Chapter 2

REVIEW OF LITERATURE
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2.1. Introduction

There is a growing concern worldwide regarding the problem of infectious disease, especially sexually transmitted diseases (STDs). STDs are a group of communicable diseases that are transmitted predominantly through sexual contact. Amongst STDs, gonorrhea continues to be a major health problem with serious health, social and economic consequences. Gonorrhea is a common and universally encountered sexually transmitted bacterial disease, caused by a gram negative diplococcus, *Neisseria gonorrhoeae*. It involves the mucousae of genital, anorectal, pharyngeal and ocular regions. The clinical spectrum of gonococcal infection extends from minimally asymptomatic mucosal colonization to frank inflammatory mucosal disease (urethritis, cervicitis, proctitis, pharyngitis, conjunctivitis) that may lead to local complications such as epididymitis, salpingitis and pelvic inflammatory disease as a result of contagious spread and even serious systemic complications such as arthritis, carditis and dermatitis. A part from causing disastrous sequelae such as infertility and ectopic pregnancy, it constitutes a major risk factor for HIV transmission through increased viral shedding from the inflamed mucosae (Wesserheit 1992, Cohen et al 1998). Therefore, there are clearer and more cogent reasons than ever before to ensure that gonococcal disease is timely diagnosed and treated effectively. Two methods for detecting *N. gonorrhoeae* are culture and nonculture tests. Culture techniques are considered the tests of choice; but nonculture techniques, which are less labor-intensive and are similar in accuracy to cultures, have replaced culture techniques in some instances. The newest nonculture technique is the nucleic acid amplification test. This test has good sensitivity (92 to
96 percent) and specificity (94 to 99 percent) compared with cultures. (Cook et al 2005). Many methods for the characterization of *N. gonorrhoeae* require cultures. The useful typing methods for determining strain relatedness include auxotyping, serotyping. DNA sequencing of the *porB* gene. The prevention of gonorrhea requires timely diagnosis and effective treatment with an appropriate antibiotic regimen, preferably in a single dose. Monitoring of antimicrobial susceptibilities of *N. gonorrhoeae* is important to investigate treatment failure and to evaluate the efficacy of currently recommended therapies. Continuing emergence of antibiotic resistant strains of *N. gonorrhoeae* and the preponderance of disease in patients in resource poor settings where many recommended antibiotics are either unavailable or expensive are the major limiting factors. Unfortunately during the last few decades, *N. gonorrhoeae* has developed resistance not only to less expensive antimicrobials such as sulphonamides, penicillin and tetracyclines but also to fluoroquinolones. Among the antimicrobials available so far, only the third-generation cephalosporins is presently recommended as first-line therapy for gonorrhoea globally. However, resistance to oral third-generation cephalosporins has also started emerging in some countries (Tapsall W 2009). Therefore, it has become imperative to initiate sustained national and international efforts to reduce infection and the misuse of antibiotics so as to prevent further emergence and spread of antimicrobial resistance. It is necessary not only to monitor drug resistance and optimise treatment regimens, but also to gain insight into how gonococcus develops drug resistance. Knowledge of mechanism of resistance would help us to devise methods to prevent the occurrence of drug resistance against existing and new drugs. Such studies could also help in finding out new drug targets in *N. gonorrhoeae* and also a possibility of identification of new drugs for treating gonorrhoea.
2.2. Taxonomy of Neisseria gonorrhoeae

The genus Neisseria was named after Albert Neisser who observed diplococci in leukocytes in urethral exudates from patients who had gonorrhea in 1879. Three years later Leistikow and Loeffler were able to cultivate the bacterium and termed it as gonococcus. Although, various species within the Neisseriaceae family can cause disease in humans and various animals, the two well-known pathogenic species for humans are *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The family of the Neisseriaceae included following four genera – *Neisseria, Moraxella, Kingella and Acinetobacter* (Vedros, Bergeys manual 1984).

*Neisseria* spp. are normal inhabitants of the nasopharyngeal and oropharyngeal mucous membrane of humans and various mammals (Knapp 1988, Morse SA et al 1996). *Neisseria meningitidis* is usually carried in the nasopharynx without causing disease (Cartwright K et al 1991). Colonization may persist for many months. One-third of the carriers have chronic carriage for more than 16 months. Carriage induces bactericidal antibodies. Since the development of such antibodies increases progressively at young age when carriage of meningococci is low, other bacteria with cross-reaction most likely induce bactericidal antibodies. *Neisseria lactamica* carriage elicits bactericidal antibodies against various meningococcal serogroups and serotypes (Cartwright et al 1991). *Neisseria gonorrhoeae* strains are always considered to be pathogenic, in contrast to *N.meningitidis*, of which only some strains can cause acute meningococcal disease. Gonococci infect those surfaces which are lined with columnar epithelial cells; endocervix, urethra, anogenital and oropharyngeal mucous membranes and conjunctiva.
*Neisseria* spp., like other gram-negative bacteria, possess an inner cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane containing proteins, phospholipids and a lipo-oligosaccharide (LOS) for *Neisseria gonorrhoeae*. (Fig 2.1) (Mandrell et al 1993).

![Fig 2.1 Structure of N.gonorrhoeae](source: www/linkinghub.elsevier.com)

Studies on the pathogenesis and epidemiology of gonorrhea have been enhanced by the availability of appropriate typing methods. Characterization of *N.gonorrhoeae* is based on auxotyping and serotyping (Morse et al 1996).

2.3. The disease and its pathogenesis:

Gonococci primarily infect columnar or cuboidal epithelium. In males the anterior urethra is the primary site of infection and in females the endocervix. Colonization may occur, often followed by infection once the mucosal membrane of the genitourinary tract, rectum, oropharynx or eyes has reached by gonococci. In most cases infection remains restricted to the initial site of colonization, although, certain gonococcal strains have the ability to spread to distant sites. The
infective dose for the male urethra is over $10^5$ gonococci and for the female cervix ranges from $10^2$ to more than $10^7$ gonococci (Jephcott et al 1997).

The first step in the pathogenesis of gonorrhea is the attachment of gonococci to mucosal surfaces and the ability of the gonococci to remain attached despite the continuous flow of body fluids. Gonococci attach to microvilli of nonciliated columnar epithelial cells. Nearly all attached gonococci are pilated. Shortly after initial attachment gonococci become more tightly anchored to the epithelial cell surface. This second stage of binding is in part mediated through fimbiae and opa (P.II) proteins (Van Putten et al 1998, Virji et al 1999). Opa also mediates the binding of gonococci to each other to form microcolonies, probably aiding the initial colonization (Fig 2.2).

After attachment there is a critical interaction between gonococci and host defenses (Cohen et al 1992). Gonococci have multiple mechanisms by which the bacteria resist innate defense as well as the defense by antibodies elicited by a previous infection. Sialylation of LOS may limit or alter complement binding. (Parsons et al 1989). The binding of cross reactive blocking antibodies to Rmp (protein III) hinders the binding of bacterial antibodies (Plummer et al 1993). *Neisseria gonorrhoeae* varies in its surface proteins, especially pili antigens, by phase variation and antigenic variation (Swanson et al 1992). In the case of pili, gonococci have an antigenic repertoire that may be as large as one million different antigenic variants. The antigenic repertoire for protein II (Opa) is smaller. The LOS composition is also changed due to antigenic variation.
Gonococci secrete an IgA protease that specifically cleaves IgA, in the hinge region. In response to environmental conditions gonococci synthesize iron-repressed gonococcal outer membrane protein, which binds lactoferrin and transferring, providing iron internalization. Cell damage is caused by LOS, shed from viable bacteria and peptidoglycan fragments liberated from lysed bacteria.

As gonococci do not produce exotoxins, tissue damage results from the bacterial cell wall components such as LOS and peptidoglycan. Both constituents induce the production of tumor
necrosis factor (TNF)-α. The inflammatory reaction is most likely responsible for the local symptoms.

A prominent feature of gonococcal infection is the intimate association with granulocytes, intracellularly; Opa+ gonococci adhere to granulocytes, passing by phagocytosis and killing (Cohen et al 1992). Also pili have antiphagocytic properties and sialylation of LOS reduces the opsonization of bacteria. The gonococci are able to resist oxidative killing inside phagocytes and may survive inside the granulocytes by binding of Por to calmodulin and up regulation of catalase production. Pili tranverse the capsule and are the most prominent adhesions of encapsulated *N. gonorrhoeae*. In addition, the integral outer membrane (OM) adhesions, Opa and Opc, are known to mediate interactions with specific host-cell receptors in appropriate phenotypes (Virji et al 2009). Lipopolysaccharide may interfere with the adhesion functions of OM proteins, but can also contribute to cellular interactions by interacting with cellular receptors. (Fig 2.3)

![Fig 2.3. Structure of Opa & Opc protein of N.gonorrhoeae](image)
Source: Pathogenic neisseriae: surface modulation, pathogenesis and infection control (Nature Reviews Microbiology 7, 274-286, April 2009)

Certain strains have the ability to spread. Strains isolated from the fallopian tube lack Opa (proteinII). Gonococci from other distant sites have the Por IA type protein. Host factors also play a role in the dissemination of gonococci. People who have terminal complement deficiencies have a high risk of developing bacteremia. (Tofte et al 1979).

2.4. Clinical manifestations of Gonococcal Infections

Gonococcal infection is generally limited to superficial mucosal surfaces lined with columnar epithelium. The area’s most frequently involved are the cervix, urethra, rectum, pharynx, and conjunctiva (Fig 2.4). Squamous epithelium, which lines the adult vagina, is not susceptible to infection by the gonococcus. Hence, gonorrhoea in young girls may be present as vulvovaginitis (Holmes, 1974). In mucosal infections, there is a brisk local neutrophilic response manifested clinically as a purulent discharge.
Fig 2.4: Clinical manifestations of *N gonorrhoeae* infection

Source: Morse in Baron, Chapter 14, Neisseria, Moraxella, Kingella and Eikenella

The main symptoms, clinical manifestations of uncomplicated gonorrhea, and complications associated with infection are shown in Tables 2.1 (Morse et al 1996). The most common symptom of gonorrhea in men is a discharge that may range from a scanty, clear, or cloudy fluid to one that is copious and purulent (Fig 2.5). Dysuria is often present. However, men with asymptomatic urethritis are an important reservoir for transmission (Holmes, et al 1974). In addition, such men and those who ignore their symptoms are at increased risk for developing complications. Endocervical infection is the most common form of uncomplicated gonorrhea in women. Such infections are usually characterized by vaginal discharge and sometimes by dysuria (because of coexistent urethritis) (Fig 2.5). About 50 percent of women with cervical infections are asymptomatic (Nielsen *et. al.*, 1975). Local complications include abscesses
in Bartholin’s and Skene’s glands (Holmes et al, 1974). Rectal infections with *N. gonorrhoeae* occur in about one-third of women with cervical infection. They most often result from autoinoculation with cervical discharge and are rarely symptomatic (Holmes et al, 1974). Rectal infections in homosexual men usually result from anal intercourse and are more often symptomatic. The symptoms and signs of gonococcal proctitis ranged from mild burning on defecation to itching and from mucupurulent discharge to frank blood in the stools. Pharyngeal infections are diagnosed most often in women and homosexual men with a history of fellatio (Holmes et al, 1974). Ocular infections can have serious consequences (corneal scarring or perforation); prompt diagnosis and treatment are therefore important (Fig. 2.5). Ocular infections (ophthalmia neonatarum) occur most commonly in newborns who are exposed to infected secretions in the birth canal. Keratoconjunctivitis is occasionally seen in adults as a result of autoinoculation (Holmes et al, 1974). Disseminated gonococcal infection presents as a few skin lesions that are limited to the extremities. These lesions start as papules and progress into bullae, petechiae, and necrotic lesions. The most commonly infected joints include wrists, ankles, and the joints of the hands and feet. (Fig 2.6) (Miller et al 2006)

![Urethral discharge, Conjunctivitis, Cervical discharge](source)

**Fig 2.5:** Clinical symptoms- urethral discharge, conjunctivitis and cervical discharge

Source: CDC/NCHSTP/Division of STD Prevention, STD Clinical Slides
**Fig 2.6: Skin lesion from patient with systemically disseminated *Neisseria gonorrhoeae***

*Source: CDC/NCHSTP/Division of STD Prevention, STD Clinical Slides*

**Table 2.1 Clinical spectrum of gonococcal infection**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Signs/ Symptoms</th>
<th>Clinical manifestations</th>
<th>Complications</th>
</tr>
</thead>
</table>
| Men      | – Purulent urethral discharge  
          – Dysuria  
          – Pain in the urethra  
          – Pain in perineum radiating into the rectum  
          – Pain and discharge from the rectum | – Urethritis  
– Balanoposthitis | – Epididymo-orchitis  
– Prostatitis  
– Phimosis  
– Paraphimosis  
– Urethral stricture  
– Lymphadenitis  
– Infertility |
| Women    | – Vaginal and/or cervical mucopurulent discharge  
          – Dysuria  
          – Lower abdominal pain  
          – Dyspareunia  
          – Pain and discharge from the rectum | – Cervicitis  
– Urethritis  
– Bartholinitis | - Bartholin apostasis/abscess  
Pelvic inflammatory disease (PID):  
– Endometritis  
– Salpingitis  
– Tubo-ovarian abscesses  
– Pelvic peritonitis  
– Perihepatitis  
– Ectopic pregnancy  
– Infertility |
| MSM      | – Pain and discharge from the rectum | – Pharyngitis  
– Proctitis  
– Ponjunctivitis | – Anorectal infection  
– Pharyngeal infection |
### 2.5 Conventional methods for the laboratory diagnosis of gonococcal infections

Various techniques are used for the identification of *N. gonorrhoeae*. There are a variety of physical and temporal conditions which affect a specimen from its acquisition to its receipt in the laboratory. The laboratory diagnosis of gonorrhea includes several interrelated aspects, each of which is important to the final results. These include specimen collection, specimen management, detection procedure, identification procedure and supplementary procedures.

#### 2.5.1 Specimen choice and collection

The specimen choice and collection method (Table 2.2) depends on the testing technique used in a laboratory and the age, sex and sexual orientation of the patient (Whittington et al 1996). Specimens should be collected with Dacron or rayon swabs because calcium alginate may be toxic to gonococci. Fatty acids inhibit the growth of gonococci; therefore, cotton swabs that do not list acceptable manufacturer specifications should not be used (Janda et al 2003). To
minimize the inhibitory effects of unknown substances in the specimen, the swabs should be inoculated directly onto growth medium or placed in swab transport medium immediately after sampling (Hansen et al 2003).

**TABLE 2.2**

Methods of collection of clinical specimens for the laboratory diagnosis of gonorrhea

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Method of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urethral</td>
<td>Express urethral exudates when patients have discharge. If there is no discharge, compress the meatus vertically to open the distal urethra and insert a thin, water-moistened swab (calcium alginate or Dacron) with flexible wire slowly (3 cm to 4 cm in males or 1 cm to 2 cm in females), rotate slowly and withdraw gently.</td>
</tr>
<tr>
<td>Urine</td>
<td>First 10 mL to 15 mL of urine is collected. Patients should not have voided for at least 2 hr. before specimen collection to increase the chance of detecting the organism</td>
</tr>
<tr>
<td>Cervical</td>
<td>Speculum is inserted into the vagina to view the cervix. A swab 1 cm to 3 cm is inserted into the endocervical canal and rotated for 10 s to 30 s to allow absorption of exudates.</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Pooled vaginal secretions, if present is collected. Vaginal wash specimens are most preferred and acceptable to prepubertal girls. If not possible, rub a</td>
</tr>
</tbody>
</table>
sterile cotton swab against the posterior vaginal wall and allow the swab to absorb the specimen

<table>
<thead>
<tr>
<th>Method</th>
<th>Process Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal</td>
<td>Specimens may be obtained blindly or, preferably, through an anoscope. A swab 2 cm to 3 cm is inserted into the anal canal. Avoiding fecal material, rotate to sample crypts just inside the anal ring; allow the swab to absorb specimen for 10 s.</td>
</tr>
<tr>
<td>Oropharyngeal</td>
<td>A sterile swab is rubbed over the posterior pharynx and tonsillar crypts, or obtain nasopharyngeal aspirate from infants.</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>Any exudate or pus present in the eye should be carefully removed with a sterile swab. A second swab moistened with saline should be used to rub the affected conjunctiva. This swab should be broken off into a vial of transport medium.</td>
</tr>
<tr>
<td>Sterile body fluids</td>
<td>Clean skin puncture site with iodine (1% to 2%, or 10% solution of povidone-iodine [1% free iodine]). If tincture of iodine is used, remove with 70% ethanol to avoid burn. Perform percutaneous aspiration for pleural, pericardial, peritoneal or synovial fluids. Use nonheparinized collection if possible.</td>
</tr>
</tbody>
</table>

1. Urethral specimens:

Discharge from the meatus is preferred for the detection of *N gonorrhoeae*. If there is no meatal exudate in postpubertal patients, an intraurethral swab can be used for the detection of
gonococci. To increase the chance of detecting the organisms, swab samples should be collected from patients who have not voided for at least 2 hrs. The swabs are suitable for smear preparation, culturing on appropriate media or for transport to other laboratories.

2. Urine:

Urine is one of the specimen types suitable for nucleic acid tests for diagnosing *N gonorrhoeae* infections in males and females. Leak-proof containers should be provided to patients for the collection of urine specimens.

3. Cervical and vaginal specimens:

Cervical specimens should be taken from young and adult females. Gonococcal infections in prepubertal girls involve the vagina and not the cervix; therefore, vaginal specimens should be collected from this age group. When the patient presents with a urethral discharge, additional urethral or meatal specimens should be taken because the sensitivity of detecting gonococci from cervical specimens is lower than that from urethral or meatal specimens. Similar to urethral swabs, cervical and vaginal swabs can be used to prepare smears, to inoculate culture media directly, for nucleic acid-based tests or for submission in transport media to distant laboratories.

In cases of suspected coinfections of *N gonorrhoeae* and *Chlamydia trachomatis*, the cervical specimen for *N gonorrhoeae* detection should be taken before the specimen for *C trachomatis*, because *N gonorrhoeae* is present in the mucus from the endocervix and *C trachomatis* is present in the cervical epithelial cells.
4. Oropharyngeal and rectal specimens:

Oropharyngeal and rectal specimens should be processed only for culture because the performance of nonculture methods is not well established for these specimen types.

5. Conjunctiva:

The purulent discharge in gonococcal ophthalmia neonatorum and conjunctivitis in adults should be used for diagnosis.

6. Body fluids:

When patients have symptoms of systemic or disseminated infections, blood and fluid from arthritic joints are suitable samples for culturing. (Lai-King et al 2005)

2.5.2 Transport of specimens & cultures

2.5.2.1 Specimens

Methods of transportation vary with the specimen and the type of test being done, but in all instances when commercially available transport systems are used, the instructions provided by the manufacturer should be followed.

Ideally, the specimens should be inoculated onto culture medium immediately after collection to preserve the viability of gonococci for isolation. If the inoculated media are being transported to a local laboratory, the plates should be held at room temperature for no more than 5 hr. in a CO₂-enriched atmosphere using a candle jar or a commercial CO₂-generating system. If long-distance shipping is required, the specimens should be inoculated onto media contained in a
CO₂-generating system, incubated for 18 hr. to 24 hr. and have visible growth on the plate before shipping. (Janda et al 2003)

CO₂-generating systems such as JEMBEC (Becton, Dickinson and Company, USA) (Janda WJ et al 2003) and InTray GC (BioMed Diagnostics, USA) preserve the viability of the organisms for longer periods of time than non-nutritive systems. (Janda et al 2003)

2.5.2.2 Cultures

Several transport systems are suitable for transporting cultures to laboratories for susceptibility testing.

Frozen cultures for transport:

Cells from a 24 hrs. agar plate are suspended in brain heart infusion broth with 20% glycerol and placed in a screw cap cryovial. The suspension can be frozen in a dry ice bath, a -70°C freezer or liquid nitrogen. The frozen cultures should be shipped on dry ice in accordance with the instructions for the Transportation of Dangerous Goods Act and Regulations.

Nonfrozen transport media:

- Transport swab systems with or without charcoal are suitable for sustaining viable gonococci for 6 hrs. to 12 hrs. at room temperature. A heavy inoculum is required for overnight transportation of pure cultures.
- The JEMBEC plate contains Martin-Lewis agar in a rectangular polystyrene plate with an inner well that holds a CO₂-generating tablet to provide a modified atmosphere for the cultures.
- InTray GC medium with preincubation.
• Chocolate agar slants with preincubation.

Most laboratory staff and clinicians use either a Gram- stained smear of infected discharge or a culture for *N. gonorrhoeae* to diagnose gonococcal infection. A thin smear of clinical specimen collected is prepared on the glass slide is gram stained and examined microscopically under an oil immersion objective (x100) for intracellular Gram-negative diplococci in polymorphonuclear leukocytes (Fig 2.7). Cells of Neisseria spp. Occur as diplococci composed of kidney shaped cells (0.8 um x 0.6 um) with adjacent sides flattened. The Gram stain, when performed by an experienced microscopist, can be regarded as highly specific in patients with cervical and urethral infections (Sparling et al 1979). Furthermore, it is inexpensive and rapid. When properly performed, the Gram stain has a sensitivity of 90 to 95% and a specificity of 95 to100% for diagnosing genital gonorrhea in symptomatic patients in males. (Jandal et al 2003). In women, Gram stain smears have a sensitivity of 50 to 70% depending on the adequacy of the specimen and patient population. An endocervical smear showing gram negative intracellular diplococci, particularly from a woman with other signs and symptoms of gonococcal infection, is highly predictive. In males with a symptomatic urethral infection and in females with cervical infection, Gram stain sensitivity, compared with that of culture, falls to 40 to 60% (Janda et al 2007). For the culture method, numerous selective media have been developed for the isolation of *N. gonorrhoeae* (Carlson et al 1980). Optimally, the best systems probably detect close to 95% of male urethral infections and 85% of female cervical infections when a single swab specimen is collected (Dans et al 1975, Carlson et al 1980). Disadvantages of culture method include loss of viability of gonococci under suboptimal conditions for transport or growth, lack of sensitivity after initiation of antibiotic treatment, failure to culture vancomycin-sensitive strains or other strains with fastidious growth.
characteristics, and the time required of up to 2 to 3 days before results are available (Dans et al 1975, Carlson et al 1980). Because of these limitations with the culture method, alternative technologies have been developed for the laboratory diagnosis of gonococcal infections.

![Image](image_url)

**Fig 2.7: Gram negative intracellular diplococci**

### 2.6 Confirmatory identification tests for *Neisseria* species.

Confirmatory tests for *N. gonorrhoeae* include carbohydrate acidification tests, chromogenic enzyme substrate tests, immunologic tests (e.g. fluorescent antibody, coagglutination tests), multitest identification systems and DNA probe tests.

#### 2.6.1 Acid production from carbohydrates

Conventional cystine-tryptic agar (CTA)

This technique contains CTA medium containing 1% carbohydrate and a phenol red pH indicator (Alexander et al 2005). The usual test battery includes CTA-glucose, maltose, sucrose
and lactose plus a carbohydrate free CTA control. The CTA medium is inoculated with a dense suspension of pure organism (18-24 hrs. old culture) and is incubated in a non-CO₂ incubator at 35°C for 24-72 hrs. There is a change in the color of the phenol indicator. This method is found to be problematic for differentiating *N. gonorrhoeae* and *N. cinerea* and is no longer used for detection of acid production from carbohydrates. (Kellogg et al 1973)

### 2.6.2 Rapid Carbohydrate tests

The rapid carbohydrate test is a non-growth dependent method for detection of acid production from carbohydrates by Neisseria species. Small volumes (0.10ml) of a balanced salts solution (0.04g of K2HPO4 per liter, 0.01 g of KH2PO4 per liter and 0.80g of KCl per liter pH 7.0) with phenol red indicator are dispensed in nonsterile tubes to which single drops of 20% filter-sterilized aqueous carbohydrates are added. A dense suspension of the aqueous carbohydrates is added to each of the carbohydrate containing tubes & the tubes are incubated for 4hr. at 35°C in a non-CO₂ incubator or water bath. This method is economical, the reagents are easy to prepare and inoculate, and the results are very clear. The RIM-Neisseria test (Rapid identification method, Remel Inc.), the Gonobio test (LA.E Production, Inc. Laval, Quebec, Canada) and the Neisseria Kwik test (MicroBIO logics, st. Cloud. Minn) are commercial utilization test and evaluations have been reported (Dillon et al 1988, Dolter et al 1990).

### 2.6.3 Chromogenic Enzyme substrate tests

Enzymatic identification system use specific substrates that after hydrolysis by bacterial enzymes yield coloured end product that is detected directly or after addition of a diazo dye coupling reagent. Enzyme activities that are detected in these systems include β-galactosidase, γ-
glutamylaminopeptidase and prolylhydroxyprolyl amino peptidase are specific for *N. lactamica* and *N. meningitidis*, respectively. The absence of these activities and the presence of prolylhydroxyprolyl aminopeptidase identify an organism as *N. gonorrhoeae*. The Gonocheck II (EY Labs, San Mateo, Calif) is a commercial system that detects all the three enzyme activities in a single tube (Dillon et al 1988). The BactiCard Neisseria (Remel Inc.) uses filter paper pads that are impregnated with substrates for the three enzymes, plus an indoxyl butyrate substrate (Janda et al 2002). Recently, gonococcal strains lacking prolylhydroxyprolyl aminopeptidase have been isolated in Denmark and United Kingdom (Alexander et al 2005, Fjeldsoe-Nielsen et al 2005).

### 2.6.4 Immunologic methods for culture confirmation

#### 2.6.4.1 Direct Fluorescent Monoclonal antibody test (DFA)

The DFA culture confirmation procedure uses monoclonal antibodies that recognize epitopes on the PorI (Protein I) outer membrane protein (OMP) of *N. gonorrhoeae*. The DFA test (*Neisseria gonorrhoeae* Culture confirmation test, Trinity Biotech, Wicklow, Ireland) is performed by preparing a smear on a DFA slide, overlaying the smear with DFA reagent and incubating the smear for 15 min. After rinsing and mounting the slide is examined with a fluorescence microscope. Gonococci appear as apple-green fluorescent diplococci. Although DFA is highly sensitive and specific, however at present many strains fail to stain with this reagent (Janda et al 1993). Some studies have showed that gonococcal strains that are negative by DFA reagent belong to a variety of serovars (Janda et al 1993). The package inserts claims 99.6% sensitivity and 100% specificity. The isolates that do not stain with the DFA reagent must be identified by another method. The advantages of this procedure are rapidity, ability to test
colonies directly from primary cultures and requires small amount of growth. The DFA test is not intended for direct identification of organisms in smears from patient specimens.

2.6.4.2 Co agglutination tests

Two co agglutination tests for identification of *N. gonorrhoeae* are currently available: the Phadebact Monoclonal GC test (Boule Diagnostics ab, Huddinge, Sweden) and the GonoGen I (New Horizons Diagnostics, Columbia, Md). The Phadebact Monoclonal GC test uses anti-PorI monoclonal antibodies bound to staphylococcal cells. The monoclonal GC test contains one reagent that reacts with serogroup WI *N. gonorrhoeae* strains and a second reagent that reacts with serogroup WI/III strains. Since a negative control reagent is not included, gonococcal isolates react with either the WI or the WI/WIII reagent, depending on the PorI epitopes expressed by an individual isolate. A suspension (0.5 Mc Farland standard) prepared in buffered saline (pH 7.2 to 7.4) is boiled and mixed with the two test reagents on a cardboard slide. Agglutination within 1 min is a positive test. Freshly subcultured serogroup WI (ATCC 19424) and serogroup WI/III (ATCC 23051) strains are recommended for quality control but are not provided with the kit. The GonoGenI co agglutination test also uses staphylococcal cells coated with anti-PorI monoclonal antibodies. This test kit contains test and control agglutination reagents, and positive and negative test control suspensions are also included. GonoGenI also uses a boiled organism suspension (McFarland standard of 3) for testing, and agglutination with the test but not the control reagent constitutes a positive test. Some gonococci may not react with these reagents, and cross reactions with other *Neisseria* species (i.e. *N. meningitidis, N. lactamica* and *N. cinerea*) and *K. denitrificans* have been reported (Janda et al 1993, Dillon et al 1988, Alexander et al 2005)
2.6.4.3 GonoGen II

GonoGen II (New Horizons Diagnostics) uses anti-PorI monoclonal antibodies conjugated to red colored metal sol particles as the detection reagent. Colonies from agar medium are suspended in a solubilizing buffer that releases PorI-antigen containing complexes from the cell wall. A drop of the antibody-sol particles is added, and the PorI antigen binds to the antibody-sol particles. After 5 mins, 2 drops of this mixture are passed through a membrane filter that retains antigen-antibody complexes. Concentration of these complexes on the filter turns the filter red, identifying the organism as *N. gonorrhoeae*. Nongonococcal isolates do not produce these, so the entire suspension passes through the filter, resulting in the filter remaining white or pale pink. *N. gonorrhoeae* strains that do not react with the conjugate are not identified and false positive reactions have been noted with some *N. meningitidis* and *N. lactamica* strains (Janda et al 1993, Alexander et al 2005).

2.6.4 Multitest identification systems

A range of commercially available biochemical kits are also widely used, including the *Neisseria* Preformed Enzyme Test (PET), Gonocheck II (EY Laboratories, RapID NH(*Neisseria-Haemophilus*) (Remel, Inc.), Vitek NH(*Neisseria-Haemophilus* identification card, bioMerieux, Inc. Hazelwood, Mo.), and API NH the Haemophilus-Neisseria identification (HNID) panel (Dade Behring, Sacramento, Calif) and the API NH (bioMerieux, Inc. La Balme-les-Grottes, France (Janda et al 1987, Janda et al 1989, Barbe et al 1994, Alexander et al 2005). The *Neisseria* PET and Gonocheck II are both single-use tubes containing chromogenic substrates that detect the presence of three preformed enzymes, each of which is indicative of a pathogenic *Neisseria* species, namely *N. lactamica*, *N. meningitidis* and *N. gonorrhoeae*. The
API NH and RapID NH, Vitek NHI kits employ a battery of tests, combining carbohydrate utilization and preformed enzymes. However, biochemical tests that detect the presence of preformed enzymes should be interpreted with caution because proline iminopeptidase (Pip)-negative *N. gonorrhoeae* have been reported (Janda et al 2007). These kits use modified conventional tests and chromogenic substrates to provide identifications within 2 to 4 hr. A new NHI card has been developed with the Vitek-2 instrument; *N. cinerea* is not included in the database of the MicroScan HNID panel resulting in misidentification as *N. gonorrhoeae* or *M. catarrhalis* (Janda et al 1989). The API NH system identifies gonococci, meningococci and *N. lactamica* within 2 hr. while other Neisseria species require additional tests for correct species identification (Barbe et al 1994, Alexander et al 2005)

2.6.5 DNA Probe test for culture confirmation

The ACCUPROBE system uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism’s ribosomal RNA to form a stable DNA:RNA hybrid. The Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in a GEN-PROBE® luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value below this cut-off is a negative result. The Accuprobe test is more sensitive and specific than biochemical or immunologic culture confirmation tests and is particularly useful for confirming problem isolates (Janda et al 1987).

A number of non-culture test methods have been evaluated for the detection of *N. gonorrhoeae*, including detection of antigen or genetic material (Granato et al 1990, Panke et
al, 1991, Hosein et al 1992, Limberger et al 1992, Miller et al 1992, Chapin-Robertson 1993, Hale et al 1993, Stary et al 1993, Vlapolder et al 1993, Morse et al 1996). The use of hybridization with DNA probes as a tool for detection of gonococci in urogenital specimens has been investigated extensively. Probes using nucleotide sequences of the pilin gene (Meyer et al 1982), the IgA1 protease gene (Koomey et al 1984), ribosomal RNA gene (Roussau et al 1989; Roussau et al 1990) and the cryptic plasmid (Totten et al 1983) have been reported. The pilin genes of the gonococci have more than one copy, but they are highly homologous with those of meningococci. All gonococci have a single copy of the IgA1 protease gene (Koomey et al 1984), and therefore detection is difficult when only few organisms are present in the clinical sample. The rRNA-derived oligonucleotide probes to different regions of the 16S ribosomal RNA have been reported to have a sensitivity and specificity of 100% (Roussau et al 1989).

2.7 DNA based methods for laboratory diagnosis of Gonococcal infections:

Nucleic acid detection techniques allow direct detection of *N. gonorrhoeae* in clinical samples and do not require viable organisms. These assays are divided into three types:

i) Direct probe hybridization to the target nucleic acid with direct detection of the hybrid

ii) Nucleic acid amplification tests (NAATs)

iii) Amplified signal probe tests, which hybridize with nucleic acid and then amplify the signal of the probe.

The direct probe tests only slightly increase the sensitivity of culture with a proficient specimen transport system, but NAATs have high sensitivity. The advantages of NAATs are that specimens may be transported from geographically distant areas and stored for several days prior
to testing in the laboratory. These samples can be stored at -70°C prior to testing. The NAATs also allow use of noninvasive specimen type such as urine, vaginal & rectal swabs. The disadvantage of using nonculture nucleic acid probe or amplification tests include unavailability of a viable isolate for antimicrobial susceptibility testing and the possibility of a positive test after treatment, since nucleic acid from organisms may persist for a period of time after successful therapy. Reports have shown that gonococcal DNA for the ligase chain reaction test remained for a mean of 1.7 days in male urine and 2.8 days in female urine, but 2.8 days in vaginal specimens (Bachmann et al 2002).

2.7.1 Direct Probe Hybridization

Two direct nucleic acid probe assays, the Gen-Probe PACE 2 and PACE 2C assays (Gen-Probe Inc. San Diego, California) are approved by Food and Drug Administration (FDA) for detecting *N. gonorrhoeae*. In the Gen-Probe assay, an acridinium ester-labeled DNA probe for specific sequence of *N. gonorrhoeae* rRNA is allowed to hybridize with any complementary rRNA in the specimen (Kluymans et al 1994). An acridinium ester hybridization protection assay detects any hybrids and chemiluminescence generated by the acridinium ester in the hybrids is detected with a luminometer, resulting in a numerical read out. The PACE 2C test detects both *N. gonorrhoeae* and *C. trachomatis* in a single test. A probe competition assay may also be used to augment the specificity of the test. The sensitivity of the assay ranged from 90.8 to 96.3% for women and 99.1 to 99.6% for men while specificity ranged from 97.5 to 100% for men and women according to the manufacturer’s package insert. Evaluations of the assay in public health settings have supported the high sensitivity and specificity of the test (Hale et al 1993, Iwen et al 1995)
2.7.2 Nucleic acid amplification techniques (NAATs)

NAATs are designed to amplify *N. gonorrhoeae* specific nucleic acid sequences from a particular gene target. Currently, there are four commercially available NAATs for the detection of *N. gonorrhoeae*:

i) based on PCR, Roche Cobas Amplicor (Roche Molecular Systems, Branchburg, NJ)

ii) based on strand displacement amplification (BD ProbeTec; Becton Dickinson, Sparks, MD)

iii) based on transcription-mediated amplification: Gen-Probe APTIMA Combo 2, which is for dual detection of gonorrhea and Chlamydia, and APTIMA AGC, which is viable for detection of only gonorrhea (both from Gen Probe, AC2; Gen-Probe)

iv) Abbott Ligase Chain Reaction (LCx) (Abbott Laboratories).

The direct detection of *N. gonorrhoeae* in specimens requires either an extremely sensitive and specific assay or a process by which a diagnostically useful gonococcal component can be amplified to a detectable level. With the advent of PCR technology, academic and commercial investigators have turned their eyes to nucleic acid amplification technologies to provide the high sensitivity and specificity that are needed to detect organisms directly in clinical specimens collected from patients and do not require viable organisms.

2.7.2.1 Roche Amplicor PCR

Roche Amplicor PCR for *N. gonorrhoeae* detects a 201 bp sequence in the cytosine methyltransferase gene. This assay showed sensitivity of above 90% for detection of
*N. gonorrhoeae* in cervical specimens & 64.8% in urine samples collected from females. (Martin et al 2000). PCR has been shown to detect gonorrhea in male urine with great accuracy with a sensitivity of 94.1% and a specificity of 99.9% in 1,291 symptomatic men and 73.1% in 721 asymptomatic men (Martin et al 2000). This assay has been used in research settings using vaginal swabs (Wiesenfeld et al 2001). False positive results have been reported (Palmer et al 2003). Roche amplicor PCR showed the sensitivity & specificity of 96.1% & 96.5% by using pooled urine samples (Lindan et al 2005) & 96.9% and 99.7% for *N. gonorrhoeae* (Gaydos et al 2010). The studies carried out on oropharyngeal showed sensitivity & specificity of 80.3% and 73.0% by PCR. (Bachmann et al 2009). The Cobas Roche Amplicor 4800 assay showed sensitivity of 92.9%, 100% and specificity of 100% for urine and swab specimens (Rockett et al 2010). A recent study carried out using pharyngeal and rectal swabs collected from MSM population showed the sensitivity of 36% and 75% (Walsh 2011).

2.7.2.2 Strand Displacement assay (SDA)

The Strand displacement amplification assay (ProbeTec) is approved for detection of gonorrhea in cervical, male urethral and female and male urine samples and have been used widely in clinical laboratories all over the world (Van Der Pol et al 2001). This technique uses readily available common enzymes (5'-3'exo DNA polymerase and HincII). The fundamental scheme for the SDA reaction is depicted (Fig 2.8). Briefly, a denatured single stranded DNA fragment binds to SDA primer containing a recognition site for HincII. Primer extension using dCTP, dTTP, dGTP and dATP produces a double stranded hemiphosphorothioate recognition site. HincII nicks the unmodified primer strand (having unmodified A residue at the recognition site) leaving the modified complimentary strand intact. DNA polymerase lacking 5'-3' exonuclease activity
extends the 3’ end at the nick and displaces the downstream fragment. Nicking and polymerization
/displacement steps cycle continuously producing single stranded complementary copies of the
target fragment (Walker et. al., 1992). Exponential target DNA amplification is achieved by
coupling sense and antisense reactions in which strands displaced from a sense reaction serves as a
target for an antisense reaction and vice versa. SDA can produce $10^6$ to $10^8$ fold amplification in
about 2hrs. and specificity depends on choice of primers to direct the DNA synthesis.

Fig 2.8: Mechanism of Strand Displacement Assay

Source: www.sciencedirect.com
SDA assays have been commercially developed to amplify target DNA in the cryptic plasmid of *N. gonorrhoeae* (Probetec, Becton-Dickinson, Franklin Lakes, NJ). Amplified products are detected by using a nonisotopic microtitre plate assay employing a biotinylated oligo deoxynucleotide probe. The combination of SDA and chemiluminiscent detection results in the specific identification of as few as 1-10 CFU. The sensitivity & specificity for various specimens (urethral, urine, endocervical) was found to be ranging from 95-100%. (Vander Pol et al 2001, Gaydos et al 2010, Wheeler et al 2005 (Table no.2.3). The sensitivity and specificity of the *N. gonorrhoeae* test were 76.2% and 100% for SDA using ano rectal swabs, Cosentino et al 2012)

### 2.7.2.3 Transcription-mediated amplification (TMA)

TMA (APTIMA Combo 2) is the most sensitive assay for detecting *N. gonorrhoeae* (Martin et al 2000, Gaydos et al 2003). TMA is a concerted; three enzyme *in vitro* reactions to carry out an isothermal replication of target rRNA via DNA intermediates. The three enzymes involved are reverse transcriptase (RT), RNase H and T7 RNA polymerase. rRNA is amplified via TMA in which the rRNA target sequences are copied into a transcription complex by using RT and then RNA polymerase is used to make numerous RNA transcripts of the target sequence from the transcription complex and this process is then repeated autocatalytically (Fig 2.9). TMA has been developed by Gen Probe Inc. (San Diego, Calif.) to detect *N. gonorrhoeae* in clinical specimens. Gen Probe’s Amplified Gonococcal Direct Test (NGDT) targets ribosomal RNA and works as an isothermal system that uses enzymatic target amplification and chemiluminescence detection of amplicons with an acridium ester-labeled DNA probe in the hybridization protection assay (HPA) in a single tube format. The entire assay can be completed in 3hrs. to 5hrs. of obtaining a processed sample. NGDT has additional advantages: it is one tube system thereby reducing the chance of
contamination of negative samples with target sequences. Since the amplified product is labile RNA, rather than harder DNA; any contaminating stray copies get readily degraded by cleaning the test area with weak bleach solution. DNA intermediates generated by NGDT during the amplification reactions are dealt with by the addition of ‘undisclosed termination reagents’ at the end of process. Although it is too early to predict the impact of NGDT on clinical practice, the benefits of the rapid diagnosis show some ray of hope.

**Fig 2.9: Transcription Mediated Amplification**

Source: Gen-Probe Inc. www.Chlamydiae.com

Table 2.3: Sensitivity & Specificity of Nucleic acid amplification tests for *N. gonorrhoeae*

<table>
<thead>
<tr>
<th>Assay and specimen</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR(COBAS Roche Amplicor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical</td>
<td>92.4</td>
<td>99.5</td>
</tr>
<tr>
<td>Female urine</td>
<td>64.8</td>
<td>99.8</td>
</tr>
<tr>
<td>Male urine</td>
<td>94.1</td>
<td>99.9</td>
</tr>
<tr>
<td><strong>Strand displacement amplification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical</td>
<td>96.6</td>
<td>98.9-99.8</td>
</tr>
<tr>
<td>Female urine</td>
<td>84.9</td>
<td>98.8-99.8</td>
</tr>
<tr>
<td>Male urine</td>
<td>98.1</td>
<td>96.8-98.7</td>
</tr>
<tr>
<td>Male urethral</td>
<td>98.1</td>
<td>96.8-98.7</td>
</tr>
<tr>
<td><strong>Transcription-mediated amplification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical</td>
<td>99.2</td>
<td>98.7</td>
</tr>
<tr>
<td>Female urine</td>
<td>91.3</td>
<td>99.3</td>
</tr>
<tr>
<td>Male urine</td>
<td>97.1</td>
<td>99.2</td>
</tr>
<tr>
<td>Male urethral</td>
<td>98.8</td>
<td>98.2</td>
</tr>
</tbody>
</table>

2.7.2.4 Oligonucleotide ligation amplification (LCR)
The ligase chain reaction-based assay Abbott LCx (Abbott Laboratories Inc. Abbott Park III) which was commercially available and was used by many laboratories is no longer available due to manufacturing issues (CDC 2002). LCR utilizes a set of four oligonucleotides for detection & the amplified products are detected immunochemically (Fig 2.10). All these assay have used multicity target sequences such as opa genes and the pil genes (Backman et al. 1987, Birkenmeyer et al., 1992; Buimer et al., 1996, Stary et. al., 1997). However, LCR has not yet found its place as a diagnostic method in a clinical laboratory.

Fig. 2.10: Flow chart of ligase chain reaction

Source: www.currentprotocols.com

2.7.3 Amplified-Signal Probe Test

The amplified-signal probe test is a hybridization of a probe with nucleic acid of the organism and then amplifies the signal of the probe. This assay (Digene Hybrid Capture II test,
Digene, Silver Spring, Md.) uses RNA hybridization probes which are specific for both genomic DNA and cryptic plasmid DNA sequences of *N. gonorrhoeae* and *C. trachomatis*. The RNA-DNA hybrids are captured in microtiter plate wells by specific antibodies, which detected by alkaline phosphatase-labeled anti RNA-DNA hybrid antibodies. The signal is amplified using a chemiluminescent substrate detected by a luminometer. The test is positive if either Chlamydia or Gonorrhoea is present, and then an organism specific test is performed. This test is approved for testing cervical samples. The sensitivity of the assay was found to be 92.2% and specificity of 99% (Darwin et al 2002, Schachter et al 1999, Vander Pol et al 2002).

2.7.4 Polymerase Chain reaction (PCR)

PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences by thermostable DNA polymerase isolated initially from *Thermus aquaticus* which has a 5' to 3' polymerase activity but lacks 3' to 5' exonuclease activity (Fig. 2.11). It uses two oligonucleotide primers which hybridize to opposite strands and flank the region of interest in the target DNA. Multiple cycles of independent steps (denaturation, annealing and extension) are carried out at specific temperatures that result in the accumulation of a specific fragment of DNA whose termini are defined by the 5' end of the primers. The primer extension products synthesized in one cycle can serve as template for the next cycle, the copy number of target DNA gets approximately doubled after every cycle, and results in the exponential increase of the target sequence over a period of time.
Specific primers and reaction conditions for the detection of the *N. gonorrhoeae* by the PCR have been developed, making it the method of choice for the fastest, most specific and sensitive for detection of an organism. The first report on the use of PCR amplification of *N. gonorrhoeae* DNA in clinical specimens was made by Ho and co-workers (1992) who employed primers based on *cppB* gene, of the cryptic plasmid (*pJD1*), for amplification followed by the digestion of the amplicons with the restriction enzyme MspI (Table 2.4). This *cppB* gene originates from the 4.2kb cryptic plasmid of *N. gonorrhoeae* which was found in whole or part, integrated in the chromosome of the gonococcal strains tested (Hagblom et al 1986). Two variants of this plasmid are characterized, one of which had a deletion of 54 bp within the *cppB* gene. Both the deleted and the undeleted version of *cppB* gene may be found as a separate copy in gonococcal genome, including the strains lacking plasmid. While selecting the primer sequences, their group did not include the possible deleted region of *cppB* gene and the two
chosen 20-mer oligonucleotide primers almost marked the entire length of the remaining \textit{cppB} gene sequence. Subsequent to the report by Ho et al (1992) numerous other PCR based with different target sequence and varying specificities and sensitivities have been described for the rapid detection of \textit{N.gonorrhoeae}. An important point of consideration is the choice of primer sequence specific to \textit{N.gonorrhoeae} as there is considerable homology (80%-90%) between the two pathogenic \textit{Neisseria} species, \textit{N.gonorrhoeae} and \textit{N.meningitidis}. It is particularly important since \textit{N. meningitidis} acne be occasionally isolated from the urogenital specimens (Hagman et 1991).

Single-copy genes encoding the outer membrane protein III, \textit{rmp gene}, \textit{opa gene}, \textit{tbpB}, \textit{tbpA} and the \textit{por} gene were the next target used in the development of the PCR assays to detect \textit{N.gonorrhoeae} (Leibling et al 1994, O’Rourke et al 1995, Cooke et al 1997) and many studies have been conducted using these genes. (Maze et al 2011, Sun et al 2008, Goire et al 2008, Tabrizi et al 2005, Klugman et al 1989, Cooke et al 1998, De Jongh et al 2008, Olesky et al 2006, BASHH et al 2005, Agarwal et al 2005). Subsequent promising approach for direct and specific identification of \textit{N.gonorrhoeae} became available based on the rRNA (ribosomal RNA) genes. Ribosomal RNA sequences are characteristics for a particular organism and can be used for fine taxonomic differentiation as well as for the identification. Especially the abundance of rRNA (103 to 104 molecules per cell) make it suitable target for the PCR assays. Based on the 16s RNA gene sequence, highly sensitive (detection limit of one cell) system was developed by Muralidhar B et al in 1994 (Muralidhar et al 1994). However, this assay was not specific for \textit{N.gonorrhoeae} and detected at least 12 different Neisseria species. Later, other PCR based – assays using 16S RNA gene sequence as the target were evaluated and were shown to be more sensitive and specific (Chui et al 2008, Van der Pol et al 2002, Farrell et al 1999 and Roymans et
al 1999) (Table 2.4). Since there is considerable sequence homology of the 23S rRNA gene among the two pathogenic Neisseria, *N. gonorrhoeae* and *N. meningitidis*, no such species specific PCR assay has been reported so far.

**Table 2.4 : PCR systems used for analysis**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Target gene</th>
<th>Chemistry of probes</th>
<th>Primers and probes</th>
<th>Hardware (company)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG&amp;GD</td>
<td><em>cppB</em></td>
<td>TaqMan</td>
<td>HO1, HO2, CppBNB</td>
<td>RotorGene (Corbett Research)</td>
</tr>
<tr>
<td>SLGD</td>
<td><em>cppB</em></td>
<td>TaqMan</td>
<td>cppB2-1, B2-2, cppB-pr</td>
<td>ABI 7700 (Applied Biosystems)</td>
</tr>
<tr>
<td>SLGD</td>
<td>16S rRNA</td>
<td>TaqMan</td>
<td>16S-f1, 16S-r2, GO-207T</td>
<td>ABI 7700 (Applied Biosystems)</td>
</tr>
<tr>
<td>PAMM</td>
<td>16S rRNA</td>
<td>FRET</td>
<td>SL67, 16S-Rev, FL4LC</td>
<td>LightCycler (Roche Diagnostics)</td>
</tr>
<tr>
<td>LVF</td>
<td><em>cppB</em></td>
<td>FRET</td>
<td>Ngon3, -4, -5FL, -6LC</td>
<td>LightCycler (Roche Diagnostics)</td>
</tr>
<tr>
<td>AMC</td>
<td><em>cppB</em></td>
<td>FRET</td>
<td>Ngon3, -4, -5FL, -6LC</td>
<td>LightCycler (Roche Diagnostics)</td>
</tr>
</tbody>
</table>

In the initial stages of the development of PCR based assays to detect *N. gonorrhoeae*, agarose gel electrophoresis patterns generated by the amplified products were used for the fastest identification of the gonococcus (Ho et al 1992). Thereafter, the sensitivity and specificity of the PCR assays were enhanced by the radioisotope as well as non-radioisotope based hybridization assays with the oligonucleotide probes defined at the species level. Besides hybridization, the specificity of the PCR assays was improved by nucleic acid sequencing (Trees et al 2000) and by subjecting the amplified products to a second round of PCR with an internal set of primers (nested primers) (Herrmann et al 1996).

In the laboratories, the assays developed for the detection of *N. gonorrhoeae* have provided excellent specificity and sensitivity with different target genes chosen. Sensitivities as
high as detecting single bacteria directly from clinical specimens have been observed (Herrmann et al 1996). However, diagnostic techniques based on PCR have suffered from two major problems, false positives due to contamination of amplified fragments from previous PCRs (amplicons) (Farrell et al 1999) and false negatives because of inhibitors present in the clinical specimens that interfere with the PCR (Ho et al 1992). The problems of false positive reactions are now being prevented by the use of aerosol resistant tips, uracil-N-glycosylase and dUTP instead of dTTP (Crotchfelt et al 1997). The problem of false negatives has been overcome by the addition of the primers that amplify a segment of the human genome coding for a 217 bp portion of the CD45RA exon and the addition of a small amount of human genomic DNA. All the PCR negative samples when tested again with this set of primers, proved amplifiable (Liebling et al 1994). A PCR study carried out using 16S rRNA gene target, cppB target, and gyr target showed that the real-time PCR assay targeting the 16S rRNA gene is a useful confirmatory assay (Chui et al 2008). A duplex Neisseria gonorrhoeae real-time polymerase chain reaction (PCR) (NGduplex) assay targeting the gonococcal porA pseudogene and multicopy opa genes was developed. The NGduplex showed the sensitivity & specificity of 100% and 99.3% for both urogenital and throat swab specimens. In addition, the 2-target system of the NGduplex assay decreases the potential for sequence-related false-negative results and can provide simultaneous confirmation of positive results. (Goire et al 2008). A study carried out in Australia using opa-based target PCR reported that it is accurate and rapid PCR assay & can be used for the detection of N gonorrhoeae in clinical specimens. (Tabrizi et al 2005)
2.8 Typing of *N. gonorrhoeae*

Typing systems have been used in epidemiological and medico legal cases, outbreak investigations, and to monitor the distribution of antimicrobial-resistant strains and track the transmission of specific strains in a population. The typing methods developed for strain differentiation include auxotyping (A), serotyping (S), plasmid profile analysis (P), *porB* sequencing, *opa* typing, pulsed-field gel electrophoresis (PFGE), ribotyping, amplified ribosomal-DNA restriction analysis, arbitrarily primed PCR and fluorescent amplified fragment length polymorphism analysis (Janda et al 2003, Dillon et al 1993, Van Looveren, et al 1999, Palmer et al 2001). Currently, auxotyping, serotyping and plasmid analysis are the most common typing methods used for such studies.

2.8.1 Auxotyping:

Auxotyping is based on the nutrient growth requirements of strains. Gonococcal strains that have specific requirements for certain nutritional growth factors are known as auxotypes and include prototrophic (none requiring), proline-requiring and arginine hypoxanthine and uracil requiring (AHU) strains. *Neisseria gonorrhoeae* can be subdivided in 35 auxotypes. Arginine, hypoxanthine and uracil auxotypes are more likely to cause asymptomatic infection and more subtle inflammatory signs than other auxotypes (Moyes et al 1993).

2.8.2 Serotyping:

Serotyping requires the use of monoclonal antibodies directed to protein I epitopes. Serotyping for *N. gonorrhoeae* is performed using a panel of monoclonal antibodies directed against epitopes on Por (protein I) (Fig 2.12). Strains are divided into two major serogroups, Por
IA and IB strains. Subdivision into serovars is based on patterns of reaction with specific protein IA and protein IB antibodies. In total there are at least 55 serovars. (Young et al 1993)

![Diagram showing serotyping systems for N. gonorrhoea based on polyvalent antisera. Source: www.cdc.gov](image)

2.8.3 Plasmid analysis:


The discriminatory index for the various typing methods was calculated (Hunter PR et al 1988). The discriminatory power of the A, S and P typing to differentiate strains for surveillance of antimicrobial-resistant N gonorrhoeae has been demonstrated (Dillon et al 1993).
2.8.4 Opa typing

Opa-typing based on restriction fragment length polymorphism of PCR amplicons of the *opa* locus has been demonstrated to be a highly discriminatory subtyping method for the investigation of outbreaks and mapping of sexual networks (Viscidi et al 2000). One of the disadvantages of this method is the lack of interlaboratory standardization of banding patterns (and the resulting difficulty for interlaboratory comparison of data). Similar to the *opa*-typing system, PFGE, ribotyping and amplified ribosomal-DNA restriction analysis are difficult to standardize for interlaboratory comparison. PFGE has high discriminatory power for differentiating *N. gonorrhoeae* strains, but the other molecular methods are not as discriminatory (Van Looveren et al 1999). One of the disadvantages of PFGE is that some PFGE banding patterns have large molecular size fragments that do not resolve well. Fluorescent amplified fragment length polymorphism analysis has discriminatory power approaching that of *opa*-typing (Palmer et al 2001). This method produces fragments that can be sized accurately using internal standards (Palmer et al 2001).

Plasmid analysis has limited value, is being used mainly to identify strains carrying plasmid-mediated resistance. Auxotyping in combination with serotyping provides higher levels of discrimination. Used together, these three methods produce adequate discriminatory indices for national surveillance. One of the shortcomings of serotyping is that some strains are nontypable.

To resolve these serotyping problems, DNA sequencing of the *porB* gene encoding the serotyping antigen, protein I or the porin, is used as an alternative method (Viscidi et al 2000,
McKnew et al 2003). DNA sequencing has the advantage over conventional serotyping because all strains are typable.

2.9 Emerging threat of drug resistance among the gonococcal isolates

In the recent past, there has been an alarming increase in the number of isolates of *N. gonorrhoeae* resistant to commonly used drugs. The spectrum of antimicrobial agents to which resistance has been demonstrated in *N. gonorrhoeae* has also become wider. The irrational and indiscriminate use of antimicrobial agents, especially in the developing countries where almost all of the antibiotics are easily available over the counter may have contributed to this trend and the situation is expected to worsen unless appropriate steps are initiated. Thus, resistance of the gonococcus to antibiotics has been the cause of much concern in recent years and has been the subject of extensive investigation.

2.9.1 Antibiotic Resistance profile in *N. gonorrhoeae*

2.9.1.1 Pre-Fluoroquinolone era

An essential element in gonorrhea control is the availability and provision of appropriate, effective antimicrobial therapy. Effective treatment not only eradicates infection in the affected individual and prevents the development of complications; it also has an important public health benefit of shortening the duration of infection, thus decreasing transmission and eliminating reservoirs of infection. However, over the past 60 years *N. gonorrhoeae* has developed resistance to multiple classes of antimicrobials.

Sulphanilamide was introduced as an antimicrobial against *N. gonorrhoeae* as early as in 1937 (Dees et al 1937, Nelson et al 1944). However, the bacteria became resistant quickly
against sulphanilamides within a span of two years. At the same time, when sulphanilamides were given as treatment for gonorrhoea, Alexander Fleming documented the ability of penicillin to inhibit the growth of *N. gonorrhoeae* in his 1929 paper describing penicillin discovery (Fleming A 2001). Thereafter, penicillin became the choice of antimicrobial for the treatment of gonorrhoea in 1943 and remained so for decades (Van Slyke et al 1943). Susceptibility profile of *N. gonorrhoeae* against penicillin and other antimicrobials was monitored throughout the world, and *in vitro* resistance to penicillin was expressed in a uniform manner in terms of minimum inhibitory concentrations (MIC). During the initial years of treatment of gonorrhea with penicillin, all the isolates had an MIC of <0.0125 mg/l (0.02 IU/l) and were considered to be sensitive to the treatment (Whittington et al 1988, Catlin et al 1982). However, *N. gonorrhoeae* began developing low level resistance to penicillin. The MIC values of *N. gonorrhoeae* isolates gradually increased to >0.12 mg/l (Whittington et al 1988, Thayer et al 1957) and gradually most of the strains became resistant to penicillin (MIC >0.5 mg/l) (Jaffe et al 1976). Due to this increase in the MIC values, it was necessary to increase the effective dose of penicillin from 50,000 units in 1945 to 4.8 million units by 1970s (Whittington et al 1988, CDC 1989). This increase in penicillin resistance was proved to be the additive effect of multiple chromosomal mutations resulting in altered penicillin binding proteins, increase in the antibiotic efflux system and probably decrease in the antibiotic uptake from the membrane (Ison et al 1996). Chromosomal mediated penicillin resistance was found to be of low level as determined by the MIC values. Simultaneous to the development of chromosomal mediated resistance, high-level plasmid mediated resistant isolates of *N. gonorrhoeae* were also observed in various countries (Whittington et al 1988, Philips et al 1976, Ashford et al 1976). These isolates were termed as penicillinase producing *N. gonorrhoeae* (PPNG) as these harboured a plasmid having a gene of
β-lactamase, the product of which conferred resistance towards penicillin. Reports of such high level penicillin resistant isolates were documented in Africa, Asia, North America, Europe and Australia (Lind et al 1997). Due to the emergence of penicillin resistant isolates (both chromosomal and plasmid mediated) of *N. gonorrhoeae*, penicillin was no longer considered an effective treatment for gonorrhoea by 1989 and, therefore, penicillin was also prohibited in most parts of the world (Whittington et al 1988, Lind et al 1997, Workowski KA et al 2006).

A few documented reports of antimicrobial susceptibility data from India suggested a slow step-wise increase in penicillin resistance (Bhallal et al 1998, Divekar et al 1999, Ray et al 2000). Low level resistance of *N. gonorrhoeae* to penicillin was observed first in India as early as 1981 in Madras, where the first case of β-lactamase isolate of *N. gonorrhoeae* was documented (Vijayalakshmi et al 1982). Thereafter, various other reports indicated the increase in the resistance profile of *N. gonorrhoeae* towards penicillin. Most of the isolates resistant to penicillin were found to be harbouring β-lactamase producing plasmid (Kulkarni et al 1983, Moorthy et al 1984). Use of penicillin for the treatment of gonorrhoea was discontinued in India in 1990 (Ray et al 2000). As a result, penicillin resistance (both chromosomal and plasmid mediated) decreased subsequently (Ray et al 2000). Thereafter resistance towards penicillin once again showed a steep rise to 42.4 and 66.7 per cent in 2000 and 2001 respectively, along with increase in isolation of penicillinase producing *N. gonorrhoea* (PPNGs) (Ray et al 2005). Study conducted by Ray et al 2005 showed high level of penicillin resistance from Hyderabad (79%) and Chennai (62.5%), while low level of resistance (20-33%) for penicillin was observed from isolates obtained from Kolkata, Nagpur and Pune (Ray et al 2005). Other studies have also reported penicillin resistance varying from 50-80% (Sethi et al 2006, Bala M et al 2008, Kulkarni et al 2011)
Coincident with the development of resistance to penicillin, gonococci also developed resistance to several other antibiotics, including tetracycline, chloramphenicol, erythromycin and streptomycin (Curtis et al 1958, Wilcox et al 1970, Ison et al 1988, Stolz et al 1975). Tetracycline was also considered as another important antimicrobial during the pre-quinolone era. Since tetracycline was not very expensive and thus was a widely used antimicrobial, and most importantly tetracycline was given as an adjunct therapy for Chlamydia trachomatis, it was not possible to evaluate the contribution of tetracycline in the management of gonorrhoea. Same was the case with azithromycin, even though it was considered to be a more expensive alternative. Gradually through constant use of tetracycline to treat such co-infections, N. gonorrhoeae acquired low-level resistance towards this antimicrobial. High-level chromosomally mediated tetracycline resistance emerged in the 1970s along with chromosomally mediated penicillin resistance (Catlin et al 1982). Plasmid mediated tetracycline resistant N. gonorrhoeae (TRNG) emerged in 1985 in Atlanta and the Netherlands and was probably a result of the acquisition on a plasmid, a tet-M determinant from streptococcal species (Ison et al 1996, Philips et al 1976, Lind et al 1997, Martin et al 2006, Morse et al 1986).

Various reports from USA also indicated the presence of both chromosomal mediated tetracycline resistance and TRNG (Whittington et al 1988, Knapp et al 1987). In 1997, 25.6 per cent of isolates from USA were tetracycline-resistant, of which 17 per cent were chromosomally mediated and 8.6 per cent were TRNG (CDC 1998). Regional data from USA showed an increase in TRNG from less than 5 per cent in 1990 to nearly 15 per cent in 1995. In the WHO Western Pacific study, TRNG were widely but unevenly distributed. In 1998, particularly high proportions of TRNG were seen in Singapore (84%), the Solomon Islands (74%) and Vietnam (35.9%), continuing a pattern observed in earlier years (Tapsall et al 2001). In all other regions
TRNG strains were also identified in the WHO South East Asia Region, and Thailand alone accounted for about 16 per cent of isolates in 1994-1997 (Tapsall J et al 2001). An additional 55 per cent of strains had chromosomal-mediated resistance. Indonesia had particularly high rates of TRNG, and virtually all *N. gonorrhoeae* isolates show one or the other forms of resistance (Djajakusumah et al 1998). In India, decreased susceptibility towards tetracycline (28% of the isolates) was reported as early as in 1971 in Mumbai, (Moses et al 1971). Bhatta et al (1998) found 28 per cent of 50 consecutive isolates in New Delhi to be TRNG. In 1997, 10 per cent of 94 isolates from Bangladesh were TRNG (Rahman et al 2002). In 2000/2001, a study conducted by Ray et al (2005) reported high percentage resistance in three centers of India (Hyderabad, Nagpur and Pune). The other studies in India also showed a varying range (10% to 46%) of resistance (Sethi et al 2006, Bala M et al 2007, Khaki et al 2007).

2.9.1.2 Quinolone era

**Quinolones in the treatment of *N. gonorrhoeae***: In 1989, in response to the increasing frequency of isolation of penicillin, tetracycline, streptomycin and spectinomycin-resistant strains of *N. gonorrhoeae* in the United States and also throughout the world, the Centers for Disease Control and Prevention (CDC) recommended the use of broad-spectrum cephalosporins or fluorquinolones for the primary treatment of uncomplicated gonorrhea (CDC 1989). Similar policies were also adopted by various national organizations in other parts of the world. The quinolones most widely used for the treatment of gonorrhoea are second generation antimicrobials such as ciprofloxacin, norfloxacin and ofloxacin (Andriole 1998). The fourth-generation quinolones, such as trovafloxacin, have been tested for the treatment of gonorrhoea,
but still have not been included in the treatment guidelines & therefore the information on resistance to this antimicrobial is not available. It was noticed that among the quinolones, fluoroquinolones had excellent oral absorption and good tissue distribution, achieved excellent interstitial fluid levels and adequate penetration into macrophages, were free from any serious toxic side effects and induced low frequency of spontaneous single-step mutations. Due to their excellent safety and tolerability, these had become popular alternatives to penicillin and cephalosporin derivatives in the treatment of various infections including gonococcal infection. Soon fluoroquinolones were regarded to be as close as possible to the ‘ideal’ antimicrobial agent, since these possessed a broad spectrum of antimicrobial activity. By 1993, ciprofloxacin was recommended as the first line therapy to treat gonorrhoea throughout the world (CDC 1993).

Quinolone resistance profile in *N. gonorrhoeae*: Initially most of the isolates of gonococci were found to be extremely susceptible to quinolones and more importantly, fluoroquinolones. Widespread use of fluoroquinolones in last 20 years and often misuse, coupled with emerging resistance, gradually compromised their utility (CDC 1998). Since the importance of proper documentation of antimicrobial susceptibility studies was understood, data became available from all parts of the world (Dicarlo et al 1998). In most of these studies, it was evident that the resistance towards fluoroquinolones, which is almost chromosomal mediated, develops in an incremental manner. Most of these studies indicate that the initial isolates which were less susceptible towards ciprofloxacin were found to have MIC values of 0.06 mg/l, which gradually increased to 1 mg/l (such strains being referred to as intermediate resistant) and later to as high as 16 mg/l (classified as resistant isolates). Such strains were referred to as quinolone resistant *N. gonorrhoeae* (QRNG). Strains with MIC value >4 μg/l were considered as high level resistance (HLR) strains. Patients infected with these isolates showing decreased susceptibility or
intermediate resistance towards ciprofloxacin, usually responded very well to 500 mg of ciprofloxacin. Later on, a few reports of treatment failure also appeared (Ison et al 1998).

The ciprofloxacin resistant isolates were reported in mid 1980s from many parts of the world (Tanaka et al 1994, Dan et al 2004). By the end of 1992, more than 40 per cent ciprofloxacin resistant isolates were documented in Japan (Tanaka M et al 1994). Thereafter, ciprofloxacin resistant strains spread very quickly from Asia to Australia, Hawaii and North America (Gorwitz et al 1993, CDC 1995, Tapsall et al 1996, Dan et al 2004). Studies from USA also indicated a rise in ciprofloxacin resistant isolates especially in California (CDC 2004). Significant ciprofloxacin resistance emerged simultaneously from the WHO Western Pacific Region (GRASP 2008) and SEAR (Tapsall et al 2001). In these countries, it was thought that the emergence of ciprofloxacin resistance was accelerated mainly because of its use for the treatment of other diseases as well. There were two reports of increasing ciprofloxacin resistance from Bangladesh (Bhuiyan et al 1999, Rahman et al 2001).

In India, the use of ciprofloxacin, as the first-line therapy for gonorrhoea started in 1990 (Tapsall 2006). It was also included in the syndromic management in cases of suspected gonorrhoea. Resistance to norfloxacin soon appeared in 1996 from New Delhi, India (Bhalla P 1998). By the end of 2000 till now, a burst in ciprofloxacin-resistant isolates was observed in India (Bhalla et al 1998, Divekar et al 1999, Bala M et al 2007, Ray et al 2000, Ray et al 2005, Bala M et al 2010, Kulkarni et al 2011). Interestingly, with the emergence of fluoroquinolone resistant strains in India, a rapid decline of PPNGs was observed (Bala M et al 2007). Similar observation was also reported from other countries indicating a penicillinase producing plasmid curing effect on an ecological scale (Tapsall et al 2006, Fox et al 1997). In most of these studies,
the molecular basis of antibiotic resistance was not investigated. In a study from India, decrease in ciprofloxacin resistant strains was observed which may have been due to ciprofloxacin not being used for treatment of gonorrhoea in India (Bala M et al 2007). Studies from developed countries such as Australia, Canada and US suggest that quinolone resistant strains were introduced sporadically over many years. Once introduced into sexual networks, these subtypes spread and eventually achieved endemic transmission. In response to the increase in ciprofloxacin resistant isolates from throughout the world, the use of this antimicrobial to treat gonorrhoea was discontinued in early 2000s from most of the countries (CDC 2004). In 2004, CDC discontinued the use of ciprofloxacin to treat gonococcal infections (CDC 2007). The use of ciprofloxacin was continued in Europe till 2004 and was discontinued only in 2005 (Martin et al 2006, BASHH 2005). Simultaneously, the use of quinolone group of antimicrobials for the treatment of gonorrhoea was also discontinued in India (Bhalla et al 1998, Bhalla et al 2002, Ray et al 2005, Sethi et al 2006, Bala M et al 2007).

2.9.1.3. Post-quinolone era

Consequent to the increase in the resistance profile of N. gonorrhoeae towards quinolones, third-generation cephalosporins, both injectable (ceftriaxone) and oral (cefixime and cefdinir), were the only available treatment recommended by the CDC and other national organizations for the gonococcal infections (WHO 2006, Ray et al 2005, BASHH 2005, Workowski et al 2006, CDC 2007). In patients allergic to cephalosporins, spectinomycin was recommended as the drug of choice. Cephalosporins were discovered in 1945. These are known to work as other β-lactams, by inhibiting the cell wall synthesis through binding and
inhibiting the action of enzymes responsible for inserting peptidoglycan cross-linkage structures into the cell wall.

Cephalosporins are known to be important antimicrobials for the last 10 years. Despite their historic reliability, resistance to cephalosporins also started developing in Asia and later on in other regions of the world as well. The resistance towards cephalosporins was documented as early as in 1996 and then later in 2000 in Japan (Yamaguchi et al 1998, Akasaka et al 2001). Several subsequent reports from Japan also indicated much higher MIC values for cephalosporins (Ito et al 2004, Tanaka et al 2006, Yokoi et al 2007, Osaka et al 2008, Tapsall et al 2008 and WHO et al 2008). Similar results were also documented from other countries like China, Hong Kong, Taiwan, Europe, US and Africa (Ye S et al 2002, Moodley et al 2006, Dillon et al 2006, GRASP 2008, WHO 2008, Tzeli E et al 2008, Wang et al 2007). A surveillance report from India, wherein isolates collected from different laboratories of India, Bangladesh, Nepal and Sri Lanka during 1999-2001, documented significant increase in the isolates with decreased susceptibility to ceftriaxone (Ray et al 2005, Bala et al 2010). In India, Bala et al (Bala et al 2007) reported nine isolates with ceftriaxone MIC of 0.064 mg/l among the 382 isolates studied during 2002-2006. All cases were treated with ceftriaxone 400 mg and there were no treatment failures observed. Some *N. gonorrhoeae* isolates demonstrating reduced cephalosporin susceptibility also has reduced susceptibility to multiple drug classes, including quinolones, macrolides, penicillins, and tetracyclines (CDC 2005). These ceftriaxone less sensitive strains almost always exhibited resistance to quinolones or quinolones and penicillin as reported from Australia, Japan and India suggesting increasing prevalence of these multi-resistant strains in these countries (Ito M et al 2005, Bala M et al 2007, Bala M et al 2008).
2.9.1.4 Potential alternatives in the treatment of *N. gonorrhoeae* infections:

Until 1980s, there was a parallel and consistent development of the new antibacterials, which were active against most of the resistant strains of bacteria. The increasing drug resistance in almost all bacteria in the recent past, has prompted scientists to look for possible alternatives such as immunotherapy, vaccination, identification of novel targets for drugs, probiotics, *etc* (Ahmad et al 2002). Attacking virulence mechanisms rather than the whole bacterial structure offers a wide range of possibilities. Although no work has been done for *N. gonorrhoeae*, in other bacteria, targets that have been investigated include receptor sites, sortases, quorum sensing signals, Shiga toxin, and staphylococcal enterotoxinB (Zopf et al 1996, Svensson et al 2001, Scott CJ et al 2002, Dong et al 2002, Nishikawa et al 2002).

Spectinomycin can also be considered as a therapeutic option for persons with gonococcal urogenital infection who cannot tolerate cephalosporins (CDC 2006). However, it would probably remain as an alternative treatment rather than a recommended one, because high levels of resistance developed when this antimicrobial was widely used in the mid-1980s (Boslego et al 1987). Azithromycin, 2 g, taken orally has been shown to be effective against uncomplicated gonococcal infection and could be thought as an option for persons who are allergic to cephalosporins. However, concerns about the development of antimicrobial resistance to macrolides with widespread use restrict current treatment recommendations to limited circumstances. Macrolides such as azithromycin and erythromycin, have also been associated with the multiple transferable resistance efflux system (Johnson et al 2003, Xia et al 2000).
2.10 Mechanism of drug resistance

*N. gonorrhoeae*, originally highly susceptible to antibiotics can adapt to adverse conditions (Johnson et al 1988). A hostile environment in which antibiotics are present may select for the multiple changes which result in resistance and treatment failure. Mechanisms of antibiotic resistance in gonococci may be conveniently grouped as those that involve reduced access of the antibiotic to the target site and those that involve alteration of the target site itself. Access of antibiotics to the target site may be limited by reduced permeability of the cell envelope caused by changes in porin proteins; active export of antibiotics from the cell by means of efflux pumps; and destruction of the antibiotic before it can interact with the target. Alteration or deletion of the target site of the antibiotic results in a reduction of its affinity for the antibiotic. Genetically, these changes may be mediated by either chromosomal or extra-chromosomal elements (plasmids). Multiple resistance determinants may co-exist in a single organism so that the level of resistance can increase incrementally and a single strain can be resistant to a number of different antibiotics.

In gonococci, chromosomally mediated resistance is generally slow to emerge and disseminate. In *N. gonorrhoeae*, the process of genetic transformation is known to be responsible for acquiring drug resistance. But such a change is visible only if many such acquisitions of the determinant take place (Johnson et al 1988). Plasmid-mediated resistance, at present limited to penicillins and tetracyclines is transmitted by means of conjugation. This process requires the presence of a conjugative plasmid to mobilize the plasmid carrying the resistance determinants. Since not all strains possess conjugative plasmids, the rate of spread of resistance may be limited to some extent. However, conjugative plasmids are also transferable during conjugation, so that some recipient strains then become donors themselves (Johnson et al 1988). Different rates of
dissemination of extra-chromosomally mediated resistance have been observed. For example, the ‘Asian’ PPNG plasmid spread more rapidly than the ‘African’ PPNG plasmid because initially only strains carrying the former determinant also contained conjugative elements. In *N. gonorrhoeae*, plasmid-mediated resistance spreads more rapidly than chromosomally mediated resistance. Amongst non-quinolone drugs, several studies have been carried out to understand the mechanism of penicillin resistance, which has been summarized below.

2.10.1 Resistance to penicillins:

The penicillins were widely used for the treatment of gonorrhoea for many years and still are in some regions. Originally, *N. gonorrhoeae* was extremely sensitive to almost all the drugs, known so far, and treatment with 150,000 units of penicillin was effective in most instances. Later on appeared and it was thought to be associated with treatment failure (Reyn et al 1958). Increasing the recommended dose of penicillin ‘temporarily alleviated the clinical problems resulting from infection with these strains, but almost inexorably levels of resistance increased and large numbers of treatment failures again occurred, even with high-dose regimens (Sparling et al 1972, Holmes et al 1974). This was an example of step-wise accrual of chromosomal changes over a period of many years. The targets of β-lactam agents are the penicillin binding proteins (PBP)s, enzymes located in the cell envelope that participate in cell wall metabolism. Alterations in PBP-2 and PBP-1 decreased their affinity for the penicillins, and thus the susceptibility of the organism. PBP-2 is encoded by the penA locus (Sparling et al 1975). Changes in other loci such as mtr and penB produce additive effects. The mtr locus mediates resistance to a wide range of antibiotics, detergents and dyes through an active efflux system (Guymon et al 1975, Hagman et al 1995). Mutations in the penB locus, which affect a porin, result in reduced permeability of the cell envelope to hydrophilic antibiotics and other
compounds (Johnson et al 1988, Gill et al 1998). *N. gonorrhoeae* also has a *porA* 'pseudogene' which is not expressed (Feavers IM et al 1998). In contrast, *N. meningitidis* expresses two porins, *PorA* and *PorB*. The combined effect of *penA* mutations and increased expression of *mtr* is shown to increase the MIC of penicillin by 120-fold (Ison CA et al 1990). Gonococci exhibiting these changes are termed chromosomally resistant *N. gonorrhoeae* (CMRNG). Reduced susceptibility to cephalosporins, tetracyclines and other agents is also mediated by chromosomal mechanisms in the concerned genes (Phillips et al 1976, Sparling et al 1975, Bygdeman et al 1984).

In addition to chromosome mediated resistance, resistance to penicillin’s is also mediated by a plasmid-borne, inducible TEM-1 type β-lactamase. β-lactamase is known to hydrolyze the β-lactam ring of penicillins, thus inactivating them. Chromosome mediated resistance is slow and incremental, on the contrary, resistance mediated by plasmid is a single step process. Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) isolates were first described in the UK and North America in 1976, and subsequently 1 year later in South Africa. (Percival et al 1976, Robins-Browne et al 1977). These first PPNG isolates contained TEM-1-type β-lactamase plasmids, termed ‘Africa’ and ‘Asia’, encoded by the Tn4transposon (Tn2), which are responsible for the transfer and dissemination of high-level penicillin resistance among gonococci. (Dillon 1987). These gonococcal plasmids appear to have been created by the direct acquisition of plasmids from other Gram-negative bacteria. Although the same TEM type of β-lactamase was present in both instances, the gene was carried on plasmids of different sizes, which became known as the ‘African’ and ‘Asian’ plasmids. Transmission of the resistance by conjugation required the presence of another mobilizing plasmid, which was already present in the Asian PPNG when it was first isolated, but was not found in the African strains until 1981.
Lactamase production (PPNG) and chromosomal changes (CMRNG) can co-exist in the same isolate. This is relevant because of the clinical use of penicillins in combination with β-lactamase inhibitors. These substances, such as clavulanic acid and sulbactam, prevent the β-lactamases from inactivating the penicillins. Combinations such as amoxicillin/clavulanic acid are widely used to treat other infections. In theory, and sometimes in practice these represent an effective oral therapy for PPNG infections, but more commonly single-dose regimens of penicillin/inhibitor combinations have failed (Lawrence et al 1985). This appears to be due to PPNG strains having a high frequency of underlying intrinsic or chromosomally mediated penicillin resistance. Chromosomally mediated resistance can be measured reliably only after the organism is ‘cured’ of its plasmid and the MICs reassessed (Tapsall et al 1987).

The isolates are sequentially grouped into mutually exclusive categories according to Clinical and Laboratory Standards Institute (CLSI) guidelines as follows:

1. Penicillinase-producing \( N.\ gonorrhoeae \) (PPNG) (β-lactamase positive)

2. Tetracycline MIC, <16 µg/ml; plasmid-mediated tetracycline-resistant \( N.\ gonorrhoeae \) (TRNG) (β-lactamase negative; tetracycline MIC, ≥16 µg/ml)

3. PPNG-TRNG (β-lactamase positive; tetracycline MIC, ≥16 µg/ml); chromosomally mediated penicillin-resistant \( N.\ gonorrhoeae \) (non-PPNG and non-TRNG; penicillin MIC, ≥2 µg/ml; tetracycline MIC, <2 µg/ml)

4. Chromosomally mediated tetracycline-resistant \( N.\ gonorrhoeae \) (non-PPNG and non-TRNG; penicillin MIC, <2 µg/ml; tetracycline MIC, ≥2 µg/ml)
5. Chromosomally mediated penicillin- and tetracycline-resistant *N. gonorrhoeae* (CMRNG) (non-PPNG and non-TRNG; penicillin MIC ≥2 μg/ml; tetracycline MIC, ≥2 μg/ml).

2.10.2 Resistance to quinolones:

The prevalence of resistance to these fluoroquinolone drugs has developed incrementally over a number of years and multiple chromosomal changes are involved. Ciprofloxacin, a fluoroquinolone, targets bacterial DNA gyrase and DNA Topoisomerase IV. Two basic mechanisms of resistance have been identified (Wiedemann *et al.*, 1994):

1. Alteration of the molecular targets of the action of fluoroquinolones, and

Fluoroquinolones target bacterial topoisomerases, enzymes ubiquitously found in all cells and known to alter the topological state of the DNA. Four topoisomerases have been identified in bacterial species. Fluoroquinolones do not inhibit either topoisomerase I or III but have a high affinity for DNA gyrase (a topoisomerase II) and topoisomerase IV. Gyrases control DNA supercoiling and relieve topological stress arising from the translocation of the transcription and replication complexes along the DNA. Topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication. Since both enzymes are required for cell growth and division, it is not surprising that fluoroquinolones are bactericidal. However, fluoroquinolones do not simply eliminate topoisomerase functions, but the trapping of gyrase and topoisomerase IV on DNA probably leads to the lethal release of double-strand DNA breaks (Drlica *et al* 1997). DNA gyrase introduces negative supercoils into the DNA molecule. The protein wraps the DNA into a positive supercoil. Then one region of the duplex is passed through the other via a DNA breakage and rejoining. Binding of a molecule of ATP drives this super coiling reaction, with
ATP hydrolysis serving to reset the enzyme for a second round of catalysis. In the absence of ATP, the gyrase enzyme removes one negative supercoil from the DNA. The active form of the enzyme is a hetero-tetramer (A2B2) encoded by gyrA and gyrB genes (Chatterji M et. al. 2000). Like DNA gyrase, topoisomerase IV is also a tetramer composed of two subunits each of parC and parE gene product (par stands for partition). The product of nearby parF may facilitate DNA dependent membrane binding of topoisomerase IV. Unlike DNA gyrase, topoisomerase IV does not wrap DNA around itself. This seems to be the principal difference between the two enzymes, since removal of a portion of gyrase A protein converts gyrase into an enzyme that has a strong decatenating activity, much like topoisomerase IV (Drlica et al. 1997). Gyrase and topoisomerase IV from Staphylococcus aureus and Escherichia coli have been purified and studied for sensitivity to fluoroquinolones (Blanche et. al. 1996). These studies revealed that the super coiling activity of S. aureus gyrase was at least 500-fold less sensitive to ciprofloxacin than that of E. coli gyrase and at least six-fold less sensitive than is the decatenating activity of the S. aureus topoisomerase IV. These data strongly support the assertion that topoisomerase IV is the primary target of ciprofloxacin in S. aureus. The decatenating activity of topoisomerase IV from S. aureus is only half as sensitive as that of topoisomerase IV from E. coli, suggesting that the differences between the gyrase molecules accounts for most of the differences between the two organisms. It is now clear that bacteria contain two topoisomerase targets of the fluoroquinolones.

In some species, such as E. coli and N. gonorrhoeae, the primary target is the DNA gyrase enzyme; in other bacteria, such as S. aureus and Streptococcus pneumonia, the primary target is generally topoisomerase IV. Since the two enzymes have different functions, it is likely that bacteria will differ in their response to the fluoroquinolones according to which enzyme is the primary target. Most of the studies done on E. coli demonstrate that the effect of fluoroquinolones
on the gyrase enzyme is to disrupt the DNA breakage-reunion reaction, thus inhibiting DNA supercoiling. If a reaction between gyrase, DNA and a fluoroquinolone drug is terminated by the addition of the sodium dodecyl sulphate, the DNA is broken in both the strands and the gyrA subunits of the enzyme are attached to 5' phosphates at the break site via tyrosine 122, the active site tyrosine. The ability of fluoroquinolones to stabilize this so-called ‘cleavable complex’ between gyrase and DNA seems to be central to their bactericidal effects. Topoisomerase IV is able to decatenate DNA before the completion of a round of replication, whereas gyrase seems to decatenate only after the round is finished.

Mutations in any one of the targets might be sufficient for acquisition of fluoroquinolone resistance. However, studies with laboratory derived mutants, using in vitro generated mutants, have shown that highly resistant clinical bacterial isolates possess more than one mechanism for antibiotic resistance. Mutations in both gyrA and parC have been shown to be common in gonococcal isolates highly resistant to fluoroquinolones and the presence of multiple mutations contributing to fluoroquinolone resistance seems to be rule rather than the exception (Belland et. al., 1994). DNA sequence analysis of the gyrA genes from resistant gonococcal isolates revealed that all mutations were located within a highly conserved region, the so called ‘quinolone resistance-determining region (QRDR) (amino acids 55 to 110 of the gonococcal gyrA protein)’ which is located at the 5' terminus of gyrA close to codon 130 coding for the active site tyrosine (corresponding to the Tyr-122 of E. coli) involved in DNA strand breakage and rejoicing. These mutations cause a simultaneous increase in the resistance towards all fluoroquinolones, but not to unrelated compounds. A similar QRDR region has also been shown to exist in the parC gene (amino acids 66 to 119 of the gonococcal parC protein) (Belland et. al., 1994). Within the gyrA subunit of N. gonorrhoeae, mutations of one or two codons, serine 91 and aspartic acid 95, give
the greatest reduction in susceptibility. When both sites undergo mutation, levels of resistance
can be two- or three-fold higher than when only one position mutates. In *N. gonorrhoeae*, as
gyrase is the primary target, *parC* mediated resistance is detectable only in *gyrA* mutants and a
high fluoroquinolone concentrations. Mutations in the *gyrB* confer low-level resistance to
nalidixic acid; high-level quinolone resistance is associated with mutations in the QRDR of *gyrA*
gene of the DNA gyrase along with the complementary mutations in the *parC* gene of the
topoisomerase IV. Resistance to fluoroquinolones could also result from reduction of quinolone
accumulation inside the bacterial cell. Number of point mutations has been characterized by
various investigators from different geographic locations. Since point mutations require
alteration in only a single nucleotide, therefore it is possible that more changes will be found
once larger and more diverse gonococcal populations are screened. The possibility of additional
point mutations with additive deleterious effects on gonococcal susceptibility may further
decrease the clinical efficacy of this group of fluoroquinolones in treating gonorrhoea.

There are two subunits of DNA gyrase and Topoisomerase IV: *gyrA* and *gyrB*, and *parC*
and *parE*, respectively. Bacterial changes in permeation routes or the effects of efflux
mechanisms that remove fluoroquinolones from inside the bacterium (Figure 2.13 A & B), or
both, can reduce the access of the antibiotic, increasing resistance; whereas spontaneous
mutations in the chromosomal genes, and rare cases of plasmid-mediated resistance promote
bacterial resistance to quinolones (File et al 2000). A reduction in the porin diffusion channels
in the outer membrane of the Gram negative bacteria cell envelope, which lead to the
cytoplasmic membrane, or an increase in the efflux mechanism activity, may decrease drug
permeation or its ability to reach bacterial targets.
Fig 2.13 A & B: Mechanism of resistance to fluoroquinolones
Source: biology.kenyon.edu

In 1998 Ison et al., a surveillance project in the United Kingdom established that ciprofloxacin, replacing penicillin, was still a highly effective agent in treating gonorrhea, but the identified drift in susceptibility most likely resulted from the increased usage of it (Ison et al 1998). This drift was attributed to *N. gonorrhoeae* isolates exhibiting chromosomal and/or
plasmid-mediated resistance to penicillin, thus using ciprofloxacin instead. Double mutations in 
gyrA (the DNA gyrase gene) have been correlated with high-level resistance (Kocagoz et al.,
1996) in isolates with MICs greater than 1mg/L, along with additional mutations in some 
isolates in the topoisomerase gene, parC.

Results of a study indicated that almost half of the N. gonorrhoeae resistant isolates were 
penicillinase producers, while the rest displayed increased resistance to penicillin; plasmid-
mediated high-level resistance to tetracycline was not observed (Ison et al., 1998). Mutations in 
the quinolone resistance-determining region (QRDR) of the gyrA gene did result in ciprofloxacin 
resistance, and certified that through a molecular-based technique, gyrA and parC could act as 
Dewi et al 2004, Tanaka et al 2004). The efflux mechanism have been associated with 
fluoroquinolones resistance in N. gonorrhoeae (Dewi et al 2004, Poole et al 2000 & 2005) and 
through acquiring the mtrRCDE operon in transformants, N. gonorrhoeae's resistance to 
hydrophobic agents is mediated (Warner et al 2008) (Fig 2.14).

Fig 2.14: Efflux pumps mechanism in N.gonorrhoeae

Source: http://www.medscape.com

### 2.10.3 Resistance to cephalosporin antibiotics:

Altered gonococcal susceptibility to cephalosporin antibiotics is chromosomally mediated and is due to the same changes that account for decreased penicillin susceptibility (Johnson et al 1988, Bygdeman SM et al 1984). There is cross-resistance between penicillin’s and early generation cephalosporin’s such as cefuroxime (Bygdeman et al 1984, Rice et al 1986). However, this is not the case for the later generation cephalosporins such as ceftiraxone and cefixime. Not all cephalosporins are hydrolyzed by the TEM-1 type β-lactamase, and therefore, some of these compounds are active against PPNG. Other β-lactamases (cephalosporinases), which are constitutively expressed by many other Gram-negative genera, have thus far not been detected in gonococci and there has been no transfer of genetic material encoding production of extended spectrum β-lactamases into pathogenic *Neisseria*. If such an event occurs, it would be
devastating for gonorrhoea treatment programmes that rely heavily on the third-generation cephalosporins. In the past five years, gonococci with decreased susceptibility to ceftriaxone have been reported though the mechanism of resistance has not been fully understood (Akasaka et al 2001). Recent data also suggest that the emergence and spread of cephalosporin resistance gonococci is quite similar to the data showing the emergence of quinolone-resistance strains (Tapsall et al 2009).

2.11 Antibiotic sensitivity pattern of *N. gonorrhoeae*, in India

Despite a high prevalence of gonorrhoea and an increasing incidence of resistant gonococcal isolates from within India, no standardized monitoring of the antimicrobial susceptibility profile has been carried out so far. Clinicians in India started reporting cases of therapeutic failure to penicillin in the late sixties. Hence a long term surveillance of *in vitro* resistance to penicillin, tetracycline and cotrimoxazole was undertaken at the STD laboratory in various hospitals throughout India. From 1969 to 1997, nearly 1,250 isolates of *N. gonorrhoeae* were tested for resistance to penicillin (WHO GASP Newsletter, 1998). It was observed that the percentage of resistant strains increased from 9% in 1970 to 22% in 1973. By the year 1976, the resistance was observed in 36% of the isolates and by 1985 nearly 50% of the isolates were resistance to penicillin. Thereafter, there has been a slight decline in the percentage of strains that are penicillin resistant. During this time, no PPNG strain was isolated or identified, indicating that in India at least the mechanism of resistance was chromosomal. In the last few years, PPNG strains appear to be a minority and the commonest penicillin resistance strains are CMRG. In India, the first report of PPNG appeared from Chennai in 1981 (Vijayalakshmi, 1982). Since then there have been some reports of the isolation of PPNG (Sharma et. al., 1984).
and its increasing incidence from North India (Bhujwala et. al., 1987), surveillance carried out in an STD Teaching and Research Centre, Safdarjung Hospital, New Delhi, from 1990 to 1995 did not detect any such isolate confirming an earlier report from Delhi itself (Agarwal et. al., 1992). In 1996, the same group isolated two PPNG isolates. Parallel to this study, another investigation revealed 5.3% of their isolates to be PPNG (Bhalla et. al., 1998). A similar situation was observed with 1141 isolates tested for resistance to cotrimoxazole. In 1997 only 6.2% of the isolates tested for resistance to the drug, but the percentage of resistant isolates doubled within a period of two years and by 1986 almost 40% of the isolates were found to be resistant to cotrimoxazole (Bhujwala et. al., 1987; WHO GASP Newsletter, 1998). During the period 1971 to 1985 a total of 559 strains were tested. In 1971 the resistance to tetracycline was only 6%; by 1986 it had increased to 20% and by 1986 to 60%. In 1997, only a few isolates were TRNG, though tetracycline resistance was common (Bhujwala et. al., 1987; GASP 1998). Tetracycline was commonly used for treatment of gonorrhea as it was inexpensive and readily available even though it was not a treatment of choice for gonorrhea. This and the fact that tetracycline could be used for the treatment of non-gonococcal urethritis caused by C. trachomatis, could account for such a high level of resistance observed in India. Chromosomal resistance for penicillin and tetracycline in gonococci may result in therapeutic failure but this can usually be overcome by increasing the dosage. In response to the increasing frequency of isolation of penicillin- and tetracycline-resistant isolates of N. gonorrhoeae in India as in other parts of the world, the WHO recommended (1989) the use of broad-spectrum cephalosporin’s or fluoroquinolones as the first line therapies for gonorrhea. Ciprofloxacin was being used as the first-line therapy for gonorrhea in India since 1990. It is also included in the syndromic management in cases of suspected gonorrhea. Resistance to ciprofloxacin appeared first in 1996 from Safdarjung
Hospital, New Delhi. In India, the use of ciprofloxacin, as the first-line therapy for gonorrhoea started in 1990 (Tapsall et al 2006). Ciprofloxacin was also included in the syndromic management in treatment of suspected cases of gonorrhoea. Resistance to norfloxacin soon appeared in 1996 from New Delhi, India (Bhalla et al 1998). *N. gonorrhoeae* strains isolated from Mumbai in 1999 showed 15.2% resistance to ciprofloxacin (Divekar et al 1999). Later quinolone resistant *N. gonorrhoeae* (MIC >1 mg/ml) in Delhi was reported to be 3.5%, 13.6 % and 22% in 1996, 1997 and 1999 respectively (Ray et al 2000) but another study carried out during the year 2000 in Delhi reported 75% resistance of *N. gonorrhoeae* to ciprofloxacin. (Bhalla P et al 2002). Later Manju Bala et al found that 67.3% and 28.2% strains were resistant and less sensitive, respectively, to ciprofloxacin (Bala M et al 2003). In a study carried out in Chandigarh also showed 78% *N. gonorrhoeae* isolates resistant to ciprofloxacin, of which 16 (35%) isolates showed high level resistance to ciprofloxacin (MIC>8 mg/ml).( Sethi et al 2006). In the last few years, an increase in ciprofloxacin resistance (95-100%) has been reported from different parts of India (Khaki et al 2007, Bala M et al 2010, Kulkarni et al 2011). Only one study has been carried out on ciprofloxacin resistant muttaions in gyrA and parC in the gonococcal isolates obtained from Delhi, India (Chaudhry et al 2002).

2.12 Rapid methods for Antibiotic profiling - an ultimate goal

The development of resistance to antimicrobial agents has posed a significant problem in the treatment and control of gonorrhoea. With the increasing emergence of antimicrobial resistance seen with *N. gonorrhoeae*, there is a need to simplify and standardize the *in vitro* antimicrobial susceptibility testing of *N. gonorrhoeae*. Moreover, the choice of antibiotics must take into account the data generated by laboratory based surveillance of gonococcal susceptibility. This surveillance
would be useful not only in deciding the correct treatment but also in helping to detect the emergence of new antibiotic resistant traits and to monitor the effectiveness of prescribed treatment. The strategy devised by the WHO has been to initiate a global Gonococcal Antimicrobial Susceptibility Programme (GASP), whereby information is obtained on gonococcal susceptibility patterns. This information is used then to develop and implement appropriate and effective treatment. To make this information reliable, the laboratories need to adopt and use standardized laboratory procedures and take part in external quality assessment programme. The disk diffusion and the agar dilution method have been the standard method recommended for quantitative monitoring of such isolates. However, these are usually laborious and impractical for routine use. The E-test method is a new technique that allows for the direct determination of minimum inhibitory concentration (MIC). It consists of a plastic carrier strip with a predefined continuous exponential antibiotic gradient immobilized on one side. It is an attractive alternative to the earlier methods for gonococcal antimicrobial susceptibility testing. PCR has played a role in determining antibiotic susceptibility data for gonorrhoea. PCR assays have been designed for the detection of antibiotic resistance genes in *N. gonorrhoeae*, such as analysis of mutations in the *gyrA* and *parC* genes for the ciprofloxacin resistant isolates. Molecular technology can provide an alternate procedure to the culture method for the surveillance of the antimicrobial susceptibility of *N. gonorrhoeae* and thus characterize *N. gonorrhoeae* isolates prevalent in India. Since the genetic basis of several antibiotic resistance mechanisms is known and the nucleic acid-based assays for them have been described, it is believed that the single tube assays using techniques like hybridization capture for the detection of wide range of resistance genes would allow their application beyond the laboratory directly in clinical practice (Piatek *et al.*, 2000). Such methods:
would facilitate an early treatment of this infectious disease and at the same time control the transmission of HIV infection among the infected individual.

2.13 HIV & Gonorrhoea

There is a strong association between the occurrence of HIV infection and the presence of other STD. During the last decade, from different epidemiological, clinical and biological and in vitro studies, overwhelming evidence has accumulated that both ulcerative and non-ulcerative STD promote HIV transmission by augmenting HIV infectiousness and HIV susceptibility via different biological mechanisms (Fleming et al 2001). Gonorrhoea can affect the anus, cervix, penis and throat. Untreated gonorrhoea can make a person with HIV more infectious, as gonorrhoea increases the number of HIV-infected cells in the genital area and in the mucous membranes of the mouth and throat. A HIV-negative person having gonorrhoea is more likely to acquire HIV if exposed to the virus (Michael Carter 2011). Prospective epidemiologic studies carried out showed that gonorrhoea in women & men is temporally associated with increased risk of acquiring HIV infection and virologic studies showed that gonococcal urethritis is associated with increased urethral shedding of cell associated HIV DNA in urethral secretions & with increased concentrations of HIV in semen which facilitated HIV transmission. Cohen et al in 1997 showed that treatment of urethral infection resulted in a dramatic decline in the HIV semen plasma viral load (Cohen et al 1997). A study conducted in Africa showed that HIV-1 shedding decreased from 42% to 21% in women treated for sexually transmitted disease including gonorrhoea. (Ghys et al 1997). GC-infected women have more endocervical CD4+ T cells providing more targets for HIV (Levine et al 1998). Several mechanisms of enhancement of HIV transmission by GC have been proposed. GC infection can enhance HIV transcription by activating HIV long-terminal repeat in transformed T cells (Chen et al 2003). In addition, GC
infection enhances HIV infection in monocyte-derived dendritic cells (MDDCs) (Zhang et al 2005). It has also been shown that infection with *N. gonorrhoeae* increases the leukocyte concentrations in the genital tract & thus increases HIV-1 shedding (Johnson 2008). The antimicrobial peptides, such as human defensin 5 and 6, promote HIV infectivity in vitro and are induced by GC infection have also been reported (Klotman et al 2008). Plasma and rectal viral load were correlated, and rectal *N. gonorrhoeae* did not increase the likelihood of detecting HIV in the rectal secretions in MSM population, including those with low or undetectable plasma viral load. Suppressing plasma viral load is likely to reduce risk of HIV transmission to insertive partners (Kelley et al 2011).