MATERIALS & METHODS
3. MATERIALS AND METHODS

3.1. Sampling Area

The study was conducted in the Integrated Counseling and Testing Centre (ICTC) of Government Primary Health Centre (PHC). The PHC was located in a rural area in Sendamangalam. Sendamangalam is one of the towns in Sendamangalam Taluk in Namakkal District in Tamil Nadu State (Plate -1). Sendamangalam is 8.4 km far from its District Main City Namakkal. It is 294 km far from its State Main City Chennai. Sendamangalam had a population of 18,085. Males constitute 50% of the population and females 50%. Sendamangalam has an average literacy rate of 64%, higher than the national average of 59.5%, male literacy is 73% and female literacy is 56%. Sendamangalam is located at 11.3000°N and 78.2333°E. It has an average elevation of 240 meters (715 feet).

3.2. Study population

In this study, 523 female subjects who attended the ICTC Clinic were randomly selected. All women who came in with a reproductive health complaint from December 2009 to December 2010 were included in this study. Women on antibiotic treatment in the preceding three weeks, pregnant women, postmenopausal women, those with benign or malignant lesions of the cervix, menstruating women and HIV patients were excluded from the study. Women who agreed to participate gave verbal informed consent and were assured of confidentiality and anonymity. Those who refused to participate were treated without prejudice. No further data were collected on women excluded from the study.

3.3. Data collection

The gynecologist carried out the clinical evaluation of each subject selected for the study. The information such as detailed sexual history, genitourinary tract complaints like vaginal discharge, vaginal itching, perineal pains and dysuria were collected. The data were entered in a questionnaire provided for each subject (Questionnaire enclosed). An interviewer-administered questionnaire was used for
delving into each participant’s medical, social and behavioral history of reproductive health-related morbidity.

3.4. Data analysis

Data were analyzed using the SPSS software (version 10). Using a chi-square test the statistical significance was defined as p<0.05. Analysis of data mainly focused on measuring the impact of certain Socio-demographic factors, symptoms, risk factors and also with a few details about their life partners.

3.5. Ethical approval

Ethical approval number Zool/ BU/ Dr.S.K / Ethics/ 2009 were obtained for this study from king institute of preventive medicine and research, Guindy, Chennai.

3.6. Collection and processing of vaginal samples

The labium was opened and moistened speculum was inserted into the vagina to separate the vaginal walls. The sterile swab was inserted 2 cm into the vagina, soak for few seconds, rotated slowly and the swab was withdrawn without touching the external part of the genitalia. The wet mount preparation was carried out for direct microscopic observation. In the wet mount preparation, a portion of the vaginal secretion was emulsified on a clean slide containing 2-3 drops of normal saline covered with cover slip and examined under the microscope using the 40 X objective lens. For culture, the swab was inoculated into the Sabouraud dextrose agar plates and incubated at 37°C for 24 - 48 hrs.


Direct smear examination was done by using 10% KOH preparation (10 g of KOH in 90 ml of distilled water). The growth of Candida spp. on SDA was confirmed by Gram staining procedure. Gram positive budding yeast cells were further confirmed by germ tube method using pooled human serum. The species identification was done by inoculating the cultures into SDA broth, Hicrome Candida agar and Corn meal agar. Other biochemical tests like sugar fermentation, sugar
assimilation and urease test was also carried out. The incubation of cultures at 45°C was also performed for the differentiation of the *Candida* species.

### 3.7. Collection and processing of cervical samples

The cervix was opened with a bivalve vaginal speculum moistened with warm water. The excess mucous discharge from the cervix was wiped with a cotton swab. A sterile Dacron swab was inserted into the cervix and soaked for about 30 seconds and gently rotated to collect endocervical exudates. The swab was withdrawn without touching the vaginal mucosa.

![Flow Chart for Laboratory Diagnosis of Gonorrhoea](image)

**Fig 4**: Flow Chart for Laboratory Diagnosis of Gonorrhoea
The collected sample was used for the smear preparation and immediately inoculated into the Modified Thayer Martin media (HIMEDIA, Mumbai, India). Inoculated plates were transferred to incubator at 36°C in a candle jar supplied appropriately with 5 to 10% carbon dioxide. Plates were examined and looked for colony characteristics of growth after 24 hrs to 48 hrs of incubation.

3.7.1. Characterization of *Neisseria gonorrhoeae*

For the identification of *Neisseria gonorrhoeae*, oxidase test was done on small pinpoint greyish colonies on Modified Thayer Martin medium and Gram stain was done on all oxidase positive colonies. For confirmation, carbohydrate degradation test was done. Isolates fermenting glucose, but not maltose and lactose were confirmed as *Neisseriae gonorrhoeae*.

3.8. Antifungal susceptibility test for *Candida* spp.

Disc diffusion method (Bauer-Kirby *et al.*, 1966)

- Muller Hinton agar was prepared, sterilized and dispensed into sterile Petri dish to a depth of 4mm.
- The plates were allowed to solidify and dried for 30 minutes in an incubator to remove excess moisture from the surface.
- Around 5-6 colonies were selected and inoculated into sterile Muller Hinton broth. The broth was incubated at 35°C for 2-5 hours.
- The size of the inoculums was standardized and their turbidity was adjusted to match the McFarland standard (1.5X10⁸CFU/ml).
• A sterile cotton swab was immersed into the standardized broth and was inoculated by swab culture in Muller Hinton Agar plate in three directions, turning the plates to 60° between each swabbing to form a lawn culture.

• The plates were kept at room temperature for 5-10 minutes to dry the excess inoculum.

• The antifungal discs such as Itraconazole (30mcg), Clotrimazole (10mcg), Fluconazole (10mcg), Ketoconazole (10mcg), Amphotericin B (20mcg) and Nystatin (50mcg) were placed carefully on the plates with flamed forceps at least 25mm away from the edge.

• A gentle press was given over the discs on the surface of the medium and they are allowed to stand at refrigeration condition for 30 minutes (Pre-diffusion time) and the plates were incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured at the end of incubation period. The radius of zones that showed complete inhibition of growth were measured and recorded as end point of complete inhibition to the nearest millimeters using zone measuring ruler (Hi-Media) without opening the lid.

Preparation of Mc Farland Turbidity Standard:

Sulfuric acid (0.18 M) - 99.5 ml

Barium chloride (0.048M) - 0.5 ml

3.9. Antibacterial susceptibility test for *Neisseria gonorrhoeae*

Disc Diffusion Method

• A standard dilution of gonococci which corresponds to a 0.5 McFarland standard and contains approximately 1.0 x 10⁸ CFU/ml was applied on chocolate agar plates to obtain a homogenous, semi confluent growth of gonococci after incubation.

• Application of suspension was made using sterile cotton swabs.
A swab was immersed in the standard suspension and the excess was removed by pressing the swab against the wall of the test tube.

The suspension was applied by stroking movements in three directions turning the agar plate by 60°C.

The agar surface was treated with the swab for 3-4 times.

Then the antibiotic discs Penicillin G (10 units) Ampicillin (10mcg), Tetracycline (30mcg), Erythromycin (15mcg), Ciprofloxacin (5mcg), Cefotoxime (30 mcg) Kanamycin (30mcg) and Spectinomycin (100mcg) were placed carefully on the plates with flamed forceps at least 25mm away from the edge.

Immediately the plate was kept in a candle extinction jar and incubated at 36°C.

Results were recorded by measuring the diameter of the zone of complete inhibition and a very small single colony detected within the growth inhibition area was neglected.

3.10. Demonstration of virulence factors of *Candida* spp.

3.10.1. Biofilm formation (Branchini *et al.*, 1994)

A loopful of organisms from the SDA plate was inoculated into a tube containing 10 ml of sabouraud's liquid medium supplemented with glucose (final concentration of 8%).

The tubes were incubated at 37°C for 24 hrs.

The broth was aspirated out and the walls of the tubes were stained with safranin.

Biofilm formation was noted as negative (0+), weak positive (1+), moderate positive (2+), or strong positive (3+).
3.10.2. Phospholipase estimation (Samaranayake et al., 1984)

- The isolates were screened for their extracellular phospholipase activity by growing them on egg-yolk agar medium and zone of precipitation was measured.

- The egg-yolk medium consists of Sabouraud dextrose agar (SDA), and 10% sterile egg yolk (the egg yolk was centrifuged at 500 g for 10 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium).

- The extracellular phospholipase activity was detected by inoculating 10 μl aliquots of the yeast suspension (approximately 108 yeast cells/ml) into the wells punched onto the surface of the egg-yolk medium.

- It was incubated at 37ºC for 48 hrs.

- The diameter of the zone of precipitation around the well was measured and Pz value (phospholipase activity) was determined by the ratio of the diameter of the colony to the total diameter of the zone of precipitation. When the Pz equaled 1, no phospholipase activity was detected in the strain. Thus, a low Pz value indicated high production of the enzyme.
3.10.3. Proteinase detection (Staib et al., 1965)

- Proteinase activity was detected by inoculating 10 μl aliquots of the yeast suspension (approximately 108 yeast cells/ml) into the wells punched onto the surface of the bovine serum albumin medium.
- The plates were incubated at 37ºC for two days.
- After incubation, the plates were fixed with 20% trichloracetic acid (20 g of trichloro acetic acid in 100 ml of distilled water) and stained with 1.25% amidoblacl.
- Decolourization was performed with 15% acetic acid (15 ml of acetic acid in 85 ml of distilled water).
- Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that could not be stained with amidoblacl, indicated degradation of the protein.
- The diameter of unstained zones around the well was measured for proteinase production.
- The proteinase activity (Pz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. When the Pz equaled 1, no proteinase activity was detected in the strain. Thus, a low Pz indicated high production of the enzyme.
Preparation of Amido black (1.25%)

- Amido black - 1.25 g
- Methanol - 90 ml
- Acetic acid - 10 ml

3.10.4. Hydrogen peroxide sensitivity (Vilela et al., 2002)

- The isolates were cultured in a 96 wells micro plate with a starter inoculum of 2x10^6 ml supplemented with 1 or 10Mm hydrogen peroxide.
- It was incubated at 37°C for 1-2 hrs and subjected to plating for on SDA for viable count.
- The viable cells were calculated in CFU and the assay was conducted in duplicate.

3.10.5. Hemolytic property (Luo et al., 2001)

- The hemolytic activity was determined by plate assay method.
- The isolates were spotted on a sugar-enriched sheep blood agar medium.
- The plates were incubated at 37°C in 5% CO₂ for 48 hrs.
- The presence of a distinct translucent halo around the inoculum was viewed with the transmitted light, indicated positive haemolytic activity.
- The diameters of the zone of lysis and the colony were measured and this ratio (equal to or larger than 1) was used as a hemolytic index.
- The plates were assayed with the duplicate plate.
3.11. Identification of drug resistance gene in *Candida albicans* by PCR

3.11.1. Isolation of Genomic DNA

- About 1.5 ml of liquid culture of yeast grown for 20-24 hrs at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) was transferred into a microcentrifuge tube. The cells were pelleted by centrifugation at 20,000 × g for 1-5 minutes.
- 200 µl of Harju-buffer was added. The tubes were immersed in a dry ice-ethanol bath for 2 minutes.
- Then it was transferred to a 95°C water bath for 1 minute.
- The last two steps were repeated and subjected to vortexing for 30 seconds.
- 200 µl of chloroform was added and vortex it for 2 minutes.
- It was subjected to centrifugation at 20,000 × g for 3 minutes at room temperature.
- The upper aqueous phase was transferred to a microcentrifuge tube containing 400 µl of ice-cold absolute ethanol. It was mixed by inversion or gentle vortexing.
- It was incubated at room temperature for 5 minutes. Alternatively, DNA was precipitated at -20°C to increase yield.
- It was subjected to centrifugation at 20,000 × g for 5 minutes at room temperature.
- The supernatant was removed with a pulled Pasteur pipette by vacuum aspiration.
- The pellet was washed with 0.5 ml of 70% ethanol
- It was centrifuged at 20,000 × g for 5 minutes at room temperature.
- The supernatant was removed and the pellets were air-dried at room temperature.
- It was resuspended in 25-50 µl of TE buffer (10 Mm Tris pH 8.0 and 1 Mm EDTA). Samples obtained directly from the plates should be resuspended in a 10 µl volume, because the yield will be smaller.
Harju- buffer preparation

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% TritonX-100</td>
<td>- 20ml TritonX-100 in 80ml distilled water</td>
</tr>
<tr>
<td>1% SDS</td>
<td>- 1gm of SDS in 100ml distilled water</td>
</tr>
<tr>
<td>100mM NaCl</td>
<td>- 584.4mg in 100ml distilled water</td>
</tr>
<tr>
<td>10mM Tris-HCl pH 8.0</td>
<td>- 1.21gm in 100ml distilled water</td>
</tr>
<tr>
<td>1mM EDTA</td>
<td>- 29.224gm in 100ml distilled water</td>
</tr>
</tbody>
</table>

70% Ethanol preparation

<table>
<thead>
<tr>
<th>Solution</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>70 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

3.11.2. Confirmation of DNA by Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in a submarine electrophoresis unit. Thirty ml of 1% Agarose gel was prepared with 1X TAE buffer (do not mix) and heated the content to get up a clear solution for casting Agarose gel. After cooling the solution, 7 µl of ethidium bromide was added into the casting system.

The gel was allowed to solidify at room temperature and then carefully disassembled from the casting system without disturbing the wells and placed in 1X TAE buffer filled electrophoresis tank (the buffer level was kept above gel). In the first lane 3 µl of DNA marker with tracking dye was added. Approximately 5 µl of genomic DNA contains 2 µl of tracking dye (0.25% of bromophenol blue and 40% sucrose) was loaded in the wells.

The power card terminals were connected at respective positions and the gel was run at 50 V, till the gel loading dye migrate more than half the length of gel. Then switched off the unit and the isolated DNA was visualized under UV transilluminator.

TAE buffer (50x) preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>– 242 g – dissolve in water</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>– 57.1 ml</td>
</tr>
<tr>
<td>EDTA (500 mM) (pH 8.0)</td>
<td>– 100 ml</td>
</tr>
</tbody>
</table>

Bring it to the volume of 1 litre and convert to 1x during usage.
3.11.3. Polymerase Chain Reaction (PCR)

All resistance gene primers were designated according to NCBI. The primers were obtained from MWG, India. The following primers were used for the amplification.

**CDRI**

F-5’AGTACTCCGTATCACCGTGC’3  
R-5’ACAGAGTGAGGGCGGCTG’3

**CDR2**

F-5’TCAAAGGGATACCGGCTGCA’3  
R-5’TGGGTTCGTCTAGCTGTTGC’3

**MDRI**

F-5’CCGGGGATGTCATTGGTTCACA’3  
R-5’CCGTCCTCGCTGTGCCCATT’3

**ERG11**

F-5’TTCCTTGGTTGTTCTGCAGCT’3  
R-5’CTTTTGAGCAGCATCACGTCTCCA’3

The PCR mix was prepared in thin walled PCR tube in a sterile laminar flow hood. The following reagents were added as follows.

Each PCR reaction mixture (20 µl) contained 2µl of template DNA 2 µl of 10X PCR buffer, 0.5 µl of (0.5 µM) each of the primers, 1 µl of 0.2 mM of each deoxynucleotide triphosphate (dNTP’S) and 1 µl of Taq DNA polymerase (Concentration is 5U/µl ) and 8.0 µl of molecular grade water.

A brief spin was given to settle down the materials, then the tubes were kept in thermocycler (Genei). After initial denaturation at 94°C for 4 minutes, the samples were subjected to 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 1 minute and extension at 72°C for 2 minutes. A final extension was performed at
72°C for 10 minutes. Following PCR, aliquots (20 μl) of the reaction mixtures were analyzed by electrophoresis on 1.5% Agarose gel containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker. Then the amplification bands for drug resistance genes were observed under UV transilluminator and detection of resistance gene with the use of 1 kb DNA marker. The molecular weight of CDR1, CDR2, MDR and ERG11 were found to be 701bp, 835 bp, 941 bp and 585 bp respectively.

3.12. Molecular identification of *Candida albicans* by (28s ribosomal RNA) Sequencing

An average yeast 28S rRNA molecule has about 855bp and has sufficient information for phylogenic analysis. The 28S rRNA gene sequence based identification of yeast is advanced, versatile and highly accurate than the conventional phenotypic and metabolic method. Even a single base pair alteration in the evolutionary conserved sequence can be detected. The genomic DNA for the *Candida albicans* were isolated by the method described in 3.10.1.

3.12.1. Polymerase Chain Reaction

The PCR reactions were carried out in 0.2 ml tubes in eppendorff Personal mastercycler. Identification of the strain was done by amplification of the partial 28S rRNA gene using 28S F 5’GTCGCCCGTCTTG3’ and R 5’TTAAGCATATCAATAA3’. The program used for the amplification was 94°C for 4 mins for initial denaturation and 35 cycles of 94°C for 1 minute, 57°C for 1 mins and 72°C for 2 minute. Final extension was made at 72°C for 10 mins. The amplified product was observed by electrophoresis on 1% Agarose gel in 0.5X TE Buffer (5mM Tris HCL and 0.5 Mm EDTA- pH 7.5).

3.12.2. Elution and purification of amplified DNA

The amplified PCR product was purified by Perfectprep Gel cleanup kit (Eppendorff AG, Germany) according to the manufacturer instruction. The purified
PCR product was used to sequence with specific forward and reverse primers. The purified PCR product was sent for 28SrRNA sequencing at Applied Biosystem Ltd.

3.12.3. Similarity searching of 28S rRNA

The identified 28S rRNA of multidrug resistant *Candida albicans* were submitted and confirmed by similarity search using BLASTN program.

3.13. PCR for the detection of plasmid borne drug resistance in *Neisseria gonorrhoeae*


- MDR strains of *Neisseria gonorrhoeae* culture were grown overnight in modified Thayer martin medium with antibiotic for isolation of plasmid.
- The growth of the organisms were scrapped off with sterile cotton swab and suspend in 1x PBS in an eppendorff tube.
- It was taken in eppendorff tubes and the cell pellet was recovered by centrifugation at 13,000 rpm for 2 minutes.
- The tube was placed in a water bath and boiled for 5 minutes.
- Then the suspension was cooled to room temperature after boiling and subjected to centrifugation at 13,000 rpm in a ultracentrifuge for 2 minutes.
- The aqueous supernatant was collected carefully and labeled for plasmid isolation. It was stored at -20°C for future use.

**PBS preparation (1x)**

Dissolved the following in 800 ml of distilled water

\[
\begin{align*}
\text{NaCl} & \, - \, 8 \text{ g} \\
\text{KCl} & \, - \, 0.2 \text{ g} \\
\text{Na}_2\text{HPO}_4 & \, - \, 1.44 \text{ g} \\
\text{KH}_2\text{PO}_4 & \, - \, 0.24 \text{ g} \\
p\text{H} & \, - \, 7.4 \\
\end{align*}
\]

Adjusted the volume to 1 litre with additional distilled water.
3.13.2. Detection of PPNG plasmid by PCR

*Neisseria gonorrhoeae* isolates showed penicillin and ampicillin resistance were selected for PPNG plasmid detection by PCR. The primers were obtained from MWG, India. The recommended reagents were prepared according to the protocol of Dillon *et al.*, 1999). Known positive control (*N. gonorrhoeae* PPNG plasmid) and negative control (distilled water) was included. The following primers were used for the amplification.

**PPNG PLASMID**

JDA 5’ – TACTCAATCGGTAATTGGTTC-3’ and

JDB 5’ – CCATATCACCGTCGGTACTG-3’

The PCR mix is prepare in thin walled PCR tube in a sterile laminar flow hood. Add the following reagents as follows. Each PCR reaction mixture contained 2.5μl of template DNA 2.5 μl of 10 X PCR buffer, 0.15 μl of each of the primers, 2 μl of deoxynucleotide triphosphate (dNTP’S) and 0.15 μl of Taq DNA polymerase, 0.75 μl of MgCl₂ and 16.8 μl of molecular grade water.

A brief spin was given to settle down the materials and then the tubes were kept in theromocycler (Genei). After initial denaturation at 94°C for 3 minutes, the samples were subjected to 30 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 5 minutes. A final extension was performed at 72°C for 5 minutes. Following PCR, aliquots of the reaction mixtures were analyzed by electrophoresis on a 1.5% Agarose gel.

**PCR buffer preparation**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Amount in 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM MgCl₂</td>
<td>- 0.304g</td>
<td>distilled water</td>
</tr>
<tr>
<td>500 mM KCl</td>
<td>- 0.373g</td>
<td>distilled water</td>
</tr>
<tr>
<td>100 mM Tris HCl (pH- 8.3 at 25°C)</td>
<td>- 12.11g</td>
<td>distilled water</td>
</tr>
</tbody>
</table>
3.13.3. Detection of TRNG plasmid by PCR

*Neisseria gonorrhoeae* isolates showed Tetracycline resistance were selected for TRNG plasmid detection by PCR. The primers were obtained from MWG, India. The recommended reagents were prepared according to the protocol of Xia *et al.*, 1995. Known positive control (*N. gonorrhoeae* TRNG plasmid) and negative control (distilled water) was included in PCR run. The following primers were used for the amplification.

**TRNG PLASMID**

RM4 5’ – CCAAATCCTTTCTGGGCT – 3’ and

G1 5’ – ATCACTCACAGTTAAT – 3’

The PCR mix was prepared in thin walled PCR tube in a sterile laminar flow hood. The following reagents were added as follows. Each PCR reaction mixture contained 1 μl of template DNA, 2.5 μl of 10 X PCR buffer, 0.05 μl of each of the primers, 1 μl of deoxynucleotide triphosphate (dNTP’S), 0.1 μl of Taq DNA polymerase, 1 μl of MgCl₂ and 19.3 μl of molecular grade water.

A brief spin was given to settle down the materials and then the tubes were kept in thermocycler (Genei). After initial denaturation at 95°C for 5 minutes, the samples were subjected to 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 3 minutes. A final extension was performed at 72°C for 7 minutes. Following PCR, aliquots of the reaction mixtures were analyzed by electrophoresis on a 1.5% Agarose gel.

3.13.4. Visualization of plasmid DNA by Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate macromolecules based on charge, size and shape. To carry out the electrophoresis, samples were placed in an electric field. The negatively charged molecules migrate towards the positive electrode (anode) and positively charged ones migrate towards the negative electrode (cathode). As DNA is negatively charged, it moved towards the positive electrode.
Agarose gel containing ethidium bromide (0.2 mg/ml) as inter-chelating agent. Then observe the amplification bands under UV transilluminator and detection of resistance gene with the use of an appropriate DNA molecular weight marker (1 kb).

The results of the PPNG bands were interpreted based on the molecular weight of markers. Bands around 3 kbp were considered as ‘Africa type’ PPNG Plasmids. The bands around 600 bp were considered as ‘Dutch type’ and around 1600 bp were considered as ‘American type’ TRNG Plasmids.

3.14. Molecular identification of *Neisseria gonorrhoeae* by *tbpB* gene sequencing

The quinolone resistant *Neisseria gonorrhoeae* isolated from women attending ICTC clinic was selected and analysed for genotyping using NG-BLAST.

3.14.1. Primary PCR of DNA extract for *tbpB* gene

Polymerase chain reaction were performed. The PCR reactions were carried out in 0.2 ml tubes in Eppendorff Personal mastercycler. Identification of the *tbpB* gene was done by amplification using *tbpB* forward 5’ – CGTTGTCGGCAGCGCGAAAAC – 3’ and *tbpB* reverse 5’ TTCATCGGTGCGCTCGCCTTG - 3’. The program used for the amplification was 95°C for 4 minutes for initial denaturation and 25 cycles of 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 1 minute. Final extension was made at 72°C for 10 minutes and cooled to 4°C. The amplified product (389 bp) was observed by electrophoresis on 1% Agarose gel in 0.5X TE Buffer. The amplified product was purified and the total amount of DNA was calculated. The purified DNA was then diluted by PCR water.

3.14.2. Sequencing of *tbpB* gene of *Neisseria gonorrhoeae*

The purified products were then amplified again in a concentration of 10-20ng/µl with a reaction volume of 20 µl using one of the primers of *tbpB* (either using reverse or forward primer of *tbpB*). The program used for the amplification was 96°C for 1 minute for initial denaturation and 25 cycles of 96°C for 20 seconds, 50°C for 5 seconds and 60°C for 3 minutes. Final extension was made at 60°C for 1 second and cooled to 4°C. The obtained products were precipitated, washed and dried. An amount
of 15 μl of formamide was added to dried pellet and denatured at 95°C for 2 minutes. The denatured product was kept in ice immediately for 5 minutes and finally transferred to DNA Sequencer for final sequencing.

3.14.3. Similarity searching of tbpB gene of *Neisseria gonorrhoeae*

The identified 16S rRNA of quinolone resistant *Neisseria gonorrhoeae* were submitted and confirmed by similarity search using BLASTN program.
Questionnaire used for primary data collection

PERSONAL PROFILE

1. Serial Number : 
2. Name and Address. : 
3. Age : 
4. Sex : Male/Female 
5. Educational status : Literate/Illiterate 
6. Occupation : coolie/ housewives/others 
7. Marital status : Married /Unmarried 
8. If married where your partner is working : local /moffusul/foreign 
9. Shift of your work : Day /Night 
10. Have you heard the term STDs. : Yes/ No 
11. Socioeconomic status : Low / Moderate / High 

SIGNS AND SYMPTOMS

12. Do you have vaginal itching : Yes /No 
13. Are you have dysuria : Yes /No 
14. Any smelling vaginal discharge? : Yes /No 
15. Micturination ? : Yes /No 
16. Any pain in abdomen ? : Yes /No 
17. Any vaginal lesion? : Yes /No 

RISK FACTORS

18. Do you have extra marital sexual behavior? : Yes /No 
19. Do you have pre-marital sexual behavior? : Yes /No 
20. Do you know the usage of condoms? : Yes/No 
21. Contraceptive method used : Copper-T/pills/ condoms/ other methods 
22. Do you use antibacterial/antifungal drugs frequently : Yes/No 
23. Prior hospitalization if any ? : Yes/No
1. வாரிகள் நாள்:

2. நிலையில் அங்கு வரும்:

3. என்று:

4. பார்வையாக:

5. காலமிக்குதல்:

6. சோதனை:

7. விண்வெளியும் அளவு?

8. விண்வெளியும் தீர்மானத்தின் தொடர்பு / மூலம் காண வேண்டும் விண்வெளியின்?

9. விண்வெளியும்:

10. பார்வையாக விண்வெளியாகப் பாதியோ சோதனைகள்?

11. பார்வையாக:

12. விண்வெளியும் அளவு தீர்மானம் வழிய வேண்டும் விண்வெளியின்?

13. காண்பதான் பாதியோ விண்வெளியும் பிரபலமான விண்வெளியின்?

14. பிரபலமான விண்வெளியும் அளவு விண்வெளியின்?

15. பிரபலமான விண்வெளியும் பி.சி.வெளியின்?

16. பிரபலமான விண்வெளியும் பி.சி.வெளியின்?

17. காண்பதான் விண்வெளியும் விண்வெளியின்?

18. விண்வெளியும் அளவு தீர்மானம் வழிய வேண்டும் விண்வெளியின்?

19. விண்வெளியும் வழிய வேண்டும் விண்வெளியின்?

20. அளவு விண்வெளியும் பி.சி.வெளியின்?

21. காண்பதான் பி.சி.வெளியின்:

22. காண்பதான் பி.சி.வெளியும் வழிய வேண்டும் விண்வெளியின்?

23. காண்பதான் விண்வெளியும் வழிய வேண்டும் விண்வெளியின்?
தகவல் செய்திகள்

பாலத்தில்லாத நீரரிவை தன்னால் கையாள உடன் / வலை கையாளில்லாத நீரறிவை கையாளத்தக்கள். பாலத்தில்லாத நீரரிவை கையாளும் தன்னால் கையாளினார் மற்றும் தன்னால் கையாளினார். பாலத்தில்லாத நீரறிவை கையாள மற்றும் சேகரிப், பாத்திர, பூத்துரைப்பிட்டு அதிசய தொகுதிகளின்படி கையாளினார். இது அரசின் ஏற்றமில்லாத

- தொகுதிகளின் மூலம் மட்டும் முதலில் முதலாம் துனை

பாலத்தில்லாத நீரரிவை தகவல் போக்கு

பாலத்தில்லாத நீரரிவை தகவல் விளக்குவரலாற். அதில் பிறகும் துடுப்பாக்கம்

- வேலைத்தொடர்
- என்று
- காலமன்னார்
- நீரரிவை
- நீர்த்தொடர்
- நீர்குழுப்பாடு

அதுவர் நீர்ப்பாடு உறுதிகள் பாலத்தில்லாத நீரறிவை கையாளும் தன்னால் கையாளும்?

தொடர்கை?

சராசரி ஆராய்ச்சி கையாளினார் வேலைத்தொடர் கையாளினார். இது கையாளும் முக்கிய

- சுருக்குத்தொடர்
- தொடர்
- என்று நீர்ப்பாடு நீர்ப்பாடு

அதுவர் தொடர்கை உறுதிகள் பாலத்தில்லாத நீரறிவை கையாளும் தன்னால் கையாளும்?

தொடர்கை?

சராசரி ஆராய்ச்சி கையாளினார் வேலைத்தொடர் கையாளினார். இது கையாளும் முக்கிய

- சுருக்குத்தொடர்
- தொடர்
- என்று நீர்ப்பாடு நீர்ப்பாடு

அதுவர் தொடர்கை உறுதிகள் பாலத்தில்லாத நீரறிவை கையாளும் தன்னால் கையாளும்?
நேர்வாரமான குழுத் படைமுறை

ஆசிரியர் பாடல் என்பது (ஆசிரியர் பாடல்) அரசு மாநில பகுதித்தொகுதிகள் குழுத் படைமுறையாகும். இது பகுதித்தொகுதிகளின் பல்வேறு வகைகளின் குழுத் படைமுறையும் (ஆசிரியர் குழுத் படைமுறையான பாரம்பரிய நூற்றாண்டு பாரம்பரியம்") குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் படைமுறை குழுத் படைமுறை என்று குழுத் 

பகுதித்தொகுதிகள் குழுத் படைமுறை

பகுதித்தொகுதிகள் குழுத் படைமுறை

பகுதித்தொகுதிகள் குழுத் படைமுறை

பகுதித்தொகுதிகள் குழுத் படைமுறை

பகுதித்தொகுதிகள் குழுத் படைமுறை

பகுதித்தொகுதிகள் குழுத் 

பகுதித்தொகுதிகள் குழுத் 

பகுதித்தொகுதிகள் குழுத் 

பகுதித்தொகுதிகள் குழுத்