CHAPTER 3

METHODOLOGY

3. METHODOLOGY:

The present study was conducted in the department of Microbiology, Assam University, Silchar, Assam, India. The duration of the study was from February 2013 to April 2015. The bacterial strains were collected from Silchar Medical College and Hospital, Silchar, Assam. This tertiary referral hospital serves around 38,26,110 number of populations of Assam (Including districts like Cachar, Hailakandi, Karimganj and neighbouring states like Meghalaya, Manipur and Tripura).

3.1 Patient population:

The patients who were admitted to different wards and those who attended the outpatient departments from February 2012 to January 2013 were selected for the study. Subjects included in the present study were diagnosed as a case of one of the following: urinary tract infection, meningitis, pyogenic infection, bacterial pneumonia, bacteremia/septicaemia or diarrheal diseases. A total number of 562 consecutive, non-duplicate clinical isolates has been studied during the period (Table 1) of which 218 from indoor patient and 344 were from outdoor patients in different wards/OPDs (Table 2).

3.2 Isolates for the study:

A total of 268 isolates were grouped under Enterobacteriaceae and obtained from the above samples and identified according to gram strain, cultural characteristics and biochemical reactions (Collee et al., 1996) (Table 3).

Table 1: Types of clinical specimens obtained for the study

Clinical specimens	Indoor (Number of isolates)	Out door (Number of isolates)	Total (Number of isolates)
Urine	102	146	248
Pus	89	131	220
Stool	10	37	47
Ear swab	2	4	6
Throat swab	4	3	7
Oral swab	4	9	13
Sputum	7	6	13
Drain tip	2	0	2
Blood	1	1	2
Pseudo pancreatic fluid	0	1	1
Wound aspirate	0	1	1
Nasal secretion	0	2	2
Total	221	341	562

Table 2: Isolates obtained from different wards

Wards/OPD	Indoor (Number of isolates)	Outdoor (Number of isolates)	Total (Number of isolates)
Surgery	97	115	212
Medicine	65	79	144
Pediatrics	8	30	38
[#]ENT	10	9	19
Chest and [##]TB	0	10	10
Orthopedics	49	10	59
Gynecology	6	10	16
Dermatology	2	4	6
[###]ICU	4	10	14
Trauma	1	0	1
Obstetrics & gynecology	11	27	38
Total	253	309	562

[#]ENT- Eye Nose Throat, [##]TB- Tuberculosis, [###]ICU- Intensive Care Unit.

3.3 Isolation and identification of isolates from clinical specimens:

For selective isolation, the clinical specimen was inoculated on Mac-Conkey agar, Blood agar and Cystine Lysine Electrolyte Deficient agar (CLED) for urine samples, followed by overnight incubation at 37^0C. The isolates were subjected to microscopic and biochemical investigation based on colony morphology.

3.3.1 Bacterial staining:

Isolated bacteria were further subjected to gram staining, which is mainly dependent on permeability of the bacterial cell wall and cytoplasmic membrane, to form dye-iodine complex. Gram positive bacteria stain purple, while gram negative bacteria stain Pink in colour.

Reagents and glass wares:

Crystal violet, Gram's iodine, 95% Ethanol, and safranine, Spirit lamp loop wire, glass slide.

Preparation of bacterial smear:

Clean grease free slide was taken for making smears

⇩

A loopful of saline was placed on centre of the slide

⇩

With a loop well isolated colony was taken from solid media and emulsified in the drop of water.

⇩

Allowed to air dry from a few minute

⇩

Then heat fix was done by holding the slide at one end over the flame.

Procedure of staining:

After the heat fix was done smear was covered with (basic dye) Crystal violet for 1 minute

⇩

Smear was rainsed under tap water

⇩

Smear was covered with (mordant) Gram's iodine for a 1 minute

⇩

Rainsed with tap water

⇩

Then decolouriser was used on bacterial smear for a few second and then gently rinsed with water

⇩

Safranine (counter stain) was added and allowed for 30 second to 1 min.

⇩

Then again rinsed with tap water and kept it for air dry

⇩

Finally observed under the low power (40x) objective and then under oil immersion (100x) objective in microscope

Quality control:

Gram positive: *Staphylococcus aureus,*

Gram negative: *Escherichia coli*

Interpretation:

Gram positive organism was stained in purple color and gram negative in pink color.

3.3.2 Motility (Hanging drop preparation)

Principle:

Microorganisms such as bacteria, because of their small size and a refractive index that closely approximates that of water, do not lend themselves readily to microscopic examination in a living, unstained state. Advantage of this method is that by this method live bacteria can be observed.

Reagents and glass wares:

Normal saline water, inoculating loop, cavity slides, cover slips and microscope.

Procedure:

A clean grease free cover slide was taken and paraffin was applied to four corner of slide

⇩

A drop of fresh culture broth was taken on coverslip with the help of inoculating loop

⇩

A cavity slide over the coverslip was placed on centre

⇩

The slide was inverted and observed under the microscope

⇩

The edge of the drop was fixed under lox power (10x), after that focus was shifted to high power (40x) and observed.

Quality control:

Motile bacteria: *Escherichia coli*

Non-motile bacteria: *Klebsiella pneumonia***.**

Results and interpretation:

Motility of bacteria is obtained at the edge of the droplet.

3.3.3 Biochemical characterization:

3.3.3.1 Catalase test:

Principle:

Catalase is produced by many bacteria which is chemically haemo-protein enzyme. These enzymes can split hydrogen peroxide into water and oxygen. Production of catalase can be observed by adding extra hydrogen peroxide on bacterial colonies and free gas bubbles indicates the catalase presence in bacteria.

Reagents and glass wares: 3% of hydrogen peroxide, glass slides, plastic loop.

Procedure:

Pure and fresh culture of bacteria were transferred on a slide

⇩

One drop of 3% hydrogen peroxide was added

⇩

Then observed for a few minute for the bubble formation.

Quality control:

Positive control: *Escherichia coli,*

Negative control: *Streptococcus pyogenes*

Interpretation: The rapid and sustained bubbles appeared in positive isolates which means the bacteria possesses the enzyme catalase.

3.3.3.2 Oxidase test:

Principle:

The enzyme cytochromes oxidase plays a vital role in the operation of the electron transport system during respiration and results the formation of water or hydrogen peroxide. The oxidase test uses certain reagent such as p-phenylene diamine dihydrochloride which act as a substitute for oxygen as artificial electron acceptors and form N-N tetramethyl para-phenylene diamine hydrochloride to indophenol blue colour compound.

Reagents and glass wares:

Platinum loop and sterile disc.

Procedure:

A sterile disc was taken and pure colonies was picked up from culture plate

⇩

With the help of platinum loop a small smear was formed on sterile disc

⇩

Colour change was obtained within 10 seconds.

Quality control:

Positive control: *Pseudomonas aeruginosa*,

Negative control: *Escherichia coli*.

Interpretation: Bacterial colonies having cytochrome oxidase activity develop a deep blue colour within 10 seconds and in a negative test the colour remain unchanged.

3.3.3.3 Indole test:

Principle:

Tryptophan is an essential amino acid that can undergo oxidation reaction under the presence of enzyme tryptophanase. This is present of peptone water of the culture medium and produces the metabolic end product indole, skatole and indole acetic acid. The aldehyde para dimethyl amino benzyldehyde reacts with the indole and produce red coloured product.

Reagents and glass wares:

Peptone water, Kovac's reagent, test tube and inoculating loop.

Procedure:

The isolates were inoculated in the test tube containing peptone water

⇩

Incubated for 24 hours

⇩

After 24 hours of incubation 0.2 ml of Kovac's reagent was added to the peptone water

⇩

Allowed to stand for few minutes.

⇩

Color changes were observed for test organism.

Quality control:

Positive control: *Escherichia coli*

Negative control: *Klebsiella pneumonia.*

Interpretation:

In a positive result a red-violet ring was formed within a minute after adding a Kovac's reagent and in negative isolates a yellow ring appeared.

3.3.3.4 Methyl red test:

Principle:

The hexose monosaccharide glucose is the major substrate oxidized by the enteric bacteria. The tested organism must produce large quantities of acid as an end product of metabolic pathway. The methyl red test is a quantitative test for acid production and requiring positive oragisms to produce strong acids from glucose through the mixed acid fermentation pathway. Because of many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phase of incubation only, organisms that can maintain this low pH after prolonged incubation overcoming the pH buffering system of the medium can be called methyl red positive.

Reagents and glass wares:

Peptone, methyl red indicator and test tube.

Procedure:

Tested isolates were inoculated in 0.5 ml of glucose phosphate broth cultures in test tubes and incubated for 24 hours.

⇩

After incubation few drops of methyl red indicator was added and mixed well

⇩

Stable red colour formation was observed.

Quality control:

Positive control: *Escherichia coli*

Negative control: *Klebsiella pneumoniae.*

Interpretation:

Development of stable red colour in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange colour between yellow and red may develop. This does not indicate a positive test and yellow colour indicates a negative test.

3.3.3.5 Voges-Proskauer test:

Principle:

The Voges-Proskauer test determines the capability of some bacteria to produce non-acidic or neutral end products such as acetyl methyl carbinol from the organic acids produced as a result of glucose metabolism. Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose is metabolised through various metabolic pathways, depending on the enzymes systems possessed by different bacteria. In the presence of atmospheric oxygen and alkali the small amount of acetyl methyl carbinol present in the medium, is converted to diacetyl, which reacts with the peptone of the broth to produce a red colour.

Reagents and glass wares:

Peptone water,5% *α-naphthol* ,40% *potassium hydroxide* and test tube.

Procedure:

Tested isolated were inoculated in a glucose phosphate broth and incubated for

24 hours

⇩

After 24 hours, at first 40% KOH and then 0.6ml of a 5% of *α-naphthol* was added in a cultured broth

⇩

Changes of colour noticed within 2-5 minutes.

Quality control:

Positive control: *Klebsiella pnuemoniae*

Negative control: *Escherichia coli.*

Interpretation:

A positive test is represented by the development of the pink colour. This indicates the presence of diacetyl, the oxidation product of acetoin. Negative results were indicated by colour less reaction for half an hour. The test should not be read after standing for over 1hour because negative VP test may produce a copper-like colour leading to a false positive interpretation.

3.3.3.6 Citrate utilization test:

Principle:

Sodium citrate is a salt of citric acid, a simple organic compound produced as one of the metabolites in the tricarboxylic acid cycle of the bacteria. In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. This ability depends on the presence of the enzyme, a citrate permease that facilitates the transport of citrate in the cell. Citrate is acted on by the enzyme citrase which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic

acid and carbon dioxide. The presence of sodium carbonate changes the indicator, bromothymol blue present in the medium from green at pH 6.9 – Prussian blue at pH 7.6.

Reagents and glass wares:

Simmon's citrate medium,test tube and inoculating loop.

Procedure:

Sterile Simmon's citrate medium were taken in a test tube in a slant position

⇩

Then tested bacteria were inoculated by means of a stab and streak into its appropriately labelled tube

⇩

All the inoculated isolated were incubated for 24- 48 hours.

Quality control:

Positive control: *Klebsiella pneumonia*

Negative control: *Escherichia coli*

Results and interpretation:

A positive test is represented by the development of a deep blue colour within 24 hours to 48 hours, indicating that the test organism has been able to utilise the citrate contained in the medium, with the production of alkaline products. A negative test is indicated by no change of colour of the citrate medium.

3.3.3.7 Urease test:

Principle:

Urea is a diamide of carbonic acid and bacteria produce this urease enzyme which hydrolyses urea and releases ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonate and increase the pH of the solution, Phenol red is incorporated in the medium which changes the colour of the solution with the presence of urease activity.

Reagents and glass wares:

Christensen's urea agar: Peptone, glucose, NaCl, Monopotassium phosphate, phenol red, agar, distilled water and pH at 6.8.

Test tubes and spirit lamp.

Procedure:

Christensen's urea agar slant was prepared in a test tube

⇩

Then pick up the pure colonies from culture plate and inoculated in the test tube slant

⇩

Incubated and the result was observed after 18 hrs.

Quality control:

Positive control: *Protues mirabilis*

Negative control: *Escherichia coli*

Interpretation:

The test tube with positive results shows purple pink colour in test tube and in case of negative the colour remain unchanged after incubation of 18 hours.

3.3.3.8 Triple sugar iron test:

Principle:

The triple sugar iron (TSI) is an example of a composite medium used widely for the identification of bacterial isolates. The TSI agar has glucose, lactose and sucrose as the sources of carbohydrates and deigned to different among different groups of the family Enterobacteriaceae. Phenol red is the acid base indicator incorporated in the medium. This indicator helps to detect carbohydrate fermentation that is indicates by a change in colour of the medium from orange red to yellow in the presence of acid. The medium can detect production of hydrogen sulphide (H_2S), Ferrous sulphate is the indicator used for the detection of H_2S which is indicated by the production of insoluble black precipitate.

Reagents and glass wares:

Triple sugar iron agar, test tube and inoculating loop.

Procedure:

Test tube by means of a stab and streak

⇩

By using the sterile technique the tested isolates were inoculated in the specific

labelled

⇩

Then the test tubes were incubated for 24 hours.

Quality control:

Alkaline slant/alkaline butt (K/K reaction): *Pseudomonas aeruginosa*

Alkaline slant/acidic butt (K/A reaction): *Shigella* spp

Alkaline slant/alkaline butt/ production of H_2S (K/A reaction and positive for H_2S): *Proteus mirabilis*

Acidic slant/acidic butt (A/A reaction): *Escherichia coli*

Acidic butt/acidicslant, production of H_2S: *Citrobacter* spp.

Results and interpretation:

Alkaline slant/alkaline butt (K/K reaction): This shows no carbohydrate fermentation and indicates that bacteria are non-fermented.

Alkaline slant/acidic butt (K/A reaction): Glucose is fermented, lactose and sucrose is not fermented which indicates the organisms is a non-lactose fermenter.

Alkaline slant /acidic butt / black precipitate of H_2S (K/A reaction and H_2S production): Glucose is fermented. Lactose and sucrose are not fermented. This is characteristic of non-lactose fermenting, hydrogen sulphide producing bacteria.

Acidic slant/acidic butt (A/A reaction): All the sugars (glucose, lactose and sucrose) are fermented. This is characteristic of lactose fermenting coliforms.

3.3.3.9 Amino acid decarboxylase and dehydrolase test (Moller's method):

Principle:

Deaminase activity that attack the carboxyl group of specific amino acids, forming amines and carbon dioxide. The amines formed are alkaline and they alter the colour of pH indicator. Each decarboxylase is specific for a particular

amino acid. Tests for lysine decarboxylase, ornithine decarboxylase and arginine dehydrolase are generally performed on the enteric bacteria. Lysine is decarboxylated to cadaverine; ornithine is dcarboxylated to purescine; arginine undergoes dehydrolase reaction to form citruline, which is then converted to ornithine in a decarboxylation.

Reagents and glass wares: Decarboxylase media: Lysine 1%, Ornithine 1%, arginine 1%, mineral oil and test tube

Procedure:

Test cultures were inoculated into the tubes containing decarboxylase media

⇩

Control test tube were taken for each organism

⇩

Media for each amino acid (Lysine 1%, Ornithine 1%, arginine 1%) to be tested

⇩

All the test tubes were overlaid with 5-10 mm of sterile mineral oil

⇩

Then each tube was incubated for 24 hours at 35˚C.

Quality control:

Arginine:

Positive control: *Enterobacter cloaceae*

Negative control: *Klebsiella pneumonia*

Ornithine:

Positive control: *Enterobacter cloaceae*

Negative control: *Klebsiella pneumonia*

Lysine:

Positive control: *Klebsiella pneumonia*

Negative control: *Enterobacter cloacea*

Interpretation:

Glucose fermentation indicates the organism is viable and the medium turn's yellow which shows the negative results and decarboxylation is indicated by a blue-purple colour in the medium

3.3.3.10 Phenyl alanine deaminase test:

Principle:

Deaminase activity of bacteria can be determined using amino acid phenylalanine which is converted into phenyl pyruvic acid.

Reagents and glass wares:

Phenylalanine, 10% ferric chloride.

Procedure:

Phenylalanine was dissolved into 1ml of sterile distilled water

⇩

Pure cultures of test organism was inoculated

⇩

Incubated at 35˚C for 24 hours

⇩

Four to five drops of 10% ferric chloride was added to the inoculate media

⇩

Finally observed the colour changes of the medium

Quality control:

Positive control: *Proteus mirabilis*

Negative control: *Escherichia coli*

Interpretation:

Positive result: Intense green colour

Negative result: No colour change

3.3.3.11 Sugar fermentation:

Principle:

This test was used to detect an organism's ability to ferment the sugars glucose, lactose, sucrose and mannitol as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous by product.

Reagents and glass wares:

Carbohydrate fermentation broth (Sucrose, mannitol, lactose and glucose), nutrient broth, Boro Cresol purple, Durham'stubes, test tubes.

Procedure:

A carbohydrate fermentation broth was prepared at pH 7.4 and distributed in four sterile test tubes for each sample

⇩

The broth contains 0.5% of carbohydrate to be tested (Sucrose, mannitol, lactose and glucose) nutrient broth and pH indicator Boro Cresol purple.

⇩

Inverted Durham's tub filled with carbohydrate fermentation broth was placed in the test tube to detect the production of gas

⇩

Bacterial cultures was inoculated in the tubes and incubated at 37˚C for overnight.

Quality control:

Fermentative: Yellow colour formation.

Non-fermentative: Purple colour.

Results and interpretation:

Colour of the media changes Yellow with gas in Durham's tube: Fermentation occurs with gas production.

Colour of the media changes Yellow without gas in Durham's tube: fermentation without production of gas.

Colour of media Purple: not fermented.

3.3.3.12 Nitrate reduction test:

Principle:

Bacteria demonstrating nitrate reduction that has capability of extracting oxygen from nitrates to form nitrite and other reduction products. The nitrite production test is detected by adding α-naphthylamine and sulphanilic acid with the formation of a red diagonium dye, p-sulfobenzene-azo-α naphthylamine.

Reagents and glass wares:

1% potassium nitrate broth (KNO_3), peptone, potassium nitrate, α-naphthylamine, acetic acid, sulphanilic acid, acetic acid, inoculating loop, spirit lamp and test tube.

Procedure:

An equal volume of reagent I and reagent II was mixed in a test tube

⇩

Then 0.1 ml of mixed reagent added in a culture broth

⇩

The colour change was observed for a few minutes.

Quality control:

Positive control: *Echerichia coli*

Negative control: *Acinetobacter bumannii*

Interpretation: After adding the mix reagents in a culture broth red colour was observed in a positive isolates and no such was observed in a negative isolates. To avoid the false negative results zinc dust was use to indicates the residue of unutilized nitrates which indicate the ture negative reaction.

3.3.3.13 Oxidative-fermentive test (O/F/ Huge and Leifson test):

Principle:

Oxidative Fermentation (O/F) test was used to differentiate those organisms that utilized carbohydrates aerobically (Oxidation), from those that utilized carbohydrates anaerobically (Fermentation), such as members of the *Enterobactericeae*. This test was developed by Hugh and Leifson so the O/F medium was known as Hugh Leifson medium.

The test organism was inoculated into two tubes of a tryptone or peptone agar medium containing glucose (or other carbohydrate) and the indicator bromothymol blue. The inoculated medium in one tube was sealed with a layer of liquid paraffin to prevent diffusion of oxygen. If the inoculated organisms utilized the carbohydrate in both the

open and sealed tubes which was indicated by change in the color of the medium from green to yellow, then the organism is fermentative.

If the inoculated organisms utilized carbohydrate only in the open tube without utilizing carbohydrate in the sealed tube (medium remains green), the organism was identified to be oxidative. Dipotassium phosphate buffer is added to further promote acid detection.

If the inoculated organism do not utilize carbohydrates, then there was no change in color of the medium in the oil-covered tube and in some cases, some organism may utilize peptone resulting in amine production and cause an increase in pH of the media changing the bromothymol blue from green to blue in the top of the open tube.

Reagents and glass wares: Hugh Leifson medium (Glucose, maltose and sucrose), liquid paraffin, test organisms.

Procedure:

Inoculum was stabbed to the bottom of the duplicate tubes containing O/F medium

⇩

A layer of liquid paraflin was added to one set of tubes for blocking air passage

⇩

Then the tubes were incubated for 24 hours at 35^0C up to 14 days and checked daily for carbohydrate utilization.

Quality control:

Oxidative control: *Pseudomonas aeruginosa*

*F*ermentative control: *Escherichia coli.*

Interpretation:

Fermentation: Bacteria that can ferment glucose give a fermentative result as indicated by acid production in both the open (aerobic) and sealed (anaerobic) tube. The acid produced changes the pH indicator, bromthymol blue, from green to yellow. The semisolid consistency of the medium also allows for detection of motility as a hazy growth away from the stab line can be visualized.

Oxidation: Non fermenting bacteria metabolized glucose via oxidative metabolism give an oxidative result as indicated by a small amount of acid production in the open tube. The acid produced changes the pH indicator, bromthymol blue, from green to yellow. After 24-hours incubation, a change in pH is observed at the surface of the open tube where growth in the presence of oxygen is observed.

No color change or reaction occurs in the oil-covered tube.

Negative result: Non-saccharolytic bacteria give a negative O/F result as indicated by no color change in the oil-covered tube and in some cases an increase in pH changing the bromthymol blue from green to blue in the top of the open tube. The increase in pH is due to amine production by bacteria that break down the peptone (protein) in the medium. Other bacteria give a negative result indicated by no growth or color change in the medium.

Hugh and Leifson's O/F basal medium:

Peptone (tryptone)	2.0 g
Sodium chloride	5.0 g
Glucose (or other carbohydrate)	10.0 g
Bromthymol blue	0.03 g
Agar	3.0 g
Dipotassium phosphate	0.30 g

Preparation of Hugh and Leifson's O/F medium: The desired amount of the commercially available Hugh Leifson media is weighted in a sterile flask and 1 litter of distilled water is added at pH 7.1. After sterilization 10% solution of carbohydrate is aseptically added to the medium to a final concentration of 1%. The sterile medium containing the carbohydrate is aliquoted aseptically into sterile test tubes and cooled unslanted as stabs.

3.4: Antibiotic susceptibility:

3.4.1 Inoculum preparation:

Pure culture was selected for preparation of inoculum. Inoculum was prepared in test tube containing 1.5 ml of sterile nutrient broth by mixing few colonies of the test strain. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units ($1.5x10^8$ CFU/ml).

3.4.2 Preparation of 0.5 McFarland standards:

Solution A was prepared by adding burium chloride ($BaCl_2$, $2H_2O$) to 100ml of distilled water. Solution B was prepared by adding 1 ml of sulphuric acid (H_2SO_4, 0.36N) to 100ml of distilled water. Then 0.5 ml solution A was added to 99.5ml of solution B, mixed well and was distributed in to test tubes with a screw cap and was kept in a dark place.

3.4.3 Disk Diffusion Method:

Isolates were inoculated within 15 min of preparation of suspension.

⇩

A sterile cotton swab was dipped into the suspension and surplus was removed by rotation of swab against the side of the tube above the fluid level.

⇩

The medium was inoculated by the even spreading of swab over the entire surface of the plate in three directions.

⇩

The inoculum was allowed to air dry for 5-15 minutes

⇩

Then commercially available antibiotic disks were applied aseptically with the help of forceps at least 24mm apart.

⇩

Plates were incubated inverted for 16-18 hr. at 37˚C.

⇩

E.coli ATCC 25922 was taken as a quality control

⇩

After incubation the diameters of the zone of inhibition was measured and compared with standard chart provided in CLSI guideline (CLSI 2013).

Table 3: Antimicrobial agents used for the study

Serial number	Antimicrobial Agents	Strength (µg)	Source
1	Ampicillin	10	Hi-Media, Mumbai, India
2	Cefepime	50	Hi-Media, Mumbai, India
3	Ciprofloxacin	5	Hi-Media, Mumbai, India
4	Gentamicin	10	Hi-Media, Mumbai, India
5	Trimethoprim/ Sulfomethoxazole	1.25/23.75	Hi-Media, Mumbai, India

3.5 Minimum inhibitory concentration (MIC) study:

MIC study was done with a special reference of trimethoprim and sulfomethoxazole antibiotics using Hi- comb MIC strip (Hi-Media). Epsilometer test (E-test) is based on the principle of disk diffusion and it carries 5x50 mm of antibiotic in a plastic strip with a continuous gradient of antibiotic immobilized on one side and MIC interpretative scale corresponding to 15 two fold MIC dilutions on the other side.

3.5.1 Preparation of inoculum:

Pure culture was selected for preparation of inoculum. Inoculum was prepared in test tube containing 1.5 ml of sterile nutrient broth by mixing few colonies of the test strain. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units ($1.5x10^8$ CFU/ml).

3.5.2 Applications of E-strips:

On the agar surface the tested organisms were swabbed and kept for 10-15 min to remove the excess water

⇩

Opened the e-test package and place in a dry petri plates

⇩

E-strips were applied on the media with forceps and MIC scale facing upwards.

⇩

Then it was incubated for 18-24 hours at 37˚C.

3.5.3 Interpretation:

After incubation an elliptical zone growth inhibition was seen around the e-strip and MIC were interpreted according to chart. MIC reading was taken only where the eclipse intersects the scale. Since e-test comprises a continuous gradient so MIC values were between two fold dilutions of the strip.

3.6 Molecular screening of Integron:

For genotypic detection of integron system two different multiplex PCR was performed by targeting *intI1* and *intI2* (Koeleman et al., 2001) (Table 4).

3.6.1 Multiplex PCR:

This PCR was performed to screen out the presence of integrase gene among the isolates.

3.6.2 Preparation of DNA template:

DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.6.3 Boiling centrifugation method:

1 ml of aliquots of the test organisms in Brain Heart Infusion Broth (Hi-Media, Mumbai, India) was centrifuged in 10,000 rpm for 10 minutes. The pellets were suspended in 100µl of sterile distilled water, heated to 95˚C in dry block for 10 minutes, cooled in ice and centrifuged at 10,000rpm for 10 minutes. These supernatants were used for PCR assay.

3.6.4 Preparation of reaction mixture:

Each single reaction mixture (25 µl) contains 1 µl of DNA suspension, 10 pmol of *int1* and *int2* primers, 12.5µl GoTaq®-Green Master mix (Promega Medison,USA).

3.6.5 Reaction condition:

The PCR conditions were 94°C for 3 min, followed by 94°C for 20 s, 54°C for 20 s, 72°C for 1 min and final extension 72°C for 5 min. 32 cycles.

Table 4: list of primers used for characterization of class I and class II integron

Primer	Nucleotide Sequence (5′ to 3′)	Product size (bp)	Reference
***Int 1* F**	CAG TGG ACA TAA GCC TGT TC	**160**	Koeleman et al., *J Clin Microbiol* 2001
***Int 1* R**	CAG TGG ACA TAA GCC TGT TC		
***Int 2* F**	TTG CGA GTA TCC ATA ACC TG	**288**	Koeleman et al., *J Clin Microbiol* 2001
***Int 2* R**	TTA CCT GCA CTG GAT TAA GC		

3.6.7 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 100bp DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.6.8 Preparation of TBE buffer:

Tris base	0.8 gm
Boric acid	5.5 gm
0.5M EDTA	4 ml
Distilled water	200 ml

3.6.9 Interpretation:

A distinct band was formed at the base pair 160 and 288 for *intI1* and *intI2* respectively.

3.7 Screening of sulphonamide resistance:

Multiplex PCR was performed with integron positive as well as integron negative isolates. Primers used for the study to amplify *sul1*, *sul2* and *sul3* (Table 5).

3.7.1 Preparation of DNA template:

DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.7.2 Boiling centrifugation method:

1 ml of aliquots of the test organisms in Brain Heart Infusion Broth (Hi-Media, Mumbai, India) was centrifuged in 10,000 rpm for 10 minutes. The pellets were suspended in 100µl of sterile distilled water, heated to 95˚C in dry block for 10 minutes, cooled in ice and centrifuged at 10,000rpm for 10 minutes. These supernatants were used for PCR assay.

Table 5: List of primers used for detection of *Sul* gene.

Primer	**Nucleotide Sequence (5' to 3')**	**Product size (bp)**	**Reference**
***Sul 1* F**	CCGATATTGCTGAGGCGG	**265**	David C.Bean et al., *J.AAC* 2009
***Sul1* R**	CCAACGCCGACTTCAGCT		
***Sul2* F**	TCGTCAACATAACCTCGGACAG	**479**	David C.Bean et al., *J.AAC* 2009
***Sul2* R**	GTTGCGTTTGATACCGGCAC		
***Sul3* F**	GAGCAAGATTTTTGGAATCG	**790**	David C.Bean et al., *J.AAC* 2009
***Sul3* R**	CATCTGCAGCTAACCTAGGGCTTTGGA		

3.7.3 Preparation of reaction mixture:

Each single reaction mixture (30 μl) contained 1.5 μl of DNA suspension, 10 pmol of each primer (*sul1*, *sul2* and *sul3*) and *Taq* DNA polymerase (Hi-Media, Mumbai, India).

3.7.4 Reaction condition:

Amplification was carried out by heating for 3 min at 95°C, followed by 34 cycles at 95°C for 20 sec, 58°C for 1 min and 72°C for 45 sec, followed by 72°C for 5 min.

3.7.5 Gel electrophoresis and analysis of PCR products:

The DNA fragments of *sul* genes amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 100bp DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.7.6 Interpretation:

A distinct band was formed at the base pair 265, 479 and 790 for *sul1, sul2* and *sul3* respectively.

3.7.6.1 Cloning of sulphonamide resistant genes:

For amplification of whole gene primer pair was designed for amplification of whole genome of *sul* gene and PCR was carried out using designed primers (Table 6) and amplified products were cloned using pGEM-T vector (Promega, Madison, USA), transformed into *E.coli, JM107.*

3.7.6.2 Preparation of reaction mixture:

25µl master mix for PCR cycle was prepared by using pfu DNA Polymerase (SRL, India), Primers 10 pmol, DNA template and deionised water.

3.7.6.3 Reaction condition:

Amplification was carried out with initial denaturation for 2 min at 94°C, followed by 35 cycles at 94°C for 15 sec, 52°C for 20 sec and 72°C for 1.3 min, followed by 72°C for 7 min.

Table 6: Primers for cloning of *sul* gene

Primer	**Nucleotide Sequence (5' to 3')**	**Product size (bp)**	**Reference**	**Primer**
Sul 1 XF	AGT TGG CGA AGT AAT CGC AAC	1300	*Sul1* whole gene	This study
Sul 1 XR	ACG CAC AGT CAA CTT ATT GGA TG			
Sul 2 YF	ATT GCC TAC TGA GCG CTG CC	1051	*Sul2* whole gene	This study
Sul 2YR	CTT CAG TTT TCT GAT GAA GCG			
Sul 3ZF	CAG CGC ATT TTT AAT GCA AAG G	1374	*Sul3* whole gene	This study
Sul 3ZR	CAA GTA CGC CAA CAC AAC TTC AG			

3.7.6.3 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 1kb DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.7.6.4 Ligation:

Purified PCR products were ligated in pGEM-T vector (Promega, Madison, USA), 20 µl per tube of ligation mixture was prepared.

3.7.6.5 Composition of ligation mixture:

2X ligation buffer	2µl
pGEM-T vector	1 µl
Target gene (Purified product)	10µl
T4 DNA ligase (Unit)	2µl

These ligated products were kept at 4˚C for overnight incubation.

3.7.6.6 Transformation of ligated products into *E.coli* JM107 competent cell:

3.7.6.6.1 Preparation of competent cell:

Overnight culture of cells were grown in LB Broth

⇩

Subculture was inoculated by diluting saturated overnight culture 1:100 into LB media

⇩

Cells were grown 37˚C until OD600 reaches ˜ 0.25

⇩

Culture was chilled in ice for 15 min to slow the growth

⇩

Cells were pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Supernatant was decanted

⇩

Cells were resuspended in 1/5 volume of original culture of ice-cold 0.1M $CaCl_2$

⇩

Cells were incubated on ice for 20 min

⇩

Cells was pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Supernatant was decanted

⇩

Cells were resuspended in 1/25 volume of original culture of ice-cold 0.1M $CaCl_2$ and 10% glycerol

⇩

100µl aliquots were made into pre-chilled micro centrifuge tubes

⇩

Stored at -80˚C.

3.7.6.6.2 Transformation Preparation:

50 ul of aliquots were taken for each transformation into 1.5ml tubes

⇩

5 µl DNA was added to the cells and gently mixed

⇩

Incubated for 1 hour on ice

⇩

For Heat shock cells were kept on dry bath at 42°c for .45 sec

⇩

Tubes were incubated on ice for 10 min

⇩

500 µl of pre-warmed SOC

⇩

Shake at 37°c for 1 hr.

⇩

Cells were palleted by centrifugation at 5000 rpm for 2 minutes

⇩

Pallet was taken from each transformation

⇩

LB plates with appropriate antibiotics (Ampicillin 10 μg/ml) were prepared

⇩

Transformants were spread over the plates

⇩

Allow plates to dry and incubated inverted at 37°c overnight.

3.7.6.6.3 Antimicrobial susceptibility of bacterial strains and clones:

Transformants were further subjected to antibiotic susceptibility against sulphafurazole (300µg), trimethoprim (5µg) and Co-trimoxazole (1.25/23.75 µl) [Hi-Media, Mumbai, India]. Minimun inhibition concentration (MIC) for sulphafurazole and trimethoprim were also determined with Hi-Comb MIC test strip [Hi-Media, India] the breakpoint used was the one defined by the CLSI.

3.7.6.6.3.1 Preparation of inoculum:

Inoculum was prepared in test tube containing 1.5 ml of sterile nutrient broth by mixing of clones. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units ($1.5x10^8$ CFU/ml).

3.7.6.6.3.2 **Disk Diffusion Method:**

Cloned were inoculated within 15 min of preparation of suspension.

⇩

A sterile cotton swab was dipped into the suspension and surplus was removed by rotation of swab against the side of the tube above the fluid level.

⇩

The medium was inoculated by the even spreading of swab over the entire surface of the plate in three directions.

⇩

The inoculum was allowed to air dry for 5-15 minutes

⇩

Then commercially available antibiotic disks were applied aseptically with the help of forceps at least 24mm apart.

⇩

Plates were incubated inverted for 16-18 hr. at 37˚C.

⇩

E.coli ATCC 25922 was taken as a quality control

⇩

After incubation the diameters of the zone of inhibition was measured and compared with standard chart provided in CLSI guideline (CLSI 2013).

3.7.6.6.3.3 Minimum inhibition concentration of cloned isolates:

On the agar surface the cloned were swabbed and kept for 10-15 min to remove the excess water

⇩

Opened the e-test package and place in a dry petri plates

⇩

E-strips were applied on the media with forceps and MIC scale facing upwards.

⇩

Then it was incubated for 18-24 hours at 37˚C.

3.8 Amplification of gene cassettes:

Gene cassettes were amplified by 59 base element PCR method outlined by Stokes *et al.,* (2001). Pair of degenerate primers HS 286-HS287 (Table 7) was designed to anneal to the relatively conserved areas at the respective ends of a generic 59-be site.

Table no 7: HS 286-HS287 primer sequence.

Primer	Nucleotide Sequence (5' to 3)	Reference
HS286	GGGATCCTCSGCTKGARCGAMTTGTT AGVC	Stokes H.W et al., *J.App Env Microl* 2001.
HS 287	GGGATCCGCSGCTKANCTCVRRCGTTA GSC	

3.8.1 Preparation of reaction mixture:

30µl master mix for PCR cycle was prepared by using Taq Polymerase (Promega, Madison, USA), Primers 10 pmol HS286 and HS287, DNA template and deionised water.

3.8.2 Reaction condition:

Amplification was carried out with initial denaturation of heating for 2 min at 95°C, followed by 32 cycles at 95°C for 20 sec, 47°C for 1.2 min and 72°C for 1.2 min, followed by 72°C for 7 min.

3.8.3 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 1kb DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.9 Amplification of whole class 1 integron;

Amplification of whole class 1 integron was done by using primer 5-CS and 3-CS (Table 8) targeting 5´conserved region and 3´conserved region of integron and amplified product further analysed through sequencing.

Table 8: Primer for amplification of whole class 1 integron:

Primer	**Nucleotide Sequence (5' to 3)**	**Reference**
5-CS	5´ GGCATCCAAGCAGCAAG 3´	Levesque C. et al., *Antimicrob.Agents.Che mother*,1995.
3-CS	5´ AAGCAGACTTGACCTGA 3´	

3.9.1 Preparation of reaction mixture:

50 µl of master mix contains 1µl (100 nm) of DNA, 10 pmol of each Primers and 25 µl of 2x high fidelity DNA Polymerase (QIAGEN, Germany), and deionised water.

3.9.2 Reaction condition:

Amplification was carried out by heating for 2 min at 95°C, followed by 32 cycles at 95°C for 20 sec, 50°C for 45 sec and 72°C for 2 min, followed by 72°C for 7 min.

3.9.3 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 1kb DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.9.4 PCR Gel product purification:

PCR product was purified by using QIAquick PCR purification Kit (QIAGEN, Germany) as per instruction of manufacturer as follows;

DNA fragments were cut from the agarose gel with a clean and sharp scalpel

⇩

Weight the gel slices was measured

⇩

3 volumes of QG buffer was added to 1 volume of gel

⇩

Incubation was done for 10 min at 50˚C until the gel was completely dissolved in buffer

⇩

Then 1 volume of isopropanol was added and placed in a QIAquick spin column

⇩

To bind DNA the sample to the QIAquick column and centrifuge for 1 min

⇩

The flow through was discarded from the same collection tubes

⇩

To wash the DNA 750µl of PE buffer was added and centrifuge for 1 min

⇩

The flow through was discarded and centrifuged the QIAquick column for an additional 1 min.

3.9.5 Sequencing of all gene:

30µl of purified PCR products were used for sequencing along with gene specific primer 20µl each (20 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nih.gov/Blast.egi).

3.9.6 Cloning of whole gene:

Amplified product were cloned using pGEM-T vector (Promega, Madison, USA), transformed into E.coli, JM107.

3.9.7 Ligation:

Purified PCR products were ligated in pGEM-T vector (Promega, Madison, USA), 20 µl per tube of ligation mixture was prepared.

3.9.7.1 Composition of ligation mixture:

2X ligation buffer	2µl
pGEM-T vector	1 µl
Template (Purified product)	10µl
T4 DNA ligase	2µl

These ligated products were kept at 4˚C for overnight incubation.

3.9.8 Transformation of ligated products into *E.coli* JM107 competent cell:

3.9.8.1 Preparation of competent cell:

Overnight culture of cells were grown with appropriate drug selection

⇩

Subculture was inoculated by diluting saturated overnight culture 1:100 into LB media

⇩

Cells were grown 37˚C until OD600 reaches ˜ 0.25

⇩

Culture was chilled in ice for 15 min to slow growth

⇩

Cells was pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Decant the supernatant

⇩

Cells were resuspended in 1/5 volume of original culture of ice-cold 0.1M $CaCl_2$

⇩

Cells were incubated on ice for 20 min

⇩

Cells was pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Supernatant was decanted

⇩

Cells were resuspended in 1/25 volume of original culture of ice-cold 0.1M

$CaCl_2$, 10% glycerol

⇩

100µl aliquots were made into pre-chilled micro centrifuge tubes

⇩

Stored at -80˚C

3.9.8.2 Transformation procedure:

50 µl of aliquots were taken for each transformation into 1.5ml tubes

⇩

5 µl DNA was added to the cells and gently mixed

⇩

Incubated for 1 hour on ice

⇩

For Heat shock cells were kept on dry bath at 42°c for .45 sec

⇩

Tubes were incubated on ice for 10 min

⇩

500 µl of pre-warmed SOC

⇩

Shake at 37°c for 1 hr.

⇩

Cells were palleted by centrifugation at 5000 rpm for 2 minutes

⇩

Pallet was taken from each transformation

⇩

LB plates with appropriate antibiotics (Ampicillin) were prepared

⇩

Transformants were spread over the plates

⇩

Allow plates to dry and incubated inverted at 37°c overnight.

3.9.8.3 Antimicrobial susceptibility of bacterial strains and clones:

Transformants were further subjected to antibiotic susceptibility against ampicillin (10 mcg), gentamicin (10 mcg), kanamycin (30 mcg), Streptomycin (10 mcg), Sulphafurazole (300 mcg), trimethoprim (5 mcg) [Hi-Media, Mumbai, India].

3.9.8.1 Preparation of inoculum:

Inoculum was prepared in test tube containing 1.5 ml of sterile nutrient broth by mixing of clones. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units ($1.5x10^8$ CFU/ml).

3.9.8.2 Disk Diffusion Method:

Cloned were inoculated within 15 min of preparation of suspension.

⇩

A sterile cotton swab was dipped into the suspension and surplus was

⇩

removed by rotation of swab against the side of the tube above the fluid level.

⇩

The medium was inoculated by the even spreading of swab over the entire surface of the plate in three directions.

⇩

The inoculum was allowed to air dry for 5-15 minutes

⇩

Then commercially available antibiotic disks were applied aseptically with the help of forceps at least 24 mm apart.

⇩

Plates were incubated inverted for 16-18 hr. at 37˚C.

⇩

E.coli ATCC 25922 was taken as a quality control

⇩

After incubation the diameters of the zone of inhibition was measured and compared with standard chart provided in CLSI guideline (CLSI 2013).

3.10 Mapping of genetic arrangement of class II integron:

In order to determine the genetic array of class II integron Hep 74 and Hep 51 primers (Table 9) was used by targeting within the variable region of the isolates harbouring class II integron

Table 9: Primer for cloning of class II integron

Primer	Nucleotide Sequence (5' to 3)	Reference
Hep 74	CGGGATCCCGGACGGCATGCACG ATTTGTA	White et al., *J Antimicrob. Agents chemother*. 2001
Hep 51	GAT GCCATCGCAAGTACGAG	

3.10.1 Preparation of reaction mixture:

25µl master mix for PCR cycle was prepared by using Taq Polymerase (Promega, Madison, India), Primers 10 pmol, DNA template and deionised water.

3.10.2 Reaction condition:

Amplification was carried out by heating for 2 min at 95°C, followed by 35 cycles at 95°C for 20 sec, 56°C for 40 sec and 72°C for 2 min, followed by 72°C for 5 min

3.10.3 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 100bp DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.10.4 PCR Gel product purification:

DNA fragments were cut from the agarose gel with a clean and sharp scalpel

⇩

Then weight the gel slices in a colourless tubes

⇩

3 volumes of QG buffer was added to 1 volume of gel

⇩

Incubation was done for 10 min at 50˚C until the gel was completely dissolved in buffer

⇩

Then 1 volume of isopropanol was added and placed in a QIAquick spin column

⇩

To bind DNA the sample to the QIAquick column and centrifuge for 1 min

⇩

Discard the flow through from the same collection tubes

⇩

To wash the DNA 750µl of PE buffer was added and centrifuge for 1 min

⇩

Discard the flow through and centrifuge the QIAquick column for an additional 1 min.

3.10.5 PCR purification of PCR product:

A total of 5 volumes of Buffer PB was added to the 1 volume of the PCR reaction and mixed well

⇩

Then it transferred to the MinElute column and centrifuge for 1min at 13,000rpm

⇩

Discard the flow through the MinElute column

⇩

750 µl of Buffer PB was added to the MinElute column and again centrifuge was done for 1 min

⇩

Discard the flow-through and place the MinElute column back in the same collection tube

⇩

Additional 1min centrifugation was done to remove the unless residual ethanol from PE buffer

⇩

Then clean 1.5 ml microcentrifuge tubes was placed

⇩

DNA elution was done with 30µl of EB buffer or water to the centre of the MinElute membrane

⇩

Centrifugation was done for 1 min at 13,000rpm.

3.10.6 Sequencing of all gene:

30µl of purified PCR products were used for sequencing along with gene specific primer 20µl each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nih.gov/Blast.egi).

3.10.7 Cloning of class II integron gene cassette array:

Amplified products were ligated on to pGEM-T vector (Promega, Medison ,USA) and transformed into *E.coli* JM107. Transformants were selected by blue white screening.

3.10.8 Ligation:

Purified PCR products were ligated in pGEM-T vector (Promega, Madison, USA), 20 µl per tube of ligation mixture was prepared.

3.10.8.1 **Composition of ligation mixture:**

2X ligation buffer	2µl
pGEM-T vector	1 µl
Template (Purified product)	10µl
T4 DNA ligase	2µl

These ligated products were kept at 4˚C for overnight incubation.

3.10.9 Transformation of ligated products into *E.coli* JM107 competent cell:

3.10.9.1 Competent cell preparation:

Overnight culture of cells were grown with appropriate drug selection

⇩

Subculture was inoculated by diluting saturated overnight culture 1:100 into LB media

⇩

Cells were grown 37˚C until OD600 reaches ˜ 0.25

⇩

Culture was chilled in ice for 15 min to slow growth

⇩

Cells was pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Decant the supernatant

⇩

Cells were resuspended in 1/5 volume of original culture of ice-cold 0.1M $CaCl_2$

⇩

Cells were incubated on ice for 20 min

⇩

Cells was pelleted by centrifugation at 2500xg for 15 minutes in at 4˚C

⇩

Supernatant was decanted

⇩

Cells were resuspended in 1/25 volume of original culture of ice-cold 0.1M $CaCl_2$, 10% glycerol

⇩

100µl aliquots were made into pre-chilled micro centrifuge tubes and stored at -80˚C

3.10.9.2 Transformation procedure:

50 ul of aliquots were taken for each transformation into 1.5ml tubes

⇩

5 µl DNA was added to the cells and gently mixed

⇩

Incubated for 1 hour on ice

⇩

For Heat shock cells were kept on dry bath at 42°c for .45 sec

⇩

Tubes were incubated on ice for 10 min

⇩

500 µl of pre-warmed SOC

⇩

Shake at 37°c for 1 hr.

⇩

Cells were palleted by centrifugation at 5000rpm for 2 minutes

⇩

Pallet was taken from each transformation

⇩

LB plates with appropriate antibiotics (Ampicillin) were prepared

⇩

Transformants were spread over the plates

⇩

Allow plates to dry and incubated inverted at 37°c overnight.

3.10.9.3 Antimicrobial susceptibility of bacterial strains and clones:

Transformants were further subjected to antibiotic susceptibility against gentamicin (10 mcg), nitilimicin (30 mcg), amikacin (30 mcg), sulphafurazole (300 mcg), trimethoprim (5 mcg), kanamicin (30 mcg) [Hi-Media, Mumbai, India].

3.10.9.1 Preparation of inoculum:

Inoculum was prepared in test tube containing 1.5 ml of sterile nutrient broth by mixing of clones. The density of suspension was standardized by dilution with sterile broth to a density visually equal to 0.5 McFarland units ($1.5x10^8$ CFU/ml).

3.10.9.2 Disk Diffusion Method:

Cloned were inoculated within 15 min of preparation of suspension.

⇩

A sterile cotton swab was dipped into the suspension and surplus was removed by rotation of swab against the side of the tube above the fluid level.

⇩

The medium was inoculated by the even spreading of swab over the entire surface of the plate in three directions.

⇩

The inoculum was allowed to air dry for 5-15 minutes

⇩

Then commercially available antibiotic disks were applied aseptically with the help of forceps at least 24mm apart.

⇩

Plates were incubated inverted for 16-18 hr. at 37˚C.

⇩

E.coli ATCC 25922 was taken as a quality control

⇩

After incubation the diameters of the zone of inhibition was measured and compared with standard chart provided in CLSI guideline (CLSI 2013).

3.11 DNA fingerprinting of study isolates:

Typing of isolated organisms was done by Enterobacterial repetitive intergenic consensus (ERIC) PCR. Primer used for the study was ERIC-F 5´ ATGTAAGCTCCTGGGGATTCAC3´, ERIC–R 5´AAGTAAGTGACTGGGGTGAGCG3´ primers (Versalovia et al., 1991).

3.11.1 Preparation of DNA template:

DNA was extracted by boiling centrifugation method (Freschi et al.,2005)

3.11.2 Preparation of reaction mixture:

25µl master mix for PCR cycle was prepared by using Taq Polymerase (Promega, Madison, USA), Primers 10 pmol each primer ERIC-F and ERIC -R, DNA template and deionised water.

3.11.3 Reaction condition:

Reaction was run under following condition: initial denaturation 95 ºC for 3 min, 30 cycles of 95 ºC for 20 sec, 46ºC for 40 sec, 72ºC for 3 min and final extension at 72 ºC for 10 min.

3.11.4 Gel electrophoresis and visualization of PCR products:

The DNA fragments of ERIC PCR products were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 1KB DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).

3.12 Plasmid incompatibility typing of the isolates:

Plasmid incompatibility types within the study isolates were determined by PCR based replicon typing.

3.12.1 PCR based replicon typing:

This was performed using 18 pairs of different basic replicon primers,by 5 multiplex and 3 simplex PCR array to recognize the FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons (Table 10) (Carattoli et al., 2005).

Table 10: Primers used for replicon typing

PRIMER	DNA SEQUENCE	SIZE
HI 1 FW	5’-GGAGCGATGGATTACTTCAGTAC-3’	471
HI1 RV	5’-TGCCGTTTCACCTCGTGAGTA-3’	
HI2 FW	5’-TTTCTCCTGAGTCACCTGTTAACAC-3’	644
HI2 RV	5’-GGCTCACTACCGTTGTCATCCT-3’	
I1 FW	5’-CGAAAGCCGGACGGCAGAA-3’	139
I1 RV	5’-TCGTCGTTCCGCCAAGTTCGT-3’	
X FW	5’-AACCTTAGAGGCTATTTAAGTTGCTGAT-3’	376
X RV	5’-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3’	
L/M FW	5’-GGATGAAAACTATCAGCATCTGAAG-3’	785
L/M RV	5’-CTGCAGGGGCGATTCTTTAGG-3’	
N FW	5’-GTCTAACGAGCTTACCGAAG-3’	559
N RV	5’-GTTTCAACTCTGCCAAGTTC-3’	
FIA FW	5’-CCATGCTGGTTCTAGAGAAGGTG-3’	462
FIA RV	5’-GTATATCCTTACTGGCTTCCGCAG-3’	
FIB FW	5’-GGAGTTCTGACACACGATTTTCTG-3’	702
FIB RV	5’-CTCCCGTCGCTTCAGGGCATT-3’	
W FW	5’-CCTAAGAACAACAAAGCCCCCG-3’	242
W RV	5’-GGTGCGCGGCATAGAACCGT-3’	
Y FW	5’-AATTCAAACAACACTGTGCAGCCTG-3’	765
Y RV	5’-GCGAGAATGGACGATTACAAAACTTT-3’	
P FW	5’-CTATGGCCCTGCAAACGCGCCAGAAA-3’	534
P RV	5’-TCACGCGCCAGGGCGCAGCC-3’	

FIC FW	5’-GTGAACTGGCAGATGAGGAAGG-3’	262
FIC RV	5’-TTCTCCTCGTCGCCAAACTAGAT-3’	
A/C FW	5’-GAGAACCAAAGACAAAGACCTGGA-3’	465
A/C RV	5’-ACGACAAACCTGAATTGCCTCCTT-3’	
T FW	5’-TTGGCCTGTTTGTGCCTAAACCAT-3’	750
T RV	5’-CGTTGATTACACTTAGCTTTGGAC-3’	
FIIS FW	5’-CTGTCGTAAGCTGATGGC-3’	270
FIIS RV	5’-CTCTGCCACAAACTTCAGC-3’	
FrepB FW	5’-TGATCGTTTAAGGAATTTTG-3’	270
FrepB RV	5’-GAAGATCAGTCACACCATCC-3’	
K/B FW	5’-GCGGTCCGGAAAGCCAGAAAAC-3’	160
K RV	5’-TCTTTCACGAGCCCGCCAAA-3’	
B/O RV	5’-TCTGCGTTCCGCCAAGTTCGA-3’	159

3.12.2 Preparation of DNA template:

Plasmid DNA was isolated from the study isolates by QIAprep® Spin Miniprep Kit and the steps were performed according to manufacturer's guidelines:

Overnight cultures of bacterial isolates were centrifuged at >8000 rpm for 3 min and pellet was collected

⇩

Bacterial cells pelleted was resuspended in 250 µl Buffer P1 and transfer to a microcentrifuge tube.

⇩

250 µl of P2 buffer was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes clear.

⇩

Then 350 µl Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times.

⇩

Centrifugation was done for 10 min at 13,000rpm

⇩

Supernatant was decanted from QIAprep spin column and again centrifuge for 30-60s, discard was flow-through

⇩

500µl PB buffer was added to the QIAprep spin column and centrifuge for 30-60s, discard the flow through

⇩

Then 750 µl PE buffer was added to the QIAprep spin column and centrifuge for 30-60s, discard the flow through

⇩

QIAprep spin column was transfer to the collection tube

⇩

Centrifugation was done for 1 min and residual wash buffer was decanted

⇩

Elute the DNA with 50µl EB buffer and placed in a 1.5 ml centrifugation tube.

3.12.3 Preparation of reaction mixture:

3.12.3.1 PCR protocol: 25µl master mix for PCR cycle was prepared by using Taq Polymerase (Promega, Medison,USA), Primers 10 pmol of each primer, DNA template and deionised water.

3.12.3.2 Reaction Condition for multiplex PCR: Amplification was carried with initial denaturation for 2 min at 95°C, followed by 30 cycles at 95°C for 20 sec, 60°C for 30 sec and 72°C for 1 min, and final extension with 72°C for 5 min.

3.12.3.3 Reaction Condition for simplex PCR: Amplification was carried with initial denaturation for 2 min at 95°C, followed by 30 cycles at 95°C for 20 sec, 52°C for 30 sec and 72°C for 1 min, and final extension with 72°C for 5 min.

3.12.3.4 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with TBE buffer.

Molecular marker of 1 kb DNA ladder (Genei, Banglore, India) was run concurrently. The gel as visualized in Gel Doc EZ imager (Bio-Rad).