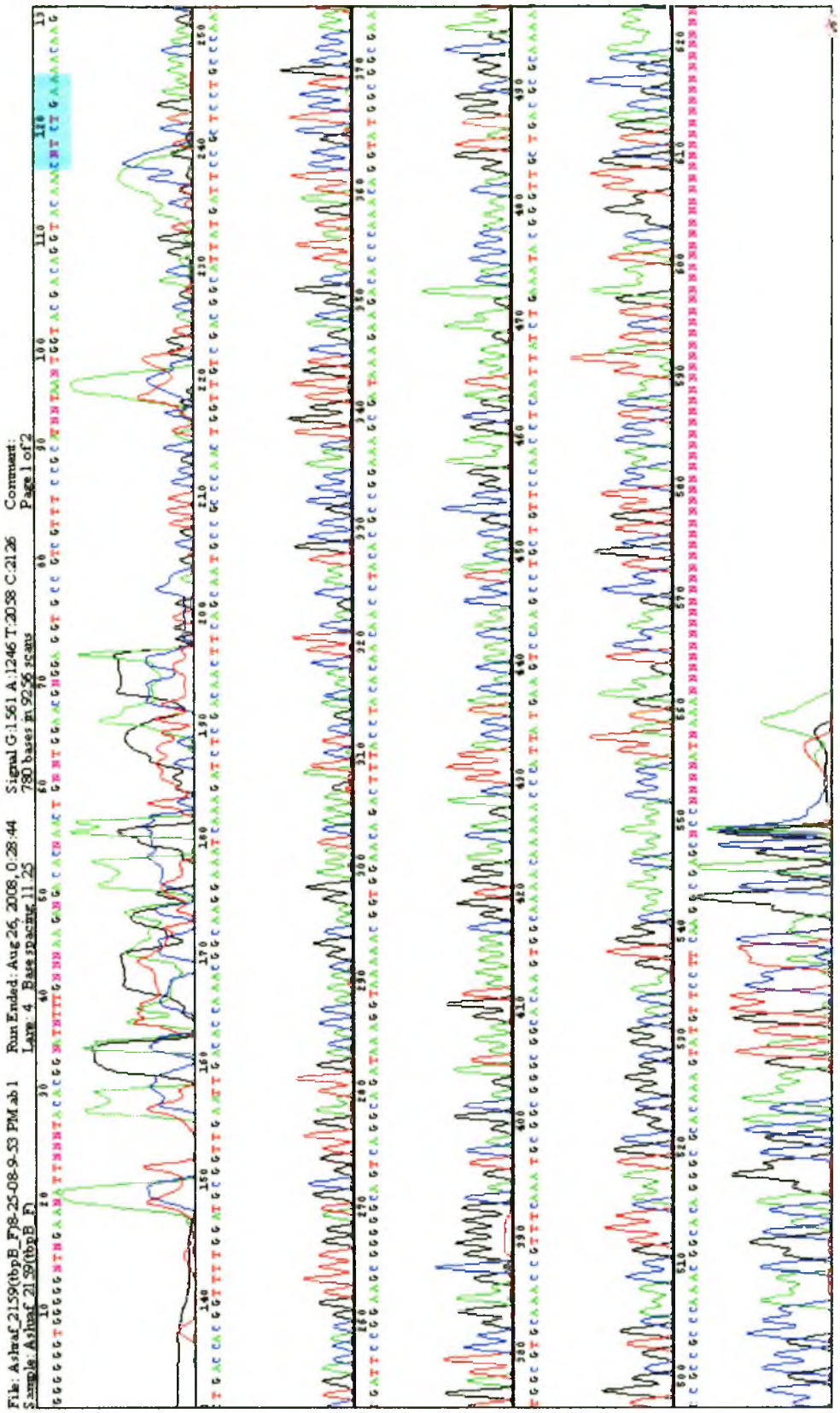
Appendix X.2: Chromatogram of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2132 (conserved region, blue highlighted: CGTCTGAA at base-86)



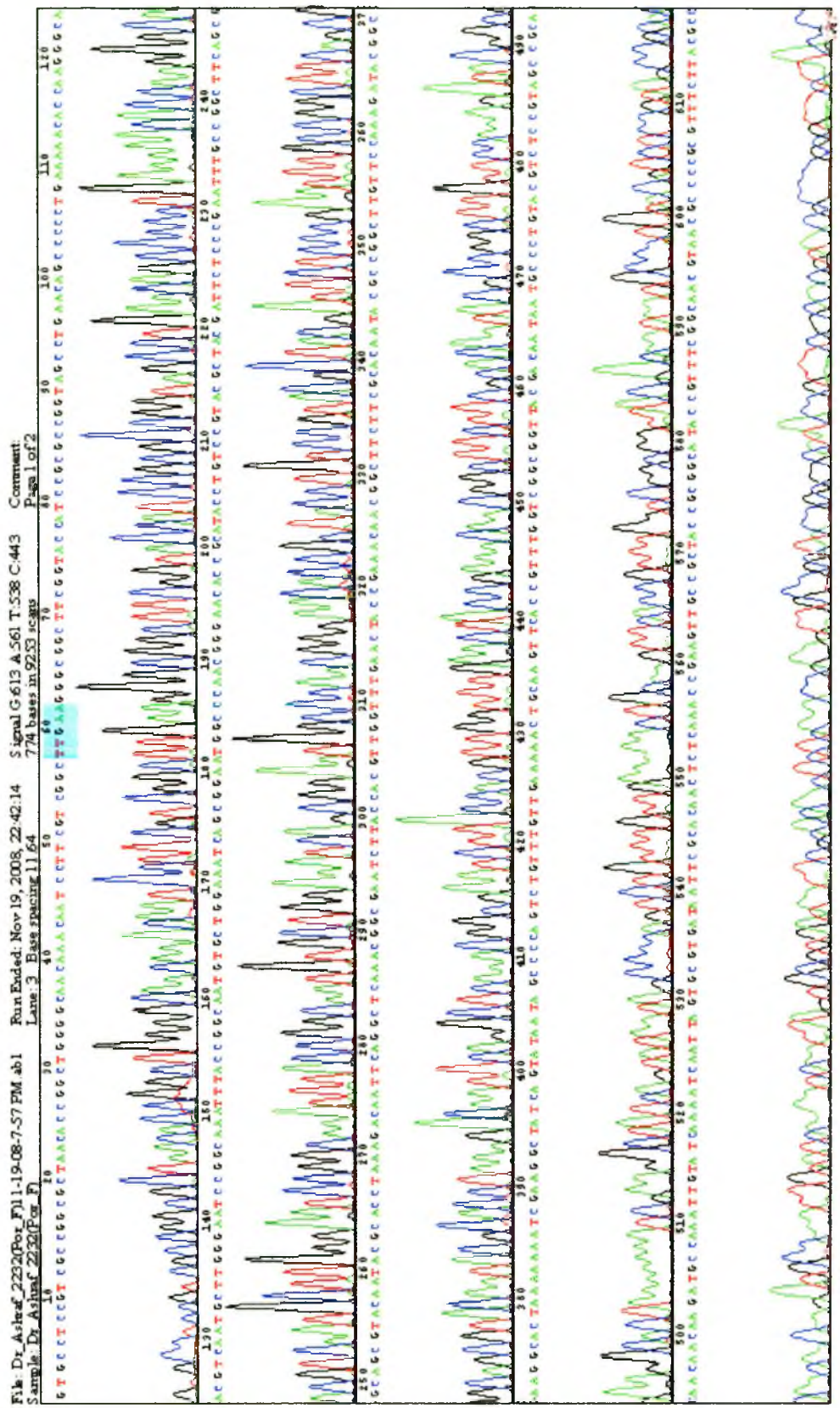
Appendix X.3: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2159 (conserved region, blue highlighted: TTGAA at base-58)



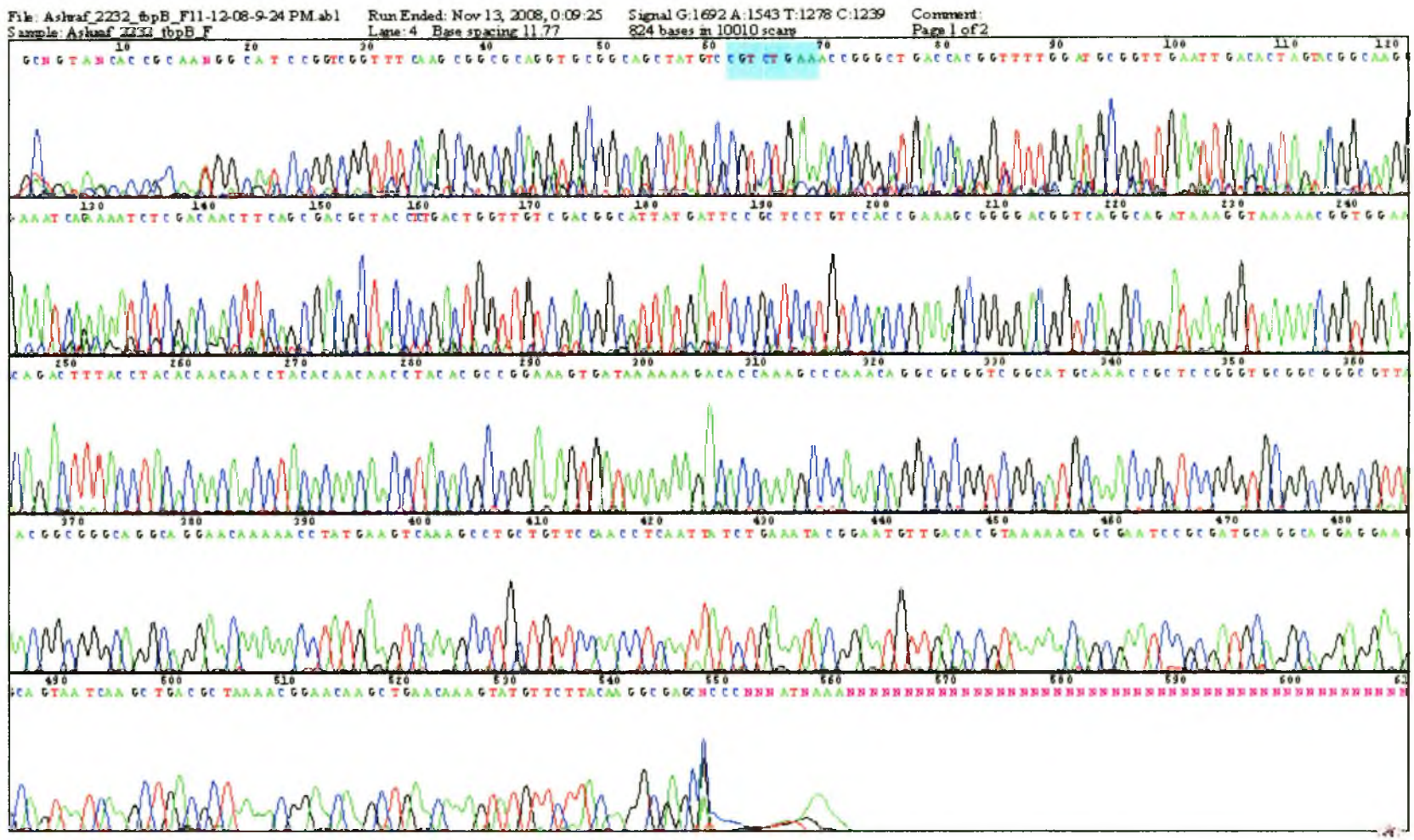
Appendix X.4: Chromatogram of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2159 (conserved region, blue highlighted: CGTCTGAA at base 117)



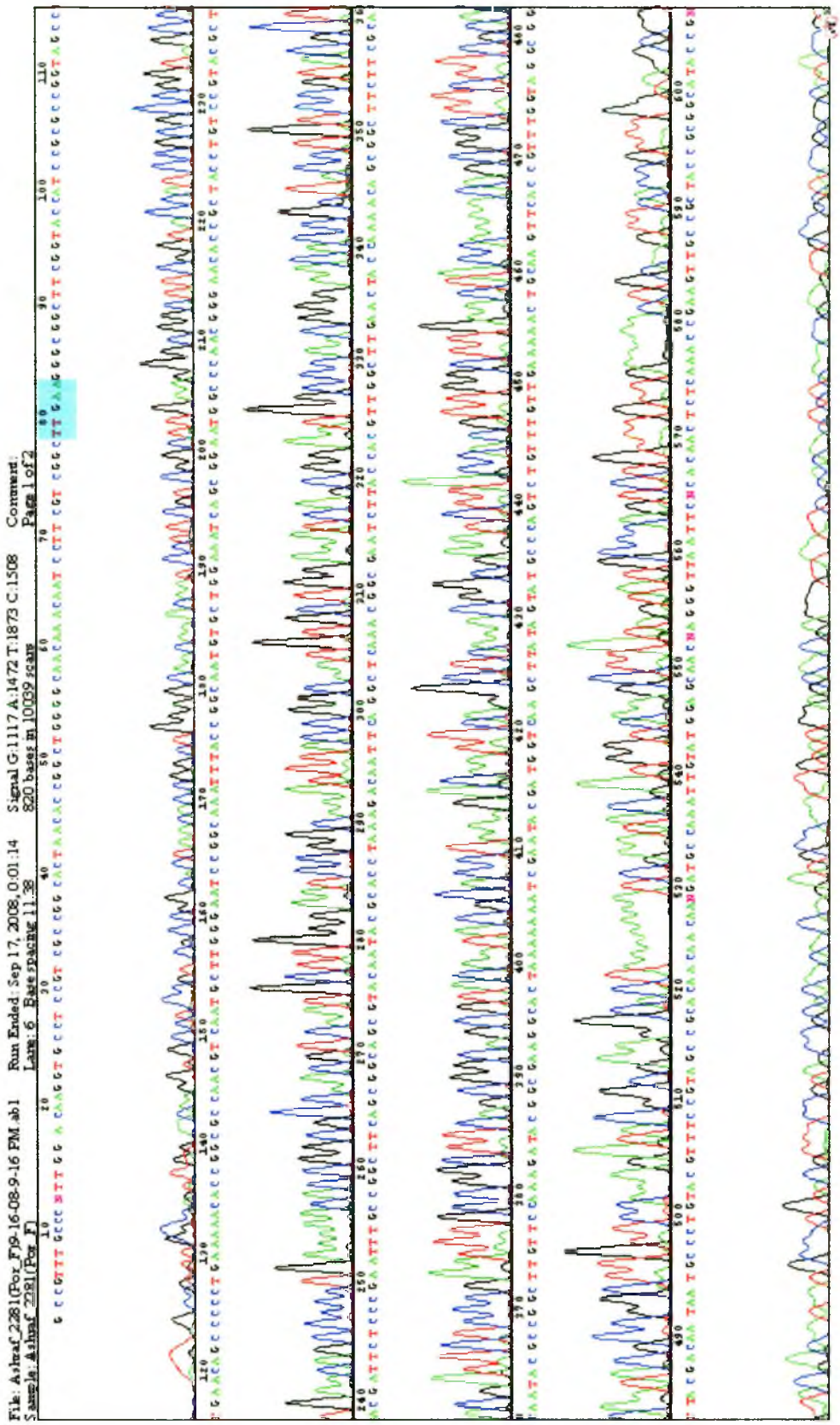
Appendix X.5: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2232 (conserved region, blue highlighted: TTGAA at base-58)



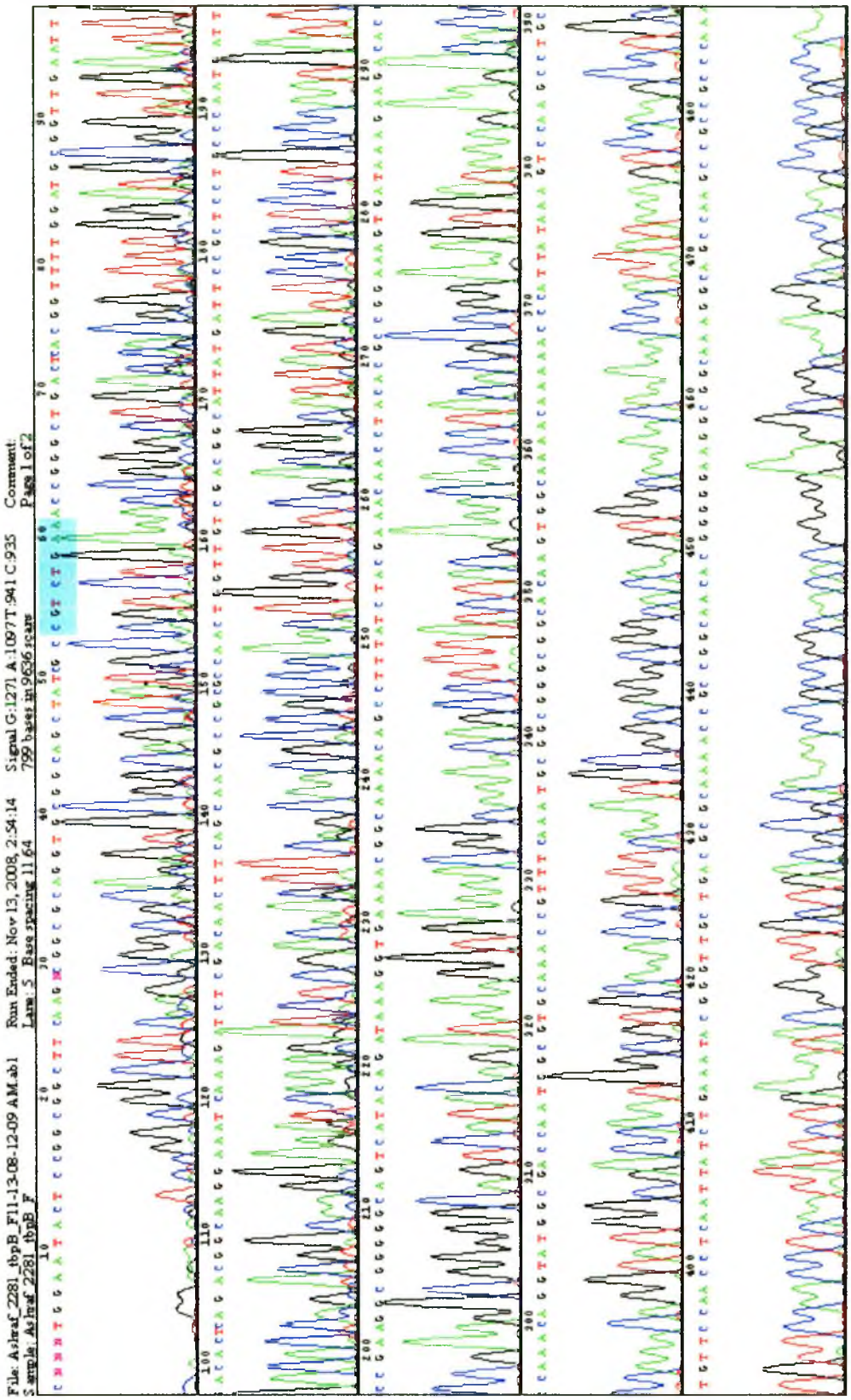
Appendix X.6: Chromatogram of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2232 (conserved region: CGTCTGAA at base-62)



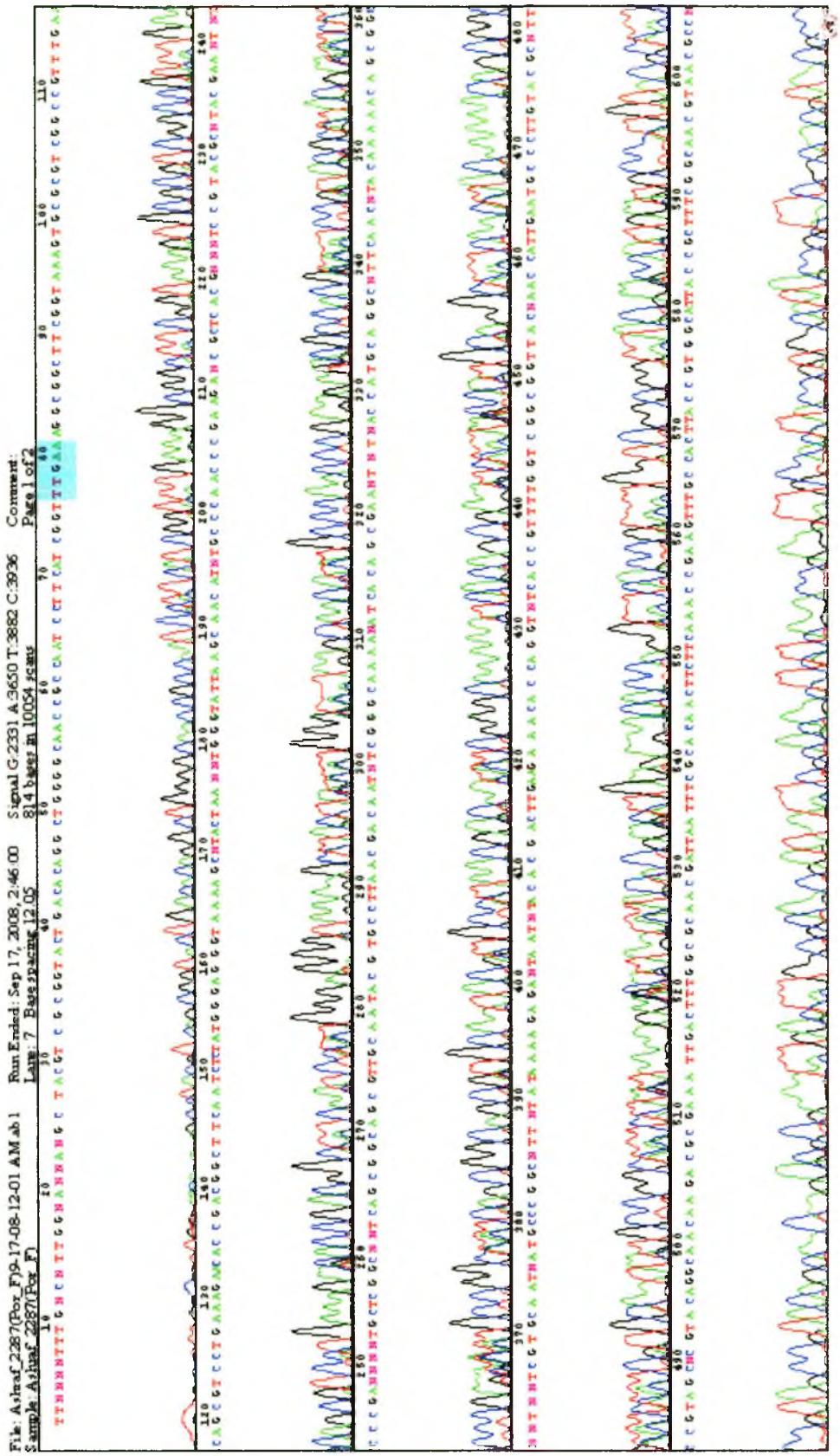
Appendix X.7: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2281 (conserved region, blue highlighted: TTGAA at base-79)



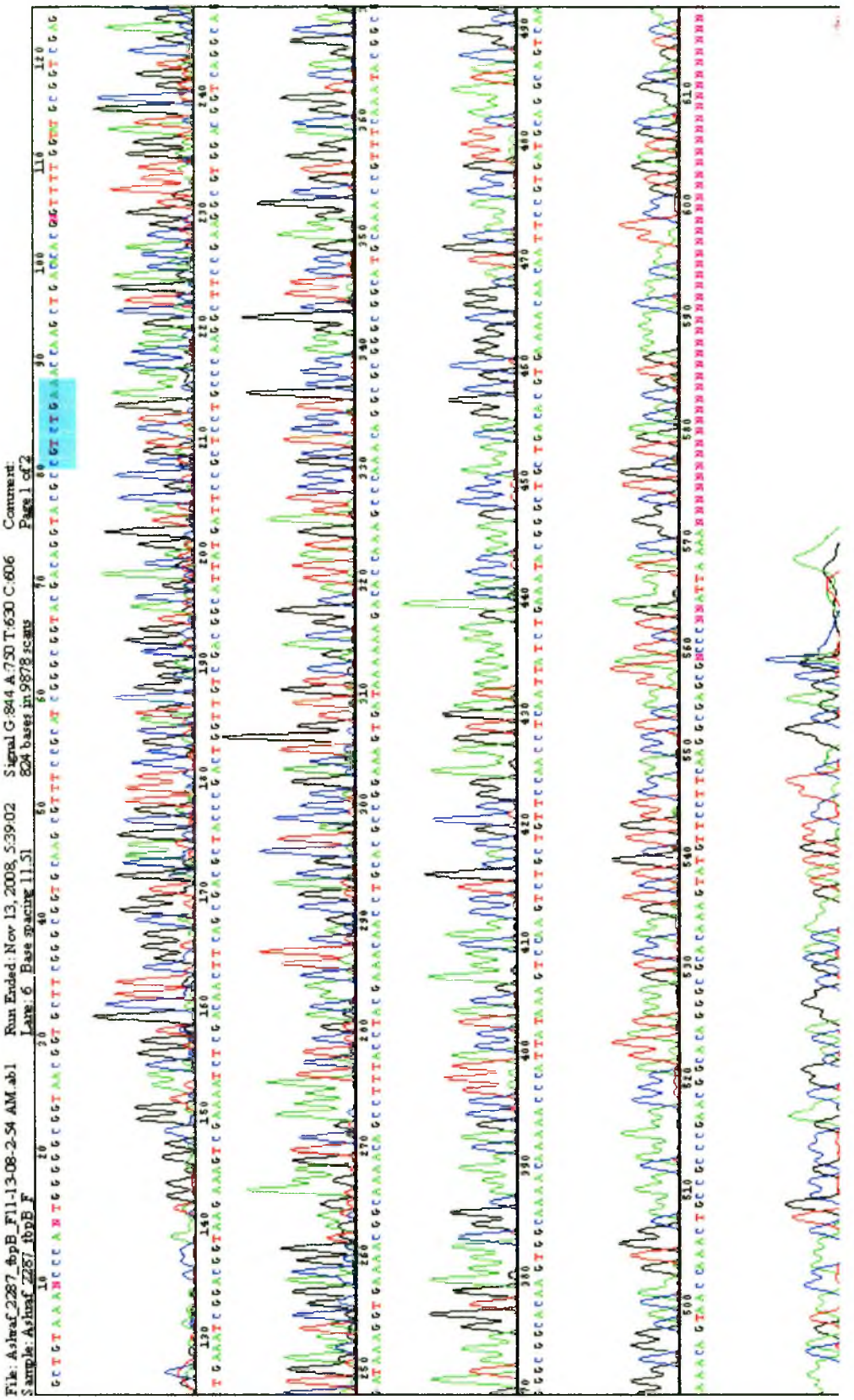
Appendix X.8: Chromatogram of base sequenee of *tbpB-F* of *N. gonorrhoeae* isolate MD-2281 (conserved region: CGTCTGAA at base-53)



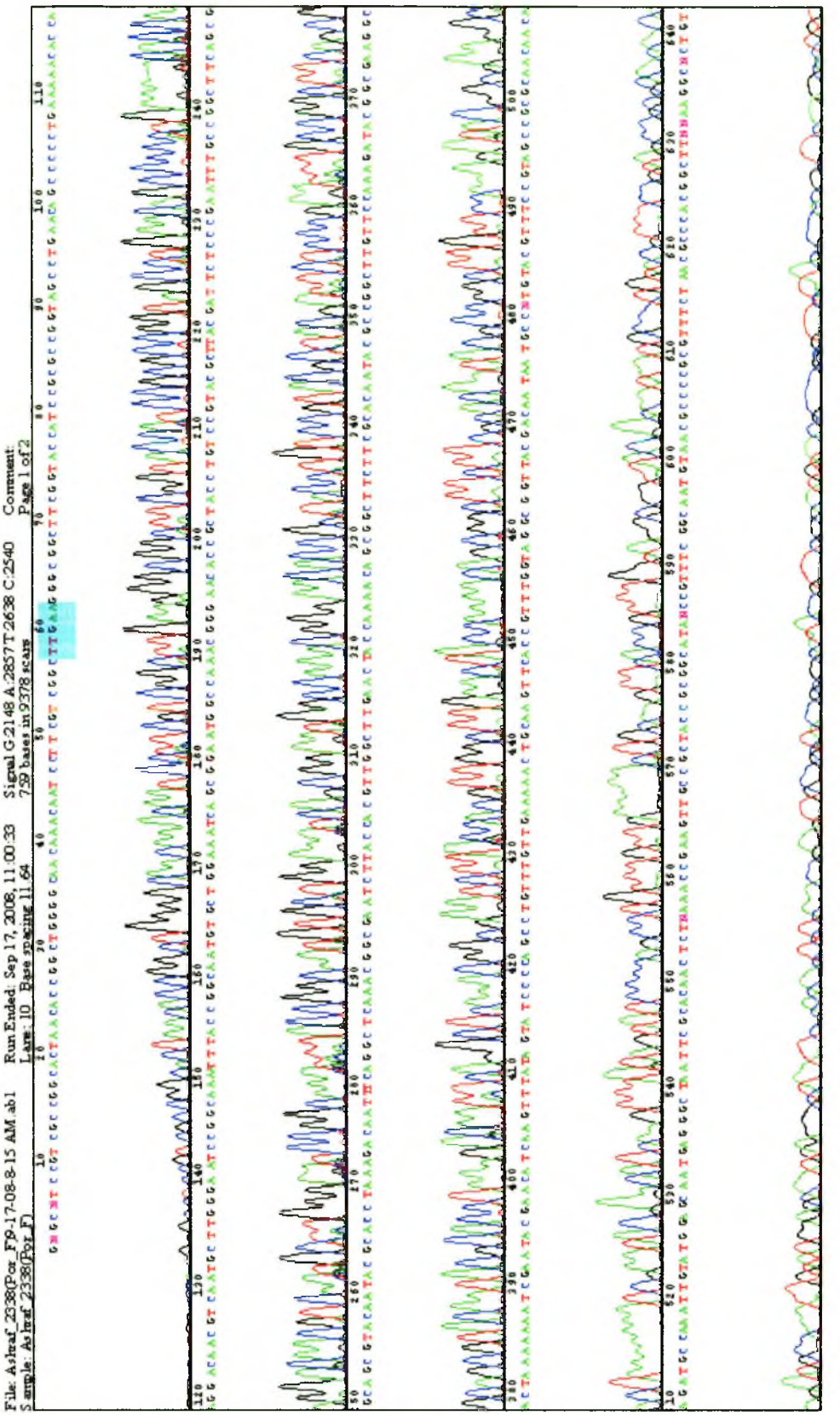
Appendix X.9: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2287 (conserved region: TTGAA at base-76)



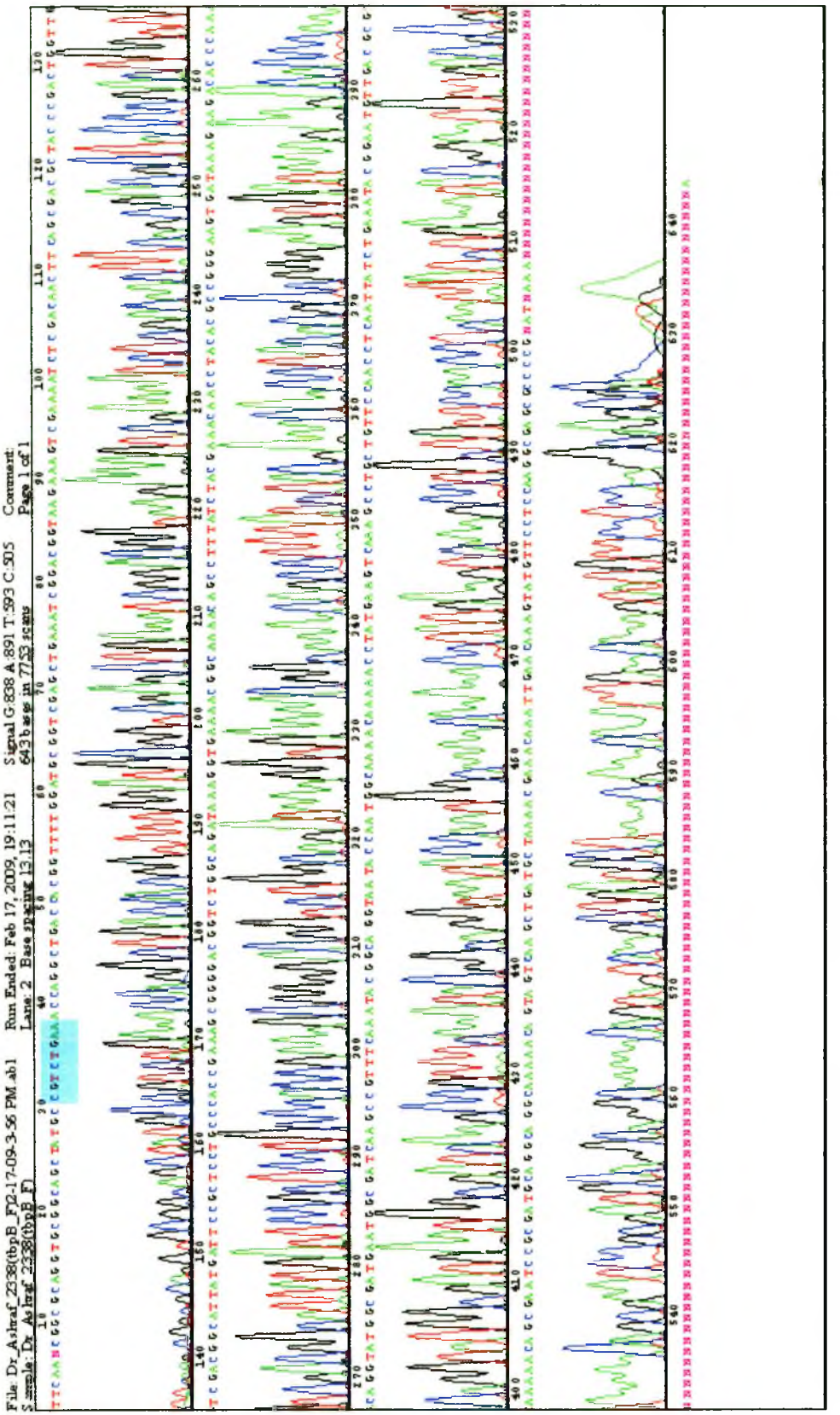
Appendix X.10: Chromatogram of base sequence of *ibpB-F* of *N. gonorrhoeae* isolate MD-2287 (conserved region, blue highlighted: CGTCTGAA at base-81)



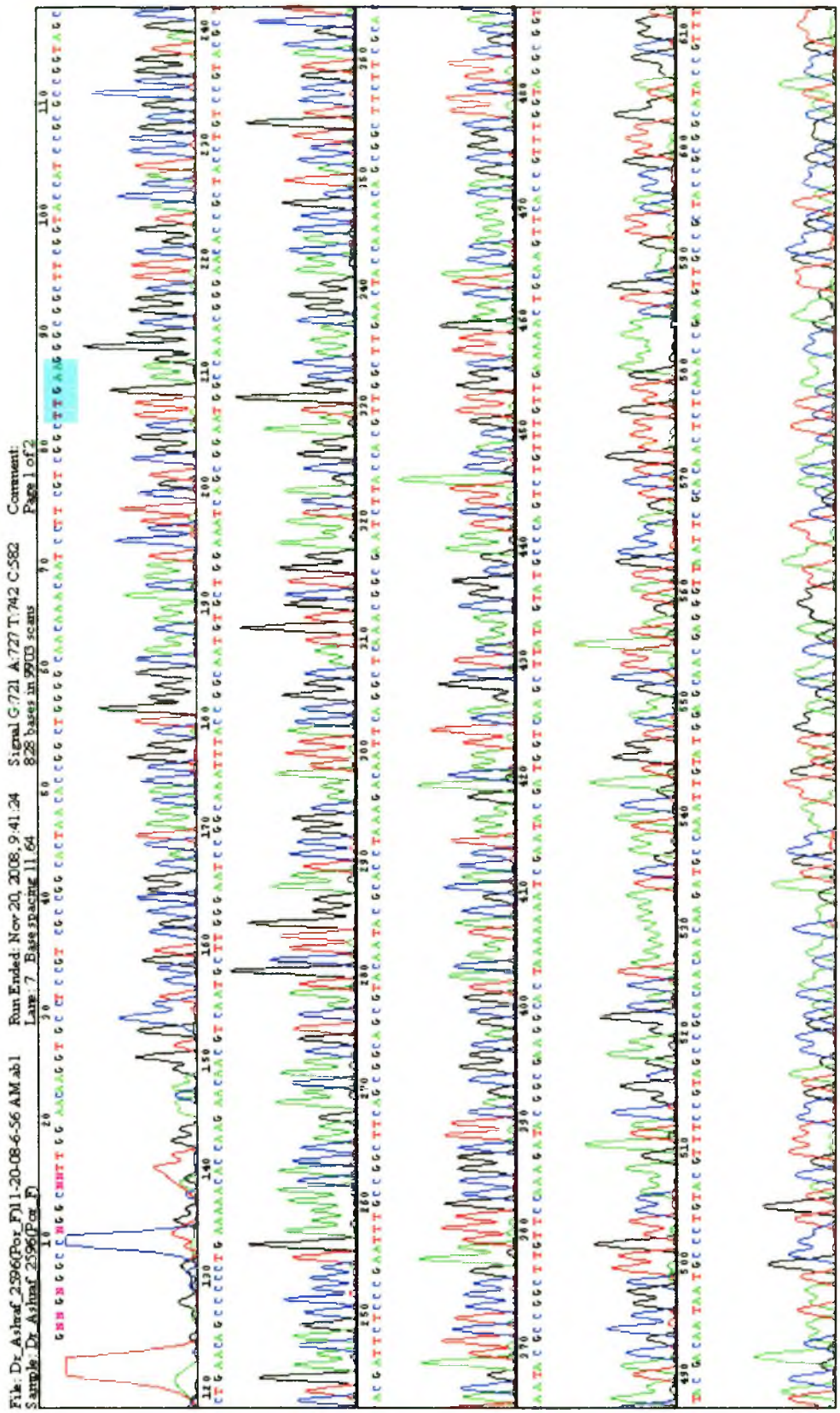
Appendix X.11: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2338 (conserved region, blue highlighted: TTGAA at base-58)



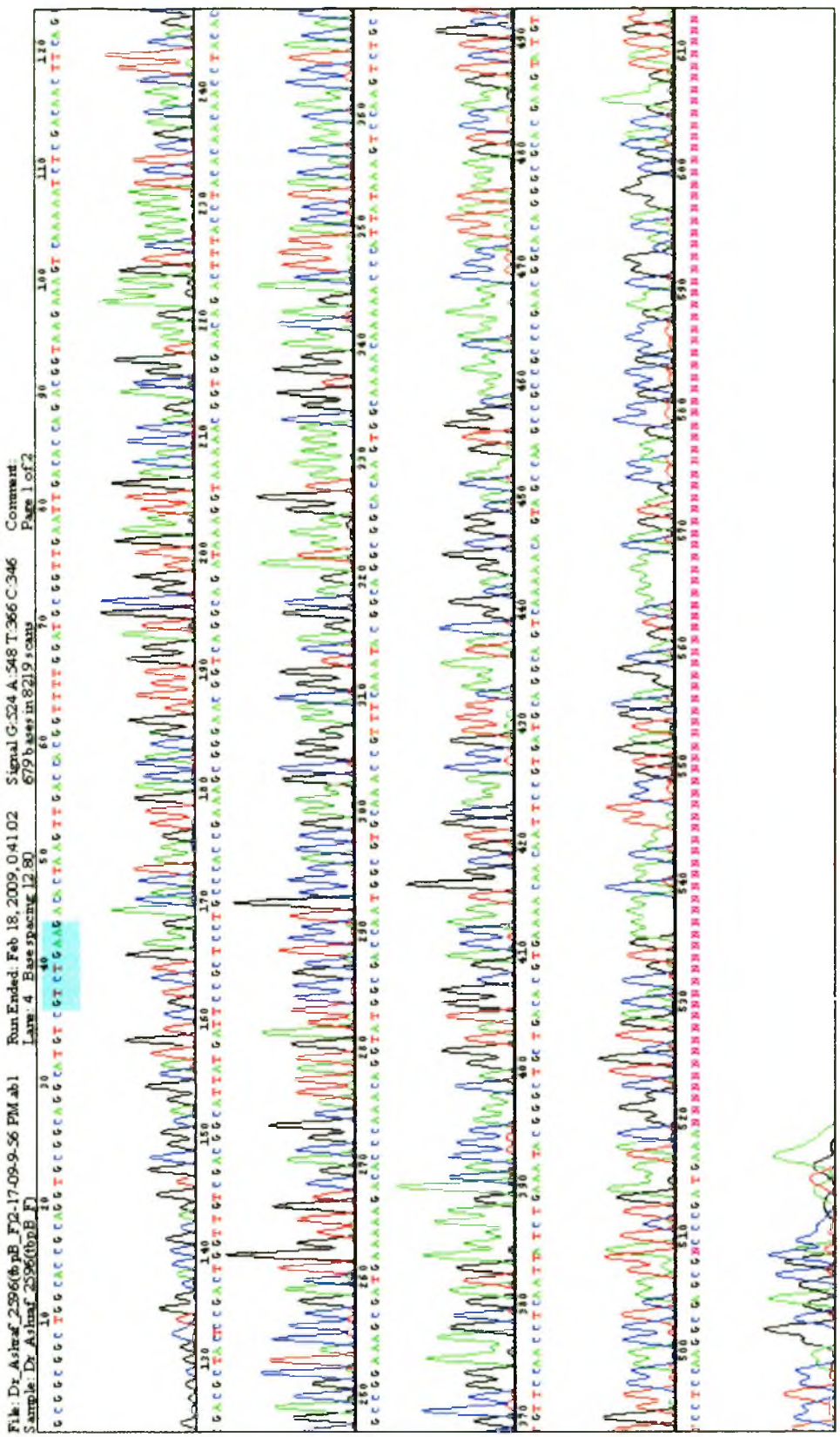
Appendix X.12: Chromatogram of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2338 (conserved region, blue highlighted: CGTCTGAA at base-30)



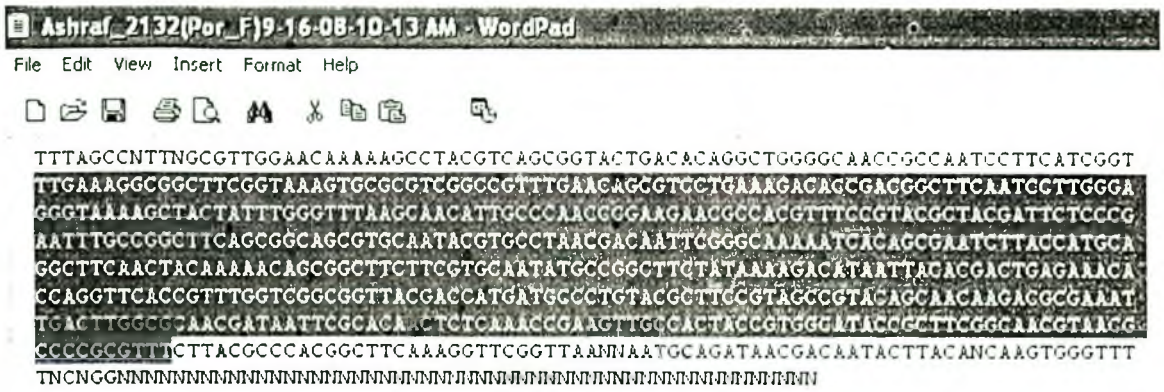
Appendix X.13: Chromatogram of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2596 (conserved region, blue highlighted: TTGAA at base-82)



Appendix X.14: Chromatogram of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2596 (conserved region: CGTCTGAA at base- 35)

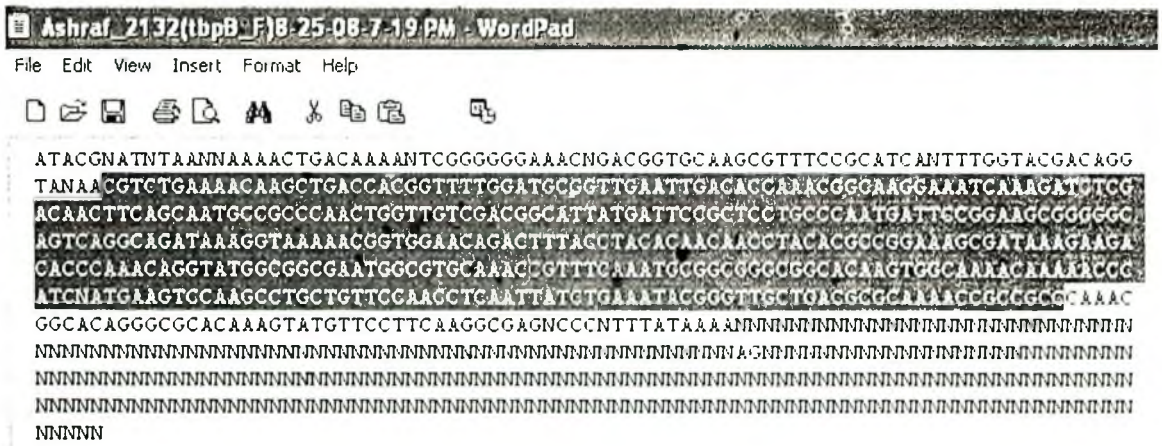


Appendix X.15: WordPad of base sequence of *por-F* of *N. gonorrhoeae* isolate MD-2132



The plan of editing:
Conserved region (TTGAA): starts at Base 81
Extension: for 490 bases
Ends at: Base 570

Appendix X.16: WordPad of base sequence of *tbpB-F* of *N. gonorrhoeae* isolate MD-2132



The plan of editing:
Conserved region (CGTCTGAA): starts at Base 86
Extension: for 390 bases
Ends at: Base 475

Appendix XI: The edited and trimmed DNA sequence data of *por* and *tbpB* genes of the *N. gonorrhoeae* isolates

Appendix XI.1: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2132

MD-2132 *por* allele sequence data & result copied from ng-mast website

TTGAAAGGCGGCTTCGGTAAAGTGCGCGTCGGCCGTTTGAACAGCGTCCTGAAAGACACCGACGGCTTCAATCCTTGGGAGGGTAAAAGCTA
CTATTTGGGTTTAAGCAACATTGCCCAACCCGAAGAACGCCACGTTTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCG
TGCAATACGTGCCTAACGACAATTCGGGCAAAAATCACAGCGAATCTTACCATGCAGGCTTCAACTACAAAAACAGCGGCTTCTTCGTGCAA
TATGCCGGCTTCTATAAAAGACATAATTACACGACTGAGAAACACCAGGTTCACCGTTTGGTCGGCGGTTACGACCATGATGCCCTGTACGC
TTCCGTAGCCGTACAGCAACAAGACGCGAAATTGACTTGGCGCAACGATAATTCGCACAACTCTCAAACCGAAGTTGCCACTACCGTGGCAT
ACCGCTTCGGCAACGTAACGCCCCGCGTTT

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 251

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
368	POR251 | TBPB 136	Angus Lo anguslo@dh.gov.hk Helen Palmer helen.palmer@luht.scot.nhs.uk Eva Tzelepi tzelepi@pasteur.gr
484	POR251 | TBPB 27	Helen Palmer helen.palmer@luht.scot.nhs.uk
502	POR251 | TBPB 165	Helen Palmer helen.palmer@luht.scot.nhs.uk Etienne Muller etiennem@nicd.ac.za Precious Magooa preciousm@nicd.ac.za
724	POR251 | TBPB 205	Bhudipa Choudhury b.choudhury@imperial.ac.uk
1356	POR251 | TBPB 340	Helen Palmer helen.palmer@luht.scot.nhs.uk
1397	POR251 | TBPB 24	
1927	POR251 | TBPB 483	Angus Lo anguslo@dh.gov.hk
2650	POR251 | TBPB 615	Etienne Muller etiennem@nicd.ac.za
2754	POR251 | TBPB 635	
2998	POR251 | TBPB 91	
3007	POR251 | TBPB 685	Etienne Muller etiennem@nicd.ac.za
3207	POR251 | TBPB 726	
3234	POR251 | TBPB 715	
3418	POR251 | TBPB 768	Paola Stefanelli paola.stefanelli@iss.it
3510	POR251 | TBPB 786	Ashraful Alam ashalam61@yahoo.com
3512	POR251 | TBPB 741	Ashraful Alam ashalam61@yahoo.com

MD-2132 *tbpB* allele data & result copied from ng-mast website

CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGGAAATCAAAGATCTCGACAACTTCAGCAATGCC
GCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGGAAGCGGGGGCAGTCAGGCAGATAAAGGTAAAAACGGTGG
AACAGACTTTACCTACACAACAACCTACACGCCGGAAAGCGATAAAGAAGACACCCAAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTT
CAAATGCGGCGGGCGGCACAAGTGGCAAAACAAAAACCCATCTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTT
GCTGACGCGCAAAACCGCCGCC

SEARCH RESULTS FOR TBPB: Sequence not found, closest similarity is 86% allele - 11, may be new allele.

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 741

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3512	POR251 | TBPB 741	Ashraful Alam ashalam61@yahoo.com

Appendix XI.2: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2159

MD-2159 *por* allele data & search result copied from ng-mast web database

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGGACAACGTCAA
TGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCAAACGGGAACACCGCTACT
GTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAATTCAG
GCTCAAACGGCGAATCTTACCACGTTGGTTTGAACTACCGAAACAACGGCTTCTTCGCACAATACGCCGGCTTG
TTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAAGGCTATCAGTATAATATCCCCAGTCTGTTTGTTGAAAA
ACTGCAAGTTCACCGTTTGGTCGGCGGTTACGACAATAATGCCCTGTACGCCTCCGTAGCCGCACAACAACAAG
ATGCCAAATTGTATGGAACATGGCGTGCTAATTCGCACAACTCTC

SEARCH RESULTS FOR POR : YOUR ALLELE NUMBER IS 49

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
63	POR49 | TBPB 32	Iona martin Stephanie.Chisholm@HPA.org.uk Helen Palmer helen.palmer@luht.scot.nhs.uk
2680	POR49 | TBPB 115	Eva Tzelepi tzelepi@pasteur.gr
2715	POR49 | TBPB 29	
2866	POR49 | TBPB 25	Elena Ilina ilinaen@gmail.com
3507	POR49 | TBPB 136	Ashraful Alam ashalam61@yahoo.com

MD-2159 *tbpB* allele data & search result copied from ng-mast web database

CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGGAAATCAAAGATCTC
GACAACTTCAGCAATGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGGAAG
CGGGGGCAGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACGCCGGAAA
GCGATAAAGAAGACACCCAAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCACA
AGTGGCAAAACAAAAACCCATTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTTGCT
GACGCGCAAAACCGCCGCCC

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 136

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
368	POR251 | TBPB 136	Angus Lo anguslo@dh.gov.hk Helen Palmer helen.palmer@luht.scot.nhs.uk Eva Tzelepi tzelepi@pasteur.gr
483	POR63 | TBPB 136	Helen Palmer helen.palmer@luht.scot.nhs.uk
3221	POR64 | TBPB 136	
3402	POR2055 | TBPB 136	
3507	POR49 | TBPB 136	Ashraful Alam ashalam61@yahoo.com
3580	POR2142 | TBPB 136	
3726	POR1144 | TBPB 136	

Appendix XI.3: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2232

MD-2232 *por* allele data & search result from ng-mast web database

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGGGCAACGTCAA
TGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCCAACGGGAACACCGCATAC
CTGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAATTC
AGGCTCAAACGGCGAATCTTACCACGTTGGTTTGAACTACCGAAACAACGGCTTCTTCGCACAATACGCCGGCT
TGTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAAGGCTATCAGTATAATAGCCCCAGTCTGTTTGTTGAA
AAACTGCAAGTTCACCGTTTGGTCGGCGGTTACGACAATAATGCCCTGTACGTCTCCGTAGCCGCACAACAACA
AGATGCCAAATTGTATCAAAATCAATTAGTGCGTGATAATTCGCAC (417-807)

SEARCH RESULTS FOR POR: Sequence not found, closest similarity is 91%
allele - 1061, may be new allele.

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 2119

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3508	POR2119 | TBPB 784	Ashraful Alam ashalam61@yahoo.com

MD-2232 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCGGGCTGACCACGGTTTTGGATGCGGTTGAATTGACACTAGTACGGCAAGGAAATCAGAAAATC
TCGACAACTTCAGCGACGCTACCTCTGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGTCCACCGAAAGCG
GGGACGGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACAACAACCTAC
ACGCCGGAAAGTGATAAAAAAGACACCAAAGCCCAAACAGGCGCGGTCGGCATGCAAACCGCTCCGGGTGCGGC
GGGCGTTAACGGCGGGCAGGCAGGAACAAAAACCTATGAAGTCAAAGCCTGCTGTTCCAACCTCAATTATCTGA
AATACGGAATGTTGACACGT

SEARCH RESULTS FOR TBPB: Sequence not found, closest similarity is 75%
allele - 646, may be new allele.

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 784

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3508	POR2119 | TBPB 784	Ashraful Alam ashalam61@yahoo.com

Appendix XI.4: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2281

MD-2281 *por* allele data & search result from ng-mast web database

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCGGCGCCAACGTCAA
TGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCCAACGGGAACACCGCTACC
TGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAATTCA
GGCTCAAACGGCGAATCTTACCACGTTGGCTTGAACTACCAAAACAGCGGCTTCTTCGCACAATACGCCGGCTT
GTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAATACGATGGTCAAGCTTATAGTATGCCCAGTCTGTTTG
TTGAAAAACTGCAAGTTCACCGTTTGGTAGGCGGTTACGACAATAATGCCCTGTACGTTTCCGTAGCCGCACAA
CAACAAGATGCCAAATTGTATGGAGCAACGAGGGTTAATTCGCACA (78-568)

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 160

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
221	POR160 | TBPB 81	Iona martin Stephanie.Chisholm@HPA.org.uk
1820	POR160 | TBPB 440	
2864	POR160 | TBPB 32	
3509	POR160 | TBPB 785	Ashraful Alam ashalam61@yahoo.com

MD-2281 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCGGGCTGACTCACGGTTTTGGATGCGGTTGAATTGACACTCAGACGGCAAGGAAATCAAAGATC
TCGACAACTTCAGCAACGCCGCGCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGA
AGGCGGGGGCAGTCATACAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGG
AAAGTGATAAAGAAGACACCCAAACAGGTATGGCGACCAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGC
ACAAGTGGCAAAACAAAAACCCATTATAAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTT
GCTGACGCGCAAAACCGCCG

SEARCH RESULTS FOR TBPB: Sequence not found, closest similarity is 82%
allele - 628, may be new allele.

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 785

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3509	POR160 | TBPB 785	Ashraful Alam ashalam61@yahoo.com

Appendix XI.5: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2287

MD-2287 *por* allele data & search result from ng-mast web database

TTGAAAGGCGGCTTCGGTAAAGTGCGCGTCGGCCGTTTGAACAGCGTCCTGAAAGACACCGACGGCTTCAATCCTTGGGAGGGTAAAA
GCTACTATTTGGGTTTAAGCAACATTGCCCAACCCGAAGAACGCCACGTTTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAG
CGGCAGCGTGCAATACGTGCCTAACGACAATTCGGGCAAAAATCACAGCGAATCTTACCATGCAGGCTTCAACTACAAAAACAGCGGC
TTCTTCGTGCAATATGCCGGCTTCTATAAAAGACATAATTACACGACTGAGAAACACCAGGTTCACCGTTTGGTCGGCGGTTACGACC
ATGATGCCCTGTACGCTTCCGTAGCCGTACAGCAACAAGACGCGAAATTGACTTGGCGCAACGATAATTCGCACAACTCTCAAACCGA
AGTTGCCACTACCGTGGCATACCGCTTCGGCAACGTAACGCCCCGCGTTT

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 251

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
368	POR251 | TBPB 136	Angus Lo anguslo@dh.gov.hk Helen Palmer helen.palmer@luht.scot.nhs.uk Eva Tzelepi tzelepi@pasteur.gr
484	POR251 | TBPB 27	Helen Palmer helen.palmer@luht.scot.nhs.uk
502	POR251 | TBPB 165	Helen Palmer helen.palmer@luht.scot.nhs.uk Etienne Muller etiennem@nicd.ac.za Precious Magooa preciousm@nicd.ac.za
724	POR251 | TBPB 205	Bhudipa Choudhury b.choudhury@imperial.ac.uk
1356	POR251 | TBPB 340	Helen Palmer helen.palmer@luht.scot.nhs.uk
1397	POR251 | TBPB 24	
1927	POR251 | TBPB 483	Angus Lo anguslo@dh.gov.hk
2650	POR251 | TBPB 615	Etienne Muller etiennem@nicd.ac.za
2754	POR251 | TBPB 635	
2998	POR251 | TBPB 91	
3007	POR251 | TBPB 685	Etienne Muller etiennem@nicd.ac.za
3207	POR251 | TBPB 726	
3234	POR251 | TBPB 715	
3418	POR251 | TBPB 768	Paola Stefanelli paola.stefanelli@iss.it
3510	POR251 | TBPB 786	Ashraful Alam ashalam61@yahoo.com
3512	POR251 | TBPB 741	Ashraful Alam ashalam61@yahoo.com

MD-2287 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCAAGCTGACACACGCGTTTTGGTATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAATCTCGACAACTTCAG
CGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAAGGCTTCCGAAGGCGTGGACGGTCAGGCAGATAAAGGT
GAAAACGGCAAAACAGCCTTTACCTACGAAACAACCTGCACGCCGGAAAGTGATAAAAAAGACACCAAAGCCCAAACAGGCGCGGGCG
GCATGCAAACCGTTTCAAATACGGCGGGCGGCACAAGTGGCAAAACAAAAACCCATTATAAAGTCCAAGTCTGCTGTTCCAACCTCAA
TTATCTGAAATACGGGCTGCTGACACGTGAAAACAACA

SEARCH RESULTS FOR TBPB: Sequence not found, closest similarity is 94% allele - 720, may be new allele.

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 786

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3510	POR251 | TBPB 786	Ashraful Alam ashalam61@yahoo.com

Appendix XI.6: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2338

MD-2338 *por* allele data & search result from ng-mast web database

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGGACAACGTCAA
TGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCAAACGGGAACACCGCTACC
TGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAATTCA
GGCTCAAACGGCGAATCTTACCACGTTGGCTTGAACTACCAAAACAGCGGCTTCTTCGCACAATACGCCGGCTT
GTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAATACGAACATCAAGTTTATAGTATCCCCAGCCTGTTTG
TTGAAAAACTGCAAGTTCACCGTTTGGTAGGCGGTTACGACAATAATGCCCTGTACGTTTCCGTAGCCGCGCAA
CAACAAGATGCCAAATTGTATGGAGCAATGAGGGCTAATTCGCACA

SEARCH RESULTS FOR POR: Sequence not found, closest similarity is 99%
allele - 581, may be new allele.

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 2258

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
3738	POR2258 | TBPB 82	Ashraful Alam ashalam61@yahoo.com

MD-2338 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCAGGCTGACCACGGTTTTGGATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAATCTC
GACAACTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCGGGGA
CGGTCTGGCAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGGAAGTGATA
AAGAAGACACCCAAACAGGTATGGCGATGAATGGCGATCAAGCCGTTTCAAATACGGCAGGTAATACCAATGGC
AAAACAAAAACCTATGAAGTCAAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGAATGTTGACGCGTAA
AAACAGCGAATCCGCGATGC

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 82

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
218	POR161 | TBPB 82	Iona martin Stephanie.Chisholm@HPA.org.uk
3513	POR2120 | TBPB 82	
3738	POR2258 | TBPB 82	Ashraful Alam ashalam61@yahoo.com

Appendix XI.7: Edited and trimmed data of *por* and *tbpB* gene alleles with ng-mast search result for *N. gonorrhoeae* isolate MD-2596

MD-2596 *por* allele data & search result from ng-mast web database

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGAACAACGTCAA
TGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCAAACGGGAACACCGCTACC
TGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAATTCA
GGCTCAAACGGCGAATCTTACCACGTTGGCTTGAACTACCAAAACAGCGGCTTCTTCGCACAATACGCCGGCTT
GTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAATACGATGGTCAAGCTTATAGTATGCCCAGTCTGTTTG
TTGAAAAACTGCAAGTTCACCGTTTGGTAGGCGGTTACGACAATAATGCCCTGTACGTTTCCGTAGCCGCACAA
CAACAAGATGCCAAATTGTATGGAGCAACGAGGGTTAATTCGCACA

SEARCH RESULTS FOR POR: YOUR ALLELE NUMBER IS 184

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
168	POR184 | TBPB 100	
536	POR184 | TBPB 153	Preshini Moodley MOODLEYP@ukzn.ac.za
1023	POR184 | TBPB 156	Angus Lo anguslo@dh.gov.hk Helen Palmer helen.palmer@luht.scot.nhs.uk
1433	POR184 | TBPB 4	
1441	POR184 | TBPB 361	Mari De Jongh maridejongh@yahoo.com
1453	POR184 | TBPB 70	nicole bilek n.bilek@imperial.ac.uk
1487	POR184 | TBPB 370	
1867	POR184 | TBPB 119	
2990	POR184 | TBPB 684	
3144	POR184 | TBPB 709	
3216	POR184 | TBPB 154	Etienne Muller etiennem@nicd.ac.za
3511	POR184 | TBPB 787	Ashraful Alam ashalam61@yahoo.com

MD-2596 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCAGGCTGACCACGGTTTTGGATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAATCTC
GACAACTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCGGGGA
CGGTCTGGCAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGGAAGTGATA
AAGAAGACACCCAAACAGGTATGGCGATGAATGGCGATCAAGCCGTTTCAAATACGGCAGGTAATACCAATGGC
AAAACAAAAACCTATGAAGTCAAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGAATGTTGACGCGTAA
AAACAGCGAATCCGCGATGC

SEARCH RESULTS FOR TBPB: YOUR ALLELE NUMBER IS 82

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
218	POR161 | TBPB 82	Iona martin Stephanie.Chisholm@HPA.org.uk
3513	POR2120 | TBPB 82	Ashraful Alam ashalam61@yahoo.com

Appendix XI.8: Edited and trimmed data of *por* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2423

MD-2423 *por* allele data & search result from ng-mast web database

TTGAAAGGCGGCTTCGGTAAAGTGCGCGTCGGCCGTTTGAACAGCGTCCTGAAAGACACCGACGGCTTCAATCCTTGGGAGGGTAAAAGCTA
CTATTTGGGTTTAAGCAACATTGCCCAACCCGAAGAACGCCACGTTTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCG
TGCAATACGTGCCTAACGACAATTCGGGCAAAAATCACAGCGAATCTTACCATGCAGGCTTCAACTACAAAAACAGCGGCTTCTTCGTGCAA
TATGCCGGCTTCTATAAAAGACATAATTACACGACTGAGAAACACCAGGTTCACCGTTTGGTCGGCGGTTACGACCATGATGCCCTGTACGC
TTCCGTAGCCGTACAGCAACAAGACGCGAAATTGACTTGGCGCAACGATAATTCGCACAACTCTCAAACCGAAGTTGCCACTACCGTGGCAT
ACCGCTTCGGCAACGTAACGCCCCGCGTTT

SEARCH RESULTS FOR POR : YOUR ALLELE NUMBER IS 251

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
368	POR251 | TBPB 136	Angus Lo anguslo@dh.gov.hk Helen Palmer helen.palmer@luht.scot.nhs.uk Eva Zelepi tzelepi@pasteur.gr
484	POR251 | TBPB 27	Helen Palmer helen.palmer@luht.scot.nhs.uk
502	POR251 | TBPB 165	Helen Palmer helen.palmer@luht.scot.nhs.uk Etienne Muller etiennem@nicd.ac.za
724	POR251 | TBPB 205	Bhudipa Choudhury b.choudhury@imperial.ac.uk
1356	POR251 | TBPB 340	Helen Palmer helen.palmer@luht.scot.nhs.uk
1397	POR251 | TBPB 24	
1927	POR251 | TBPB 483	Angus Lo anguslo@dh.gov.hk
2650	POR251 | TBPB 615	Etienne Muller etiennem@nicd.ac.za
2754	POR251 | TBPB 635	
2998	POR251 | TBPB 91	
3007	POR251 | TBPB 685	Etienne Muller etiennem@nicd.ac.za
3207	POR251 | TBPB 726	
3234	POR251 | TBPB 715	

Appendix XI.9: Edited and trimmed data of *tbpB* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2443

MD-2443 *tbpB* allele data & search result from ng-mast web database

CGTCTGGAAACAGTAATCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGGAAATCAAAAATCTCGACA
ACTTCAGCAACGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCGGGGACGGTCTGGCAG
ATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGGAAGTGATAAAGAAGACACCCAAACAG
GTATGGCGATGAATGGCGATCAAGCCGTTTCAAATACGGCAGGTAATACCAATGGCAAAACAAAAACCTATGAAGTCAAAG
CCTGCTGTTCCAACCTCAATTATCTGAAATACGGAATGTTGACGCGTAAAAACAGCGAATCCGCGA

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 72

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
160	POR91 | TBPB 72	Iona martin Stephanie.Chisholm@HPA.org.uk Bhudipa Choudhury b.choudhury@imperial.ac.uk

Appendix XI.10: Edited and trimmed data of *tbpB* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2642

MD-2642 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACAGTAATCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGGAAATCAAAGATCTCGACAA
CTTCAGCAACGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCGGGAACGGTCAGGCAGA
TAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGAAAGTGATAAAAAAGACACCCAAACAGG
TATGGCGACCAATGGCGTGCAAACCGTTTCAAATACGGCGGGCGGCACAAGTGGCAAAACAAAAACCCATTATAAAGTCCA
AGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGCTGCTGACGCGCAAAAACAGCGAATCCGC

SEARCH RESULTS FOR TBPB : Sequence not found, closest similarity is 91% allele - 747, may be new allele.

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 788

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user

Appendix XI.11: Edited and trimmed data of *tbpB* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2683

MD-2683 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACAGTAATCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGGAAATCAAAGATCTCGACAA
CTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCGGGAACGGTCAGGCAGA
TAAAGGTAAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGAAAGTGATAAAAAAGACACCAAAGCAGG
TACGGCGGCGAATGGCGTGCAAACCGTTTCAAATACGGCAGGCGGCACAAGTGGCAAAACAAAAACCTATAAAGTCCAAGT
CTGCTGTTCCAACCTCAATTATCTGAAATACGGGCTGCTGACACGTGAAAACAACAATTCCGTGAT

SEARCH RESULTS FOR TBPB : Sequence not found, closest similarity is 89% allele - 648, may be new allele.

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 789

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user

Appendix XI.12: Edited and trimmed data of *tbpB* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2725

MD-2725 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAACCAAGCTGACCACGGTTTTGGATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAATCTCGACAACT
TCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAAGGCTTCCGAAGGCGTGGACGGTCAGG
CAGATAAAGGTGAAAACGGCAAAACAGCCTTTACCTACGAAACAACCTGCACGCCGGAAAGTGATAAAAAAGACACCAAAG
CCCAAACAGGCGCGGGCGGCATGCAAACCGTTTCAAATACGGCGGGCGGCACAAGTGGCAAAACAAAAACCCATTATAAAG
TCCAAGTCTGCTGTTCCAACCTCAATTATCTGAAATACGGGCTGCTGACACGTGAAAACAACAATT

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 81

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user
221	POR160 | TBPB 81	Iona martin Stephanie.Chisholm@HPA.org.uk

Appendix XI.13: Edited and trimmed data of *tbpB* gene allele with ng-mast search result for *N. gonorrhoeae* isolate MD-2443

MD-2797 *tbpB* allele data & search result from ng-mast web database

CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGGAAATCAAAGATCTCGACAACT
TCAGCAATGCCGCTCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGGAAGCGGGGGCAGTCAG
GCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACGCCGGAAAGCGATAAAGAAGACACCCAA
ACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCACAAGTGGCAAAACAAAAACCCATTATGAA
GTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTTGCTGACGCGCAAAACCGCCGCC

SEARCH RESULTS FOR TBPB : Sequence not found, closest similarity is 77% allele - 300, may be new allele.

SEARCH RESULTS FOR TBPB : YOUR ALLELE NUMBER IS 790

This allele has been found in the following STs
(please contact strain owner for further details) -

ST	profile	user

Appendix XII: Closely similar Allele data from www.ng-mast.net

por Allele: 581

(99% similar to new *por* allele 2258 of *N. gonorrhoeae* isolate MD-2338)

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGGACAACGTCA
ATGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGAATGGCCAAACGGGAACACCGCTA
CCTGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAAT
TCAGGCTCAAACGGCGAATCTTACCACGTTGGCTTGAACTACCAAAACAGCGGCTTCTTCGCACAATACGCCG
GCTTGTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAATACGAACATCAAGTTTATAGTATCCCCAGCCT
GTTTGTTGAAAAACTGCAAGTTCACCGTTTGGTAGGCGGTTACGACAATAATGCCCTGTACGTTTCCGTAGCC
GCGCAACAACAAGATGCCAAATTGTATGGAGCAAGGAGGGCTAATTCGCACA

por Allele: 1061

(91% similar to new *por* allele 2119 of *N. gonorrhoeae* isolate MD-2232)

TTGAAGGGCGGCTTCGGTACCATCCGCGCCGGTAGCCTGAACAGCCCCCTGAAAAACACCAAGGACAACGTCA
ATGCTTGGGAATCCGGCAAATTTACCGGCAATGTGCTGGAAATCAGCGGGAATGGCCAAACGGGAACACCGCT
ACCTGTCCGTACGCTACGATTCTCCCGAATTTGCCGGCTTCAGCGGCAGCGTACAATACGCACCTAAAGACAA
TTCAGGCTCAAACGGCGAATCTTACCACGTTGGTTTGAACTACCGAAACAACGGCTTCTTCGCACAATACGCC
GGCTTGTTCCAAAGATACGGCGAAGGCACTAAAAAAATCGAAGGCTATCAGTATAATAGCCCCAGTCTGTTTG
TTGAAAAACTGCAAGTTCACCGTTTGGTCGGCGGTTACGACAATAATGCCCTGTACGCCTCCGTAGCCGCACA
ACAACAAGATGCCAAATTGTATGGAACATGGCGTGCTAATTCGCACAACTCT

tbpB Allele: 11

(86% similar to new *tbpB* allele 741 of *N. gonorrhoeae* isolate MD-2132)

CGTCTGAAAACAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAAACGGCAAGGAAATCAAAGATCT
CGACAACTTCAGCAATGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGGA
AGCGGGGGCAGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACGCCGG
AAAGCGATAAAGAAGACACCCAAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGG
CACAAGTGGCAAAACAAAAACCCATTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGG
TTGCTGACGCGCAAAACCGCCGGCA

tbpB Allele: 300

(77% similar to new *tbpB* allele 790 of *N. gonorrhoeae* isolate MD-2797)

CCGTCTGAAACCGGGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGGAAATCAAAGATC
TCGACAACTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCCGA
AGGCGGGGGCAGTCATACAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCG
GAAAGTGATAAAGAAGACACCCAAACAGGTATGGCGACCAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCG
GCACAAGTGGCAAAACAAAAACCCATTATAAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGG
GCTGCTGACGCGCAAAACTGCCGAC

tbpB Allele: 628

(82% similar to new *tbpB* allele 785 of *N. gonorrhoeae* isolate MD-2281)

CGTCTGAAAACACTAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGAAAATCAAAGA
TCTCGACAACTTCAGCAACGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAATGATTCC
GAAGGCGGGGGCAGTCATACAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGC
CGGAAAGTGATAAAGAAGACACCCAAACAGGTATGGCGACCAATGGCGTGCAAACCGTTTCAAATGCGGCGGG
CGGCACAAGTGGCAAAACAAAAACCCATTATAAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATAC
GGGCTGCTGACGCGCAAAACTGCCG

tbpB Allele: 637

(86% similar to new tbpB allele 787 of *N. gonorrhoeae* isolate MD-2596)

CGTCTGAAAACGGTAAGATGCAAAAAGAATTGGATGTGGTCGAGCTGGAATCGGACGGTAAGAAAGTCAAAAA
TCTCGACAACTTCAGCTCTCTTTCTAAGACTGGTTGTCGACGGTATTATGATTCCGCTCCTGCCCACCGAAAG
CGGGAACGGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACGCCGGAA
AGCGATAAAGAAGACACCCAAACAGGTATGGCGGCGAATGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCA
CATGTGGCAAAACAAAAACCCATTATGAAGTTCTTGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTT
GCTGACGCGCAAAACCGCCGGCAAC

tbpB Allele: 646

(75% similar to new tbpB allele 784 of *N. gonorrhoeae* isolate 2232)

AGGAAGCGAGGAACATGGTCGTAGCTGTTTGTGATGGGGAGAAAAAAGAGAAGGACGGCAAGGTAATCAAAAA
TCTCGACAACTTCAGCGACGCTACCCGACTGGTTGTCGACTTCTACTCTGAATCCGCTCCTGTCCATCGAAAG
CGGGGACGGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACACAACAACCTACACAACAACC
TACACGCCGGAAAGTGATAAAAAAGACACCAAAGCCCAAACAGGCGCGGTCGGCATGCAAACCGCTCCGGGTG
CGGCGGGCGTTAACGGCGGGCAGGCAGGAACAAAAACCTATGAAGTCGAAGCCTGCTGTTCCAACCTCAATTA
TCTGAAATACGGAATGTTGACACGT

tbpB Allele: 648

(89% similar to new tbpB allele 789 of *N. gonorrhoeae* isolate MD-2683

GTCTGGAAACAGTAATCTGACCACGGTTTTGGATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAAT
CTCGACAACTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGTCCACCGAAAGCG
GGAACGGTCAGGCAGATAAAGGTAAAAACGGTGGAACAGACTTTACCTACGAAACAACCTACACGCCGGAAAG
CGATGAAAAAGACACCAAAGCAGGTACGGCGGCGAGTGGCGTGCAAACCGTTTCAAATACGGCAGGCGGCACA
AGTGGCAAAACACAAACCTATAAAGTCCAAGTCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTTGCTGA
CGCGCAAAACTGCCGACAACACGAT

tbpB Allele: 720

(94% similar to new tbpB allele 786 of *N. gonorrhoeae* isolate MD-2287)

CGCCGTCTGAAACCAAGCTGACCACGGTTTTGGATGCGGTCGAGCTGAAATCGGACGGTAAGAAAGTCGAAAA
TCTCGACAACTTCAGCGACGCTACCCGACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCAAGGCTTCC
GAAGGCGTGGACGGTCAGGCAGATAAAGGTGAAAACGGCAAAACAGCCTTTACCTACGAAACAACCTACACGC
CGGAAAGTGATAAAAAAGACACCAAAGCCCAAACAGGCGCGGGCGGCATGCAAACCGTTTCAAATACGGCGGG
CGGCACAAGTGGCAAAACAAAAACCCATTATAAAGTCCAAGTCTGCTGTTCCAACCTCAATTATCTGAAATAC
GGGCTGCTGACACGTGAAAACAACA

tbpB Allele: 747

(91% similar to new tbpB allele 788 of *N. gonorrhoeae* isolate MD-2642)

CGTCTGAAACGGTAAGCTGACCACGGTTTTGGATGCGGTTGAATTGACACCAGACGGCAAGGAAATCAAAAAT
CTCGACAACTTCAGCAACGCCGCCCAACTGGTTGTCGACGGCATTATGATTCCGCTCCTGCCCACCGAAAGCG
GGACGGTCAGGCAGATAAAGGTGAAAACGGCAAAACAGCCTTTATCTACGAAACAACCTACACGCCGGAAAGC
GATGAAAAAAACACCCAAGCCCAAACAGGCGCGGGCGGCGTGCAAACCGTTTCAAATGCGGCGGGCGGCACAA
GTGGCAAAACAAAAACCCATTATGAAGTCCAAGCCTGCTGTTCCAACCTCAATTATCTGAAATACGGGTTGCT
GACGCGCAAAACCGCCGGCAACAC

Appendix XIII: Bangladesh: A brief identity

Bangladesh is a small country of 147,570 sq km land area inhabiting 144,319,600 populations and is included in the Southeast Asia (SEA). (Source: Bangladesh Bureau of Statistics, web: http://www.bbs.gov.bd/)Bangladesh now has a rising economy with per capita income of about 750 US Dollars annually in 2010 (http://thedailystar.net/story.php?nid=140346).

Above: Map of the Southeast Asia including Bangladesh (Greenish-red)) surrounded by India, Myanmar and the Bay of Bengal; ***Below***: Position of Bangladesh in the world map

Appendix XIV: Bangladesh map showing sites (✸) of isolation of *N. gonorrhoeae*