Chapter 4
MATERIALS AND METHODS
MATERIAL AND METHODS

4.1 Materials

4.1.1 Chemicals and Biochemicals

Fine chemicals and biochemicals were purchased from the following standard commercial sources such as: Sigma Chemical Co., St. Louis, USA; Genetix, Delhi, India; Bangalore Genie India Pvt. Ltd., Bangalore, India; Himedia laboratories, Mumbai, India Ltd., Mumbai, India; Becton Dickinson, USA. Agarose, low melting agarose & ethidium bromide were purchased from Bangalore Genie India Pvt. Ltd., Bangalore, India. DNA ladder & loading dye were purchase from Fermentas International Inc. US). Culture media & chemicals including GC Agar Base, Isovitalex, GC supplement, Hemoglobin powder, VCNT, Sodium chloride, Nutrient Broth, Glycerol were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India; BBL, Cockeysville, MD, USA). Amplified PCR products were eluted from the agarose gel by using the Qiagen (Qiagen GmbH, Hilden, Germany).

4.1.2 Antibiotics

Nitrocefin-containing disc (Cefinase) were purchased from BBL, Cockeysville, MD, USA, ciprofloxacin 5µg discs, ofloxacin 5µg discs, enoxacin 10µg discs, gatifloxacin 5µg discs, lomefloxacin 10µg discs, norfloxacin 5µg discs were purchased from Hi media laboratories Mumbai, India. Other antibiotics tested were: penicillin 10 units, tetracycline 30µg, spectinomycin 100µg, ceftriaxone 30µg, cefixime 5µg. The E-test strips ciprofloxacin, ofloxacin, gatifloxacin and norfloxacin, penicillin, tetracycline, spectinomycin, ceftrixone, cefixime were purchased from AB BIODISK (AB BioDisk, Solna, Sweden). Lomefloxacin E-test strips were purchased from Hi
Media Laboratories, India. Enoxacin antibiotic powder of stated potency was purchased from Sigma, Sigma-Aldrich Co, USA. Concentrations of enoxacin tested ranged from 0.001 to 128 μg/ml.

4.1.3 Enzymes, Primers & Solutions for purification of PCR products

Taq DNA Polymerase was purchased from Bangalore Genei India Pvt. Ltd., Bangalore, India. Primers were designed as per the primers mentioned by Tanaka et al (2000) and purchased from Bioresource, Biotech Pvt Ltd, India. The list of primers used in this study is shown below (Table 4.1). All the solutions required for the purification of PCR products were supplied by the manufacturer (Qiagen GmbH, Hilden, Germany)

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyr A</td>
<td>gyrA-F</td>
<td>CGGCGCGTACTGTACGCGATGCA</td>
</tr>
<tr>
<td></td>
<td>gyrA-R</td>
<td>AATGTCTGCCAGCATTTCATGTAGA</td>
</tr>
<tr>
<td>par C</td>
<td>ParC-F</td>
<td>ATGCCGATATGGGTGGAC</td>
</tr>
<tr>
<td></td>
<td>ParC-R</td>
<td>GGCAAACAGCAATCCGGCA</td>
</tr>
<tr>
<td>mtrR</td>
<td>MtrA-1-F</td>
<td>AAAACGCCATTATGAGAAAA</td>
</tr>
<tr>
<td></td>
<td>MtrA-2-R</td>
<td>TTACCAAAAGGCTTATAT</td>
</tr>
<tr>
<td></td>
<td>MtrA-3-F</td>
<td>CGGATGCCGACGGTACGAC</td>
</tr>
<tr>
<td></td>
<td>MtrA-4-R</td>
<td>TGGCGTAAAAGGGTTCCAAG</td>
</tr>
</tbody>
</table>
4.1.4 Bacterial Strains

*N. gonorrhoeae* isolates were isolated from male and female patients attending sexually transmitted disease clinic at Safdarjang hospital Delhi, NARI clinics, Pune & from institutional STI-operational research project carried out at government hospital at Mumbai, Hyderabad and Nagpur.

4.1.5 Reagents for identification tests & media for growth of *N. gonorrhoeae*

Crystal violet, Gram’s iodine, Decolourization solution, Safranin was purchased from Hi media laboratories, Mumbai, India.

Oxidase discs & Hydrogen peroxide pellets (Himedia laboratories, Mumbai, India ), API detection system (Biomerieux ,SA) ; Phadebact® Monoclonal GC test (Boule Diagnostics AB, Hudding, Sweden), Gonocheck II test (E-Y Laboratories, San Mateo, Calif), Serotyping-Phadebact GC serovar test (Boule Diagnostics AB, Hudding, Sweden), Auxotyping - Alanine Arginine, Biotin, Cysteine HCl, Fe(NO₃)₃, Glutamine, Glycerol , Glucose, Glutamate, Histidine, Hypoxanthine, Lactate, Lysine HCl, Methionine, Oxaloacetate, Pantothenate Ca²⁺ Proline, NAD, Sodium acetate, Serine, Thiamine HCl , Thiamine pyrophosphate, Uracil (Hi media Laboratories, Mumbai, India).

All the media were sterilized by autoclaving at 15 lbs/in² at 121°C for 15 mins. All the reagents required for serotyping were supplied by the manufacturer.
4.2. Methods

4.2.1 Collection of specimens

Clinical specimens were collected from male and female patients attending sexually transmitted disease clinic at Safdarjung hospital Delhi, NARI clinics, from institutional STI-operational research project carried out at government hospital at Mumbai, Hyderabad and Nagpur. Two urethral / cervical swabs were collected from male and female patients with symptoms of urethral / cervical discharge. One swab was directly inoculated on to Modified Thayer Martin selective agar (Appendix I) in Pune, Mumbai, Hyderabad, and Nagpur and on selective media with saponin lysed blood agar with VCNT inhibitors & chocolate agar with Columbia agar base (Appendix I) at safdarjung hospital, New Delhi. The plates were incubated at 37°C in a 5% CO₂ with 45% moisture. The second swab was used for microscopic examination.

4.2.2 Microscopic identification of *N. gonorrhoeae*

Before inoculating the urethral /cervical/vaginal specimens collected from male & female patients directly onto the growth medium, a smear for microscopy was made. To obtain a thin and homogeneous film, the swab was rolled onto a clean slide and the smear was allowed to dry before it was Gram stained. With the smear side up, the dried smear was fixed by passing the slide rapidly three times over a flame. Overheating was avoided as it would distort the cellular morphology. The fixed smear was then covered with crystal violet for 30 secs. The excess stain was poured off and the slide was washed with water. Gram’s iodine solution was applied for 30 secs followed by decolourization solution and washing with water to stop the decolourisation. Thereafter, the sample was counterstained with safranin for 30 secs, washed the slide with running water and gently blotted the slide with absorbent paper. Using a bright-light microscope and immersion oil, the slide
was examined under the 100x objective for the presence of pink coloured gram-negative diplococci and polymorphonuclear leukocytes (Fig 4.1) (Murray et al 2007)

![Gram stain showing gram negative intracellular diplococci](image)

**Fig 4.1** Gram stain showing gram negative intracellular diplococci

### 4.2.3 Primary culture of *N. gonorrhoeae* on Modified Thayer Martin medium, Saponin lysed blood agar plus GC supplements and VCNT inhibitors

The inoculation of Modified Thayer Martin medium, Saponin lysed blood agar plus VCNT inhibitor plates with swabs was carried out aseptically for the presence of *N. gonorrhoeae* in the clinical specimens. A swab was inoculated onto a 1 cm circular area of the agar plate by rubbing the whole surface of the swab, including the tip, onto the inoculum site. A sterile nichrome wire, bent at angle of 30°, was then used to streak out the inoculum over the surface of the plate. Inoculated plates were immediately incubated at 37°C in 5% CO₂ and 45% moisture (Fig 4.2). This was provided by placing the plate in a candle extinction jar with a white, unscented, nontoxic candle. The jar was then placed in the incubator at 37°C. The humidity was maintained by placing wet paper towels. Plates were then examined after 18 to 24 hours for the growth of *N. gonorrhoeae* and the colonies were identified (Fig 4.3) (Murray et al 2007)
Fig 4.2 Candle extinction jar with inoculated MTM agar plates. Only white candles must be used; colored ones may inhibit growth

Fig 4.3 Growth of *N.gonorrhoeae* on modified Thayer Martin medium
4.2.4 Presumptive identification of gonococcal isolates

When *N. gonorrhoeae* was suspected in a primary culture, a presumptive identification was made by performing a Gram Stain, oxidase test & super oxal test. (Murray et al 2007)

4.2.4.1 Gram Staining

Gram staining was performed on the single suspect colony from the primary culture to confirm if the organism cultured was a Gram-negative diplococcus. This was achieved by emulsifying a single colony in a small drop of saline on a glass slide. A smear was prepared which was allowed to dry, then heat-fixed and Gram-stained as described above (4.2.2). A 24 hour culture showed typical Gram-negative diplococci. (Murray et al 2007)

Controls: Positive Control: *Staphylococcus aureus*: 25923

Negative Control: *Escherichia coli*: 35218

4.2.4.2 Oxidase test

A loop full of test culture was placed on an oxidase disc (Hi-Media, Mumbai). The disc was observed for appearance of purple colour within 30 seconds indicates positive test and the observations recorded (Fig. 4.4). (Murray et al 2007)

Control: An uninoculated disc was used as negative control and oxidase disc inoculated with *N. gonorrhoeae* ATCC-49226 was positive control.

![Fig 4.4 Oxidase test: Positive reaction (purple Colour) - *N. gonorrhoeae*](image)

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4.2.4.3 Super oxal test

The catalase test was done by transferring a small portion of the culture with a clean glass rod on to a slide with 3% hydrogen peroxide (H₂O₂), which is placed under cover of a petri plate to avoid aerosols. If the bacteria produce catalase, it will split hydrogen peroxide and oxygen gas will be evolved. The evolution of gas causes bubbles to form and is indicative of a positive test. (Collee et al., 1989)

Control: An uninoculated slide having H₂O₂ drop on its surface is used as negative control. Slide with 3% H₂O₂ and N. gonorrhoeae 49226 standard strain culture on glass slide was used as positive control.

Fig 4.5: Superoxal test showing positive and negative reaction

4.2.5 Definitive identification of N. gonorrhoeae

Definitive identification could be performed by a number of methods. Colonies which were oxidase-positive, super oxal positive and microscopically resemble Gram-negative diplococci were subcultured (to ensure a pure culture) onto a Thayer Martin medium, & saponin lysed blood agar (LBA) medium which contained 0.5% glucose. The cultures were then incubated overnight at 37°C in air containing 5% CO₂. (Murray et al. 2007)
4.2.5.1 Rapid Carbohydrate utilization test (RCUT) performed on isolates at Safdarjung hospital

The overnight growth on the LBA medium (Appendix I) was subcultured in 1.5 ml buffered salt indicator solution (Appendix I) such that the solution contains approximately $10^9$ organisms per ml. This was prepared by suspending two full 3 mm loopfuls of culture into the BSIS and mixing well with a micro pipette. With the same micro pipette, 100 ul of the bacterial suspension was delivered into each of the five wells of a microtitre plate (or small tubes) which were labeled horizontally across the plate. 25 ml of the appropriate 10% carbohydrate solution was then added to each of the wells containing glucose (G), lactose (L), maltose (M), and sucrose (S). No carbohydrate was added to the control well labeled ‘C’. The plate was tapped gently and then incubated at 37°C in air. Reactions were read after 2 to 4 hours of incubation. (Moyes A et al 1994)

4.2.5.2 API system performed on all isolates isolated & received at NARI: (API NH BioMérieux).

A heavy suspension (McFarlands 4) of pure culture was prepared in 0.9 % saline. The suspension was inoculated into the 13 test wells, which included a pencillinase well, four carbohydrate utilization wells (glucose, fructose, maltose and sucrose) and eight biochemical wells (L-ornithine (ornithine decarboxylase), urea (urease), 5-bromo-3-indoxyl caprate (lipase), 4-nitrophenyl phosphate (alkaline phosphatase), 4-nitrophenyl β-d-galactopyranoside (β-galactosidase), proline 4-methoxy-β-naphthylamide (proline arylamidase), γ-glutamyl 4-methoxy-β-naphthylamide (γ-glutamyl transferase), 1-tryptophan (indole)). Following 2 h incubation at 35 °C the profile was recorded (Fig 4.6). (Alexander et al 2005)
4.2.5.3 Gonocheck-II (EY laboratories, San Mateo, Calif)

Each test kit consisted of a Gonocheck-II tube (for species differentiation) and a tube of EY-20 (diazonium salt derivative). Kits were removed from refrigeration and allowed to come to room temperature before use. The chromogenic substrates were rehydrated with five drops of PBS into which five to seven colonies were emulsified. Tubes were incubated at 37°C for 30 min. Following incubation a colour change to either blue (hydrolysis of 5-bromo-4-chloro-3-indolyl β-galactoside) or yellow (hydrolysis of γ-glutamyl-β-nitroanilide) was recorded. In the absence of a colour change the primary lid was removed, the secondary lid replaced and the tube inverted. If a red colour (hydrolysis of l-proline 4-methoxynaphthalamide) was observed this was identified presumptively as *N. gonorrhoeae* (Fig 4.7). (Dillon et al 1988)

Fig: 4.6 API strip used for identification of *N. gonorrhoeae*

Fig: 4.7 Gonocheck II test showing positive and negative reaction
A quality control test of Gonochek-II was performed at the time of receipt of the product according to the manufacturer instructions.

4.2.5.4 Phadebact system performed on all isolates isolated & received at NARI:

The Phadebact Monoclonal GC OMNI test kit contained one vial of gonococcal control reagent, one vial of gonococcal OMNI reagent, and 40 disposable slides. The control reagent contained rabbit nonimmunoglobulin bound to nonviable staphylococci. The OMNI reagent contained mouse monoclonal antibodies IA and IB bound to nonviable staphylococci. The (gonococcal) OMNI reagent is a defined mixture of mouse monoclonal antibodies to different epitopes on a gonococcus-specific membrane protein, protein I.

A few colonies were removed from the primary or subculture plate to make a light suspension in 0.9% saline (pH 7.4), equivalent to a 0.5 McFarland standard, according to the directions of the manufacturer. No attempt was made to ensure pure or viable cultures, since OMNI tests may be performed on nonviable cultures. The cotton plugged test tubes (12 by 75 mm) containing the suspensions were heated in a boiling water bath for 5 min and then cooled to room temperature prior to testing. The boiled colony suspension (1 drop) was then placed on each of two wells on the disposable slide; 1 drop each of control and OMNI reagents was placed adjacent to the suspension. Each suspension and its adjacent reagent drop were thoroughly mixed with a separate applicator stick, and the slide was then rocked for a maximum of 60 sec or until agglutination was apparent (Fig. 4.8) (Moyes A et al 1994)

A positive result consisted of an agglutination reaction with the OMNI reagent and no reaction with the control reagent. A negative result was defined as the absence of agglutination in both OMNI and control reagents.
Fig: 4.8 – Procedure for Phadebact Monoclonal GC OMNI test

4.2.6 Preservation of frozen stocks of *N. gonorrhoeae* at low temperature

*N. gonorrhoeae* isolates were preserved in cryovials containing nutrient broth with 20% glycerol at -70°C. A heavy inoculum of the bacteria was made in 1.0 ml labeled sterile cryovial containing nutrient broth with glycerol (Appendix I). The suspension was mixed well and then stored in a freezing box at -70°C. Recovery of the cultures was carried out by removing the
cryovials from the freezer and at the same time not allowing the cultures to thaw. Using the micropipette, a small sample of the frozen suspension was removed aseptically and inoculated on a suitable medium. The cryovial was then immediately stored back in the freezer. (WHO 2007)

4.2.7 *N. gonorrhoeae* culture on GC agar without antibiotics

GC agar without antibiotics (Appendix I) was used for culture of *N. gonorrhoeae* to check the antibiotic susceptibility profile. On the day of the test, plates were dried by inverting, with the lid removed in an incubator at 35°C for one hour.

4.2.8 Chromogenic Cephalosporin (Nitrocefin) Test

A nitrocefin-containing disc was hydrated with a drop of distilled water and inoculated with at least five colonies. Red coloration of the discs within three minutes indicated the presence of β-lactamase. (WHO 1999).

4.2.9 Susceptibility Testing using the Kirby Bauer disc diffusion technique

The list of antimicrobial agents used in the study are given in the Table (4.1). The antibiotics used in the group were those currently recommended by the WHO. Two main techniques were used for antimicrobial susceptibility testing, Kirby Bauer Disc Diffusion Technique (Bauer *et al.*, 1966) and the E-test method for the determination of the minimum inhibitory concentration (MIC). Antibiotic susceptibility testing was performed as per the reliability and reproducibility guidelines of Clinical Laboratory Standards (CLSI) and quality assurance guidelines of World Health Organization (WHO). (CLSI 2006)

**Procedure:** The antibiotic discs, GC agar base and GC growth supplement were obtained from Hi-Media laboratories Mumbai, India. GC agar base and 1% defined growth supplement. GC agar plates were prepared as per the manufacturer’s instructions and checked for sterility by
incubating the plates overnight at 37º C. The quinolones & the other antibiotics included in this study are shown below (Table 4.1)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Disc concentration (µg/Disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quinolones</strong></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>5</td>
</tr>
<tr>
<td>Ofloxacin (Of)</td>
<td>5</td>
</tr>
<tr>
<td>Norfloxacin (Nx)</td>
<td>10</td>
</tr>
<tr>
<td>Lomefloxacin (LOM)</td>
<td>5</td>
</tr>
<tr>
<td>Gatifloxacin (GAT)</td>
<td>5</td>
</tr>
<tr>
<td>Enoxacin (EN)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Cephalosporins</strong></td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone (TX)</td>
<td>30</td>
</tr>
<tr>
<td>Cefixime (IX)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Penicillins</strong></td>
<td></td>
</tr>
<tr>
<td>Penicillin (PG)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
<td></td>
</tr>
<tr>
<td>Tetracycline (TC)</td>
<td>30</td>
</tr>
<tr>
<td><strong>Aminocyclitol</strong></td>
<td></td>
</tr>
<tr>
<td>Spectinomycin (SC)</td>
<td>100</td>
</tr>
</tbody>
</table>
• Antibiotic discs were kept at room temperature for 1 h before use.

• Inoculum size was maintained as per the turbidity standards of near “confluent growth”.
  A suspension of pure growth (18-24 hr. old culture) was prepared in 2.5 ml sterile normal saline in a sterile test tube.

• The turbidity was adjusted to Mc Farland 0.5.

• 2 ml of gonococcus suspension was prepared in 0.85% Normal saline & the suspension was dispensed in the plate.

• The plate was tilted in all directions so that the whole plate surface becomes wet.

• The remaining fluid was removed from the plate with a micro pipette and was put back in the test tube.

• The plates were inverted & kept in the incubator at 37 °C for 30 min. to dry.

• With the help of sterile forceps the antibiotic discs were placed on the GC agar plate and the discs were pressed slightly with proper spacing to avoid overlapping and properly pressed for required diffusion.

• On the back of plate the name of the antibiotic or a code word for the antibiotic was written.

• Only seven antibiotics were added on one plate. The remaining antibiotics were added on the second plate.

• A gap of 2.5 cms was kept between the two discs.

• The plates were incubated at 37 °C in a candle jar with 5% CO₂ and 45% moisture (similar incubation for gonococcus culture).

• The plates were placed after 18-24 hour incubation.
The diameter of the clear zone was measured by placing the ruler on the inoculated surface of the petridish (The side on which the code word for the antibiotic was written).

Depending upon the zone size diameter, the isolates were labeled as susceptible, intermediate susceptible and resistant. The size of the zone was interpreted according to the CLSI guidelines (2006).

ATCC 49226, WHO A-E, H and J strains were used as controls.

4.2.10 Susceptibility Testing using the Etest method

A suspension of pure growth (18-24 hr. old culture) was prepared in 2.5 ml sterile normal saline in a sterile test tube.

The turbidity was adjusted to Mc Farland 0.5.

2 ml of gonococcus suspension was prepared in 0.85% Normal saline & the suspension was dispensed in the plate.

The plate was tilted in all directions so that the whole plate surface becomes wet.

The remaining fluid was removed from the plate with a micro pipette and was put back in the test tube.

The plates were inverted & kept in the incubator at 37 °C for 30 min. to dry.

Meanwhile the Etest strips were removed from the -20°C freezer and allowed to equilibrate at room temperature for about 30 mins. Moisture condensing at the outer surface of the strips was allowed to dry completely. Then the Etest package of the respective antibiotic was opened.
• With a pair of forceps the handle of the strip was gripped and applied directly onto the dried GC agar plate, ensuring that the MIC scale was facing upwards and that the concentration maximum was nearest to the rim of the plate.

• Care was taken that the whole length of the strip was in complete contact with the agar surface. Residual air pockets were removed by gently pressing the strips with forceps from the minimum concentration upwards. The inoculated plate was incubated for 18 hrs at 37°C in the 5% CO₂ atmosphere with high humidity.

• After the required period of incubation, when bacterial growth became distinctly visible, MIC value which is the point of intersection between the inhibition ellipse edge and the E-test strip was recorded.

• The MIC of all the isolates for enoxacin (Sigma chemical, USA ) was determined by using agar dilution method using GC agar base & vitamin growth supplement (Hi media Laboratories, India). The antibiotic enoxacin was obtained as powder from the manufacturer. Concentrations of enoxacin tested ranged from 0.001 to 128 ug/ml. Based on MIC values, the strains were labeled as susceptible, intermediate susceptible and resistant, as per the CLSI guidelines.(2006). Four WHO reference strains (A, K, L,G) received under WHO Gonococcal Antimicrobial Surveillance Programme from WHO GASP South East Asia Regional Reference Laboratory, New Delhi , one ATCC strain (49226) and 8 stored strains, sensitive to all 6 antibiotics, were used as control strains.

4.2.11 Auxotyping and Serotyping

The medium for auxotyping was prepared (Appendix I). The isolates were inoculated on each medium and observed for their requirements for arginine, proline,
uracil and hypoxanthine. Serotyping was performed using the Phadebact Monoclonal GC kit.

4.2.12 Discriminatory index

The discriminatory index for antimicrobial susceptibility testing, auxotyping and serotyping was calculated as described previously (Hunter PR et al 1998) to distinguish between unrelated strains.

4.2.13 Chromosomal DNA isolation

Chromosomal DNA from sixty four gonococcal isolates was extracted by using Qiagen kit (QIA amp® DNA Blood minikit, Qiagen GmbH, Hilden, Germany) according to the kit manufacturer protocol. The procedure is as follows:

- Protease K was diluted with solvent provided in the kit and 2.5 ml was distributed in two bottles and stored at -20°C for 6 months.

- Protease K (20ul), culture suspension (200ul), buffer AL (200ul) was added in the empty sterile tube.

- The suspension was vortexed for 60 sec and inactivated in a water bath at 56°C for 15 mins.

- 200 ul of absolute alcohol was added & the suspension was vortexed & all the contents were loaded in the column tubes provided in the kits.
Then the suspension was centrifuged at 8000 rpm for 1 min at 21°C and the column was transferred to an empty collection tube provided in the kit and 500 ul of buffer AW1 was added to the tube. The tube was again centrifuged at 8000 rpm for 1 min at 21°C.

The column was transferred in a new transfer tube provided in the kit and 500 ul of buffer AW2 was added to the tube.

The tube was centrifuged at 14000 rpm for 3 mins at 21°C.

The extracted chromosomal DNA was stored at -20°C until further processing.

4.2.14 PCR of the gyrA and parC genes of quinolone resistant isolates

The oligonucleotide primers for the PCR amplification of the gyrA and parC genes (Table 4.1) were as previously reported by Tanaka et. al (2000). These primers amplified gyrA gene from nucleotides 160 to 438 (278 bp product), corresponding to the amino acids 54 to 146. The 266 bp amplified product of parC gene represented the nucleotides 166 to 420, which corresponds to amino acids 56 to 140 (Belland et. al., 1994). Chromosomal DNA was extracted as described above (4.2.13) and the PCR amplification was performed in 25 ul of a reaction mixture which contained 2.5ul of 10X Taq polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5mM MgCl2, 0.01% gelatin), 1 ul of each of the two primers (reverse and forward), 2.5 ul of deoxynucleoside triphosphates (New England Biolabs, Inc., USA), 0.4ul of Taq DNA polymerase (BangaloreGenei India Pvt. Ltd., India) and 10 ul of template DNA. Thermal cycling was performed after an initial incubation at 94°C for 5 mins. Thirty five cycles were performed for each reaction. Each cycle consisted of 30sec. at 93°C, 1 min at 52°C and 1 min at 72°C, followed by final extension for 10 mins at 72°C.
4.2.14.1 Preparation for the PCR reaction for gyra and par C

For 1x (25 ul)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
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<td>10x buffer</td>
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<tr>
<td>dNTPs (100mM)</td>
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</tr>
<tr>
<td>gyra-F</td>
<td>1 ul</td>
</tr>
<tr>
<td>gyra-R</td>
<td>1 ul</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.4 ul</td>
</tr>
<tr>
<td>H2O</td>
<td>7.6 ul</td>
</tr>
<tr>
<td>DNA</td>
<td>10 ul</td>
</tr>
</tbody>
</table>

4.2.15 PCR of the mtrR genes of quinolone resistant isolates

Primers used in the amplification of mtrR coding region (mtr1: 5’-aaagcctgtgagatac-3’ and mtr2: 5’-gtagtccagaggttcat-3’) and the intervening region between mtrRA and mtrC genes (mtr3: 5’-cagctgcgtagtac-3’ and mtr4: 5’-tgggtagatttc-3’) were designed by Bioresource Biotech India. PCR amplification was performed in 25 ul of a reaction mixture which contained 2.5 ul of 10X Taq polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5mM MgCl2, 0.01% gelatin), 1 ul of each of the two primers (reverse and forward), 2.5 ul of deoxynucleoside triphosphates (Bangalore Genei India Pvt. Ltd., India), 0.4 ul of Taq DNA polymerase (Bangalore Genei India Pvt. Ltd., India), and 10 ul of template DNA. Thermal cycling was performed after an initial incubation at 94°C for 5 mins. Thirty cycles were performed for each reaction. Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by final extension for 5 mins at 72°C.
4.2.15.1 Preparation for the PCR reaction for \textit{mutR} \\

For 1x (25 ul) 

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>dNTPs (100mM)</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>mutR-1</td>
<td>1 ul</td>
</tr>
<tr>
<td>mutR-2</td>
<td>1 ul</td>
</tr>
<tr>
<td>mutR-3</td>
<td>1 ul</td>
</tr>
<tr>
<td>mutR-4</td>
<td>1 ul</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.4 ul</td>
</tr>
<tr>
<td>H\textsubscript{2}O</td>
<td>5.6 ul</td>
</tr>
<tr>
<td>DNA</td>
<td>10 ul</td>
</tr>
</tbody>
</table>

4.2.16 Agarose gel Electrophoresis 

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1x TAE buffer as described by Sambrook \textit{et al}, 2006. Required amount of agarose (depending upon the percentage) was added to beaker (2 to 4 times the volume of the solution) containing distilled water. The agarose was melted by microwave (2mins at maximum). 50x TAE was added to give a final concentration of 1x TAE. An aliquot of ethidium bromide was removed & 2 ul was added to the agarose solution. The agarose solution was then poured on to gel loading system fitted with comb. The agarose was allowed to polymerize for 30 mins. The comb was then removed and the gel was immersed in 1x TAE buffer in horizontal electrophoresis tank. The DNA samples were mixed with 1/6 volume of 6x loading buffer and electrophoresed at 5V/cm. A 100 bp DNA ladder was run in parallel as size standard. The bands were visualized using
short wave (300 nm) transilluminator and photographed with a gel documentation system. The characterization of the gyrA, parC and mtrR genes was done by comparing the electrophoretic mobility of the PCR products with 100 bp DNA ladder.

4.2.17 Purification of DNA:

Plasmid DNA was extracted by using Qiagen DNA purification kit (Minielute ® PCR purification kit, Qiagen GmbH, Hilden, Germany).

- 20ul of PCR reaction product was added to 100ul of buffer (PB)
- The mixture was suspended in a MinElute column provided in the kit and then centrifuged for 1 min
- The flow through was discarded and the MinElute column was kept back in the same tube.
- To wash, 750 ul buffer PE was added to the MinElute column and the tube was centrifuged for 1 min.
- The flow through was discarded and the MinElute column was kept back in the same tube. The column was again centrifuged for an additional 1 min at maximum speed.
- The MinElute column was placed in a clean 1.5 ml microcentrifuge tube.
- DNA was eluted by adding 10ul buffer EB (10mM Tris-Cl, pH-8.5) to the center of the membrane and the column was kept for 1 min and then it was centrifuged for 1 min.
- A nanodrop reading of the purified DNA was taken and then processed for sequencing.
4.2.18 DNA Sequencing

The purified DNA was sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions at the sequencing facility at molecular virology department at NARI. The amount of purified PCR product required for the addition for sequencing depended on the nanodrop reading for each isolate. The purified PCR product was subjected to sequencing using specific primers.

4.2.19 Sequence Analysis

Nucleotide and amino acid sequence analysis was performed using BLAST programs & raw data was analyzed using Seq Scape, Applied Biosystem. Mutations were identified by comparing translated amino acid sequences to reference gyrA (GenBank accession no. U08817), parC sequence (GenBank accession no. U08907) and mtrR (GenBank accession no. Z25796).

4.2.20 Phylogenetic analysis

Phylogenetic analysis was done based on amino acid sequence using Maximum likelihood statistical method and Jones-Taylor-Thornton (JTT) model for the 64 isolates of N. gonorrhoeae for gyrA, parC and mtrR gene sequences.

4.2.21 Statistical Analysis

The data was analyzed using SPSS 15.0 for windows (SPSS Inc. 1989-2006). The difference between the proportion of strains showing mutations in gyrA, parC & mtrR was analyzed using Z test for proportion.