3. Methodology

The present study was conducted in the Department of Microbiology, Assam University, Silchar, Assam, India. The duration of the study was November 2011 to April 2013. The bacterial strains were collected from Silchar Medical College and Hospital, Silchar, Assam. This tertiary referral hospital serves around 38,26,110 number populations of Assam [Including districts like Cachar, Hailakandi, Karimganj, North Cachar Hills (Dima Hasao)] and neighbouring states like North Tripura, Mizoram, Meghalaya and Manipur.

3.1 Patient population:

The patients who were admitted to different wards and those who attended the outpatient departments from November 2011 to April 2013 were selected for the study. Subject 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 of 643 consecutive non duplicate different clinical specimens have been studied during the period (Table 2) of which 405 from indoor patient and 258 were from outdoor patients in different wads/OPDs (Table 3).

3.2 Isolates for the study:

A total of 663 isolates were obtained from the above samples which included members of *Enterobacteriaceae* family and non fermenting gram negative rods (Table 4). All the isolates were identified according to gram stain, cultural characteristics and biochemical reactions (Collee et al., 1996).

Table 2: Types of clinical specimens obtained for the study

Clinical specimens	Indoor (n=405)	Outdoor (n=238)	Total (n=643)
Urine	187	121	308
Pus	203	28	231
Stool	1	21	22
Ear swab	0	9	9
Throat swab	1	11	12
Oral swab	0	17	17
Sputum	7	13	20
Ascitic fluid	0	1	1
Drain tip	3	0	3
Blood	0	3	3
Conjuctivial scrapping	0	2	2
Pseudo pancreatic fluid	0	1	1
Wound aspirate	1	0	1
Bile	1	0	1
Urethral discharge	1	0	1
Nasal Scrapping	0	11	11
Total (n)	405	238	643

Table 3: Clinical specimens obtained from different Wards/OPD's

Wards/OPDs	Indoor (n= 405)	Outdoor (n= 238)	Total (n= 643)
Surgery	207	21	228
Medicine	63	131	194
Paediatrics	40	29	69
ENT*	0	30	30
TB**	3	0	3
Burn	7	0	7
Orthopaedics	31	6	37
Gynaecology	40	18	58
Skin	0	3	3
ICU***	13	0	13
Trauma	1	0	1

*__ENT__- Eye Nose Throat; **__TB__- Tuberculosis; ***__ICU__-Intensive care unit

Table 4: Different isolates obtained in the study

Name of organisms	**Isolated from indoor patients (n=373)**	**Isolated from outdoor patients (n=290)**	**Total number(n=663)**
Escherichia coli	144	83	227
Pseudomonas aeruginosa	101	107	208
Pseudomonas Spp.	40	19	59
Klebsiella pnuemoniae	35	45	80
Klebsiella oxytoca	16	20	36
Vibrio cholerae	0	3	3
Acinetobactor baumanii	12	4	16
Proteus vulgaris	13	4	17
Proteus mirabilis	12	5	17
Total (n=)	373	290	663

3.3 Isolation and identification of isolates from clinical specimens:

The specimens were first inoculated onto Blood agar, MacConkey agar and CLED agar (Urine isolates) and incubated at 37°C overnight. Colony morphology and cultural characteristics were observed microscopically.

Identification of gram negative rods was done by Gram staining, motility, and standard biochemical reactions.

3.3.1 Gram staining:

A thin smear of the culture was prepared and allowed to air dry and fix

↓

The smear was overlaid with crystal violet for 1 min.

↓

Rinsed with distilled water

↓

The smear was flooded with Gram's iodine for 1min.

↓

Rinsed with distilled water

↓

Ethyl alcohol was added drop by drop until no violet color appears in rinse

↓

Rinsed with distilled water immediately

↓

The smear was overlaid with safranin for 30 Sec.

↓

Rinsed with distilled water

↓

Air dried

↓

Observed under oil immersion objective for characteristic Gram stain reaction

3.3.1.1 Interpretation:

Gram positive organism stained **purple**

Gram negative organism stained **pink red**

3.3.2 Motility (hanging drop preparation)

A clean cover slip was taken and paraffin or Vaseline was applied to four corners of the cover slip.

A drop of broth culture was placed on the cover slip with the help of inoculating loop.

A clean grease free cavity slide (cavity down) was placed over the cover slip so that the drop is placed in the center.

The slide was inverted and observed under the microscope.

The edge of the drop was first located under low power (10X), after that the focus was shifted to high power (40X) and observed.

3.3.3 Biochemical tests:

3.3.3.1 Catalase test: Catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

With the help of applicator stick a small portion of a well isolated colony that is 18-21 hours old was transferred on to a clean slide

One drop of 3% hydrogen peroxide was added

Formation of rapid and continuous bubble was observed

3.3.3.1.1 Interpretation:

Positive reaction: Production of rapid and continuous gas bubbles

Negative reaction: Lack of gas bubble formation

3.3.3.1.2 Quality control:

***Staphylococcus aureus*:** Positive

***Streptococcus pyogenes*:** Negative

3.3.3.2 Oxidase test:

This test depends on the presence in bacteria of certain oxidases that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye-tetramethyl-*p*-phenylene-diamine.

Oxidase disc (Hi-Media, Mumbai, India) was placed in a clean petriplate.

With the help of melting capillary tube a speck of culture was rubbed in the disc at once.

3.3.3.2.1 Interpretation:

Positive reaction: Formation of intense deep purple color, appearing within 5-10 seconds.

Negative reaction: Absence of coloration or by coloration later than 60 seconds.

3.3.3.2.2 Quality control:

***Pseudomonas aeruginosa*:** positive

***E. coli*:** Negative

3.3.3.3 Indole test:

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium.

Culture was inoculated in the medium containing peptone and NaCl, and incubated at 37°C overnight.

0.5 ml of Kovac's indole reagent (Hi-Media, Mumbai, India) was added to it and shaken gently.

3.3.3.3.1 Interpretation:

Positive reaction: Red ring at the interface of reagent and broth

Negative reaction: No color development.

3.3.3.3.2 Quality control:

***E. coli*:** Positive

***K. pneumoniae*:** Negative

3.3.3.4 Methyl red test:

This test is used to detect the production of sufficient acid during the fermentation of glucose and the maintenance of condition such that the pH value remains below 4.5.

Culture was inoculated in MR-VP medium (Hi-Media, Mumbai, India) and incubated at 37°C for overnight.

Five drops of methyl red indicator (Hi-Media, Mumbai, India) was added to the medium.

It was mixed properly and reading was taken immediately.

3.3.3.4.1 Interpretation:

Positive reaction: Formation of bright red color.

Negative reaction: Formation of yellow color.

3.3.3.4.2 Quality control:

***E. coli*:** Positive

***K. pneumoniae*:** Negative

3.3.3.5 Voges proskauer test:

Many bacteria ferment carbohydrates with the production of acetyl methyl carbonyl or its reduction product 2, 3 butylene glycol.

Culture was inoculated in MR-VP medium (Hi-Media, Mumbai, India) and incubated at 37°C for overnight.

⬇

40% KOH and 5% of α-naphthol, (Hi-Media, Mumbai, India) was added to the medium.

⬇

It was mixed properly and reading was taken immediately.

3.3.3.5.1 Interpretation:

Positive reaction: Pink red color at the surface of the medium

Negative reaction: Yellow color at the surface of the medium

3.3.3.5.2 Quality control:

***K. pneumoniae*:** positve

***E. coli*:** negative

3.3.3.6 Simmons citrate reactions: This test is performed to detect whether bacteria is able to utilize citrate as sole source of carbon.

Slant of Simmon's citrate agar (Hi-Media, Mumbai, India) was prepared in a test tube.

⬇

With the help of a straight wire the culture was stabbed in the bud and streaked on the slant.

The test tube was incubated at 37°C for overnight.

3.3.3.6.1 Interpretation:

Positive reaction: Growth with intense blue color in the slant

Negative reaction: absence of growth and no color change in the medium (remains green)

3.3.3.6.2 Quality control:

***K. pnemoniae*:** positive

***E. coli*:** negative

3.3.3.7 Urease test: Bacteria produce urease enzyme that splits urea in to alkaline end product.

A well isolated 18-24 hours colony was selected and streaked on to the christensen's agar slant

Incubated at 35°C for 18-24 hours.

3.3.3.7.1 Interpretation:

Positive reaction: Pink color in the slant

Negative reaction: No color change in the medium (remains yellow)

3.3.3.7.2 Quality control:

***Proteus mirabilis*:** Positive

***E. coli*:** Negative

3.3.3.8 Triple sugar iron test: The medium contains proteins sources that permit the growth of the most bacterial strains. Lactose, sucrose and glucose are present as well as phenol red indicator. Glucose is in concentration of one tenth that of the other carbohydrates. Ferrous sulphate is present as an indicator of hydrogen sulphide (H_2S) production.

Slant of TSI agar (Hi-Media, Mumbai, India) was prepared.

⬇

With the help of straight wire the culture was stabbed in the bud and streaked on the slope.

⬇

The tube was incubated at 37°C for overnight.

3.3.3.8.1 Interpretation:

The result was interpreted according to the reaction obtained in the test (Table 5).

Table 5: Summary of the TSI reactions

Reaction	Carbohydrate fermented	Typical organisms
Alkaline slant/alkaline butt (K/K reaction)	No carbohydrate formation. Bacteria are non-fermented.	*Pseudomonas aeruginosa*
Alkaline slant/acidic butt (K/A reaction)	Glucose is fermented, lactose and sucrose is not fermented. Organism is non-lactose fermenter.	*Shigella* Spp., *Vibrio* Spp.
Alkaline slant/acidic butt/black precipitate of H2S (K/AH2Spositive reaction)	Glucose is fermented; lactose and sucrose are not fermented. Characteristics of non-lactose fermenting, H_2S producing bacteria.	*Salmonella* Spp., *Proteus* Spp.
Acidic slant/acidic butt (A/A reaction)	Glucose, lactose and sucrose are fermented. Characteristics of lactose fermenting coli form.	*Escherichia coli, Klebsiella* Spp., *Enterobacter* Spp.

3.3.3.9 Phenyl alanine deaminase test: Deaminase activity of bacteria can be determined using amino acid phenylalanine which is converted in to phenyl pyruvic acid.

Phenylalanine was dissolved in to 1 ml 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 ($FeCl_3$) was added to the inoculated media

Result was observed.

3.3.3.9.1 Interpretation:

Positive: Intense green color

Negative: No color change

3.3.3.9.2 Quality control:

***Proteus mirabilis*:** Positive

***E. coli*:** Negative

3.3.3.10 Amino acid decarboxylase and dehydrolase test (Moller's method): The decarboxylase are enzymes that attack the carboxyl group of specific amino acids, forming amines and carbon dioxide. The amines formed are alkaline, and they alter the color 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 decarboxylated to putrescine; arginine undergoes dehydrolase reaction to form citruline, which is then converted to ornithine in a decarboxylation.

Test cultures were inoculated into the tubes of decarboxylase media for each amino acid (1% lysine, 1% ornithine, 1% arginine) to be tested.

A control tube for each organism was also taken

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

Incubated for 24 hours at 35°C

3.3.3.10.1 Interpretation:

- Glucose fermentation indicates the organism is viable and the medium turns yellow
- Decarboxylation is indicated by a blue-purple color in the medium
- All the negative tubes should be incubated again for another 24 hours and reading should be taken thereafter.

3.3.3.10.2 Quality control: All readings were taken at 24 hours.

ARGININE:

Enterobacter cloacae: Positive (purple), alkaline

Klebsiella pneumoniae: Negative (Yellow), acidic

Ornithine:

Enterobacter cloacae: Positive (purple), alkaline

Klebsiella pneumoniae: Negative (Yellow), acidic

Lysine:

Klebsiella pneumonia: Positive (purple), alkaline

Enterobacter cloacae**:** Negative (Yellow), acidic

3.3.3.11 Sugar fermentation: 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 byproduct.

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 the pH indicator phenol red.

Inverted durham tubes 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.

3.3.3.11.1 Interpretation:

Color of the media changes to pink with gas in Durham's tube: Fermentation with production of gas.

Color of the media changes to pink without gas in Durham's tube: Fermentation without production of gas

Color of media remains yellow: Not fermented.

3.3.3.12 Oxidative-Fermentation test (O/F test/ Hugh and Leifson test):

Inoculum was stabbed in duplicate tubes containing O/F medium

To one of the tubes a layer of the liquid parafilm was added to a depth of about 10 mm above the medium to seal it from the air

The tubes were incubated initially for 24 hours at 37°C

Yellow coloration in the media was noted

3.3.3.12.1 Interpretation:

An oxidative organism produces acid from the glucose of the unsealed tube only whereas a fermentative organism produces acid both in sealed and unsealed tubes.

3.3.3.12.2 Quality control:

P. aeruginosa: Oxidative

***E. coli*:** Fermentative

3.3.3.13 Nitrate reduction test:

This test detects the ability of organisms to produce enzyme nitrate reductase which reduces nitrates to nitrites.

A loopful of growth from peptone water was inoculated in nitrate broth (0.1 % KNO_3) and incubated at 37°C for 24 hours.

After 24 hours 1 ml of nitrate reagent A (α - naphthalamine) was added and then 1 ml of reagent B (sulfanilic acid) was added.

Result was observed.

3.3.3.13.1 Interpretation:

Positive reaction: Development of brick red color in the media

Negative reaction: No color development

3.3.3.13.2 Quality control:

***E. coli*:** Positive

***Acinetobacter*:** Negative

3.4 Detection of Extended spectrum β-lactamases (ESBLs):

ESBL production was performed according to CLSI guidelines with an initial screening followed by phenotypic confirmation by combined disc diffusion method (CLSI 2011).

3.4.1 Screening of ESBLs

The screening test of the isolates were done using two antibiotics namely Cefotaxime and ceftazidime (Hi–Media, Mumbai, India) at a concentration of 1μg/ml in Mueller Hinton Agar by agar dilution method.

3.4.2 Preparation of stock solution: 20mg of each antibiotic namely Cefotaxime and ceftazidime (Hi–Media, Mumbai, India) were dissolved in 2ml of sterile triple distilled water in separate vials to make a final concentration of 10mg/ml.

3.4.3 Preparation of screen agar plate: 1μl/ml of each antibiotic from stock solution was added in to molten cool Mueller Hinton agar (Hi-Media, Mumbai, India). The agar and antibiotics were mixed thoroughly and poured into Petri dish. The agar was allowed to solidify at room temperature and stored at 4–8°C.

3.4.4 Inoculation of test isolates in screen agar plates: Dilution of inoculum was adjusted to 10^6 CFU/ml. 2μl of the inoculum was applied to the surface of the agar with the help of a micropipette. A control plate (without any antimicrobials) was inoculated first followed by the screen agar plates. *Klebsiella pneumoniae* ATCC700603 and *Escherichia coli* ATCC25922 were used as positive and negative control respectively.

3.4.5 Incubation of screen agar plates: Inoculated plates were allowed to stand in room temperature until the moisture in the inoculum spots were absorbed into the agar. The plates were incubated in inverted position at 37°C for 12 to 16 hours.

3.4.6 Interpretation: The isolates that grown in any of the antibiotic containing medium was suspected to be ESBL producer and subjected to confirmatory test.

3.5 Confirmatory tests for ESBL production

3.5.1 Combined disc diffusion test

This test was carried out for all the screened positive isolates against cefotaxime (30µg) and ceftazidime (30µg) antibiotic discs with and without clavulanic acid (10µg).

3.5.2 Antibiotic discs: Cefotaxime (30µg), ceftazidime (30µg), ceftazidime/clavulanic acid (30/10µg) and cefotaxime/clavulanic acid (30/10µg) disc (Hi–Media, Mumbai, India) were obtained commercially.

3.5.3 Inoculation:

Dilution of inoculum was adjusted to 10^6 CFU/ml. A sterile cotton swab was dipped in to the suspension and rotated safely against the upper inside wall of the tube to remove the excess fluid. It was then streaked evenly onto the entire agar surface of the MHA plate 2-4 times, turning the plate at 60° angle between each streaking. The inoculums were allowed to dry to 10-15 min. with lid in place. The above mentioned antibiotic discs were applied using aseptic technique.

3.5.4 Quality control:

Klebsiella pneumoniae ATCC700603 and *E. coli* ATCC25922 were used as positive and negative control respectively.

3.5.5 Interpretation:

A $\geq$ 5mm increase in zone diameter for any antimicrobial tested in combination with clavulanic acid against its zone when tested alone confirms ESBL production.

3.6 Antibiotic susceptibility testing

3.6.1 Disk Diffusion Method:

Mueller Hinton Agar plates were prepared with a depth of about 4mm.

⬇

Pure culture was selected for preparation of inoculum. 3-4 similar colonies were selected and transferred into peptone water. Incubated at 35°C for 2-8 hrs., till light to moderate turbidity was achieved. The turbidity of the suspension was adjusted to MacFarland's standard 0.5 (1.5×10^8 CFU/ml)

⬇

A sterile cotton swab was dipped in to the suspension

⬇

The soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid

⬇

It was streaked evenly onto the entire agar surface of the plate three times, turning the plate 60°angle between each streaking

⬇

The inoculum is allowed to dry to 5–15 min. with lid in place

⬇

Commercially prepared antibiotic discs (Table 6) were applied using aseptic technique

⬇

The discs were placed with centres at least 24mm apart

⬇

Incubated immediately at 37°C and examined after 14–16 hrs.

⬇

E. coli ATCC 25922 and *E. coli* ATCC 35218 were taken as quality control strains for the testing of various β-lactam, non β-lactam antibiotics.

⬇

The zone of inhibitions was measured in millimetre with standard chart provided in CLSI guideline (CLSI 2011).

Table 6: Antimicrobial agents used for the study

Antimicrobial agents	Strength (µg)	Source
Amikacin	30	Hi-Media, Mumbai, India
Ciprofloxacin	30	Hi-Media, Mumbai, India
Gentamicin	10	Hi-Media, Mumbai, India
Cefopodoxime	10	Hi-Media, Mumbai, India
Cefoxitin	30	Hi-Media, Mumbai, India
Ceftriaxone	30	Hi-Media, Mumbai, India
Cefepime	30	Hi-Media, Mumbai, India
Tigecycline	15	Hi-Media, Mumbai, India
Imipenem	10	Hi-Media, Mumbai, India
Meropenem	10	Hi-Media, Mumbai, India
Aztreonam	30	Hi-Media, Mumbai, India
Trimethoprim/sulfomethoxazole	1.25/23.75	Hi-Media, Mumbai, India

3.6.2 Minimum inhibitory concentration (MIC) study

MIC study was done using expanded spectrum cephalosporin like: cefotaxime, ceftazidime, ceftriaxone, cefepime and Carbapenems like imipenem, meropenem, ertapenem and Monobactam like aztreonam by agar dilution method according to CLSI guidelines (CLSI 2011).

3.6.2.1 Preparation of stock solution:

10 mg of each antibiotics namely cefotaxime, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, Ertapenem and aztreonam (Hi–Media, Mumbai, India) were dissolved in 5 ml of sterile triple distilled water in separate vials to make a final concentration of 2 mg/ml.

3.6.2.2 Preparation of agar dilution plates:

A series of dilution of eight β- lactam antibiotics ranging 256µg/ml were made in Mueller Hinton agar plates (Table 7).

3.6.2.3 Preparation of the agar plate:

Appropriate dilutions of each antimicrobial solution were added to the corresponding amount of molten Muller Hinton agar (Table 7). The agar and antibiotics were mixed thoroughly and poured into Petri dish in a depth of 4mm. The agar was allowed to solidify at room temperature and stored at 4°C.

3.6.2.4 Preparation of inoculum:

Pure culture was selected for preparation of inoculum. 3-4 similar colonies were selected and transferred into peptone water (Hi–Media, Mumbai, India) and incubated at 35°C for 2-8 hrs. The turbidity of the suspension was adjusted to MacFarlands standard 0.5 (1.5×108 CFU/ml).

3.6.2.5 Inoculation of test isolates in antibiotics agar plates: 2µl of the inoculum was applied to the surface of the agar with the help of a micropipette (spot inoculation). A control plate (without any antimicrobials) was inoculated first followed by the screen agar plates.

3.6.2.6 Quality control:

Klebsiella pneumonia 700603 and *E. coli* 25922 were used as positive and negative control respectively.

3.6.2.7 Incubation of antibiotics agar plates: Inoculated plates were allowed to stand in room temperature until the moisture in the inoculums spots were absorbed into the agar. The plates were incubated in inverted position at 37°C for 16 hrs.

3.6.2.8 Interpretation: The minimum inhibitory concentration was recorded as the lowest concentration of antimicrobials agent that completely inhibited the growth disregarding a single colony or a faint haze caused by the inoculum. The results were interpreted according to CLSI criteria for MIC break points in to sensitive, intermediate and resistant.

3.6.2.9 Preparation of stock solution: 2mg/ml stock solution of each antibiotic was prepared.

Table 7: Preparation of plates for MIC

Amount of antibiotic solution added (µl)¥ (Original stock solution concentration of 2mg/ml)	**Volume Mueller Hinton agar (ml)**	**Final concentration of antibiotic in 20 ml of medium used in each plate (µg/ml)**
512	19.488	256
256	19.744	128
128	19.872	64
64	19.936	32
32	19.968	16
16	19.984	8
8	19.992	4

¥Only a single antibiotic is used in each plate

3.7 Genotypic characterization of ESBL:

For genotypic characterization of ESBLs two multiplex PCR was performed by targeting different ESBL gene types namely *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{OXA-10}$, *bla*$_{OXA-2}$, *bla*$_{PER}$, *bla*$_{VEB}$, and *bla*$_{GES}$ (Table 8).

3.7.1 Multiplex PCR1:

PCR was performed for all isolates which were confirmed phenotypically to be positive for ESBL production.

For partial gene PCR amplification, primers (Table 8) specific for targeting the genes namely, TEM, SHV, CTX – M, OXA – 2, OXA – 10 were used for reaction with bacterial DNA as template.

3.7.1.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.7.1.2 Boiling centrifugation method:

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

3.7.1.3 Preparation of reaction mixture:

Each single reaction mixture (25 µl) contained 1.5 µl of template DNA (100ng/µl), 1 µl of each primer (10picomole), 12.5 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.7.1.4 Reaction condition: Reactions were run under the following conditions: initial denaturation 95°C for 5 min, 32 cycles of 95 °C for 1min, 54°C for 1min, 72°C for 1min and final extension at 72°C for 7 min.

Table 8: Oligonucleotides used as primers for amplification of different ESBL genes

Primer pairs	Target	Sequence(5' -3')	Amplified Product size (bp)	Reference
TEM – F TEM – R	TEM	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	867	Bert et al., 2002, J. A C. 50:11 – 18
SHV – F SHV – R	SHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	Colom et al., 2003, FEMS Microbiol let, 223: 147 – 151
CTX – M – F CTX – M – R	CTX – M – 1,-2,-9 group	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	Lee et al., 2005, J. A. C. 56:122 – 127.
OXA – 10 – F OXA – 10 – R	OXA – 10 group	TCAACAAATCGCCAGAGAAG TCCCACACCAGAAAAACCAG	276	Bert et al., 2002, J. A C. 50:11 – 18
OXA – 2 – F OXA – 2 – R	OXA – 2 group	AAGAAACGCTACTCGCCTGC CCACTCAACCCATCCTACCC	478	Bert et al., 2002, J. A C. 50:11 – 18
PER-F PER-R	PER-1	AAT TTG GGCTTA GGG CAG AA ATG AAT GTC ATT ATA AAA GC	920	Lee et al., 2005, J. A. C. 56:122 – 127.
GES-F GES-R	GES-1, -2, IBC-1	AGTCGGCTAGACCGGAAAG TTTGTCCGTGCTCAGGAT	863	Lee et al., 2005, J. A. C. 56:122 – 127.
VEB-F VEB-R	VEB-1	CATTTCCCGATGCAAAGCGT CGAAGTTTCTTTGGACTCTG	650	Lee et al., 2005, J. A. C. 56:122 – 127.

3.7.1.5 Gel electrophoresis and analysis of PCR products:

The DNA fragments of ESBL genes amplified by PCR were identified by agarose gel electrophoresis. 5 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with Tris Borate EDTA (TBE) buffer.

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

3.7.1.6 Interpretation:

Presence of bands of molecular weight of 276 bp, 392 bp, 478 bp, 550 bp, and 867 bp confirm the presence of OXA-10, SHV, OXA-2, CTX-M, and TEM ESBL genes respectively.

3.7.2 Multiplex PCR2:

PCR was performed targeting the genes namely PER, VEB and GES primers (Table 8).

3.7.2.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.7.2.2 Preparation of reaction mixture:

Each single reaction mixture (25 µl) contained 1.5 µl of template DNA (100ng/µl), 1 µl of each primer (10picomole), 12.5 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.7.2.3 Reaction condition

Reactions was run under following condition: initial denaturation 95°C for 2 min, 32 cycles of 95°C for 20 Sec., 50°C for 1min, 72°C for 1min and final extension at 72°C for 5 min.

3.7.2.4 Gel electrophoresis and analysis of PCR products:

The DNA fragments of ESBL genes amplified by PCR were identified by agarose gel electrophoresis. 5 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

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

3.7.2.5 Preparation of TBE (10X):

Tris base	-10.8gm
Boric acid	-5.5gm
0.5M EDTA	-4ml
Distilled water	-200ml

3.7.2.6 Interpretation:

Presence of bands of molecular weight of 920 bp, 863 bp and 650 bp confirm the presence of PER, GES and VEB group of ESBL genes respectively.

3.8 PCR product purification:

PCR products were purified using Gene JET PCR product purification kit [Thermo Scientific, Lithuania]. Following steps were used:

1:1 volume of Binding Buffer was added to the PCR product

(Volume: Volume)

800 µl of the solution was transferred to the Gene JET purification column and centrifuged it for 60 seconds. The flow-through was discarded.

600 µl of Wash Buffer (diluted with ethanol) was added to the Gene JET purification column and centrifuge for 60 seconds.

The flow through was discarded and column placed in the same collection tube

Empty Gene JET purification column was centrifuged for additional 60 seconds to completely remove any residual wash buffer.

The Gene JET purification column was transferred to a clean 1.5 ml micro centrifuge tube

50 µl of Elution Buffer was added to the centre of the Gene JET purification column membrane, and centrifuged for 60 seconds.

The Gene JET purification column was discarded and the centrifuge tube containing purified DNA was collected in to fresh micro centrifuge tube

Purified DNA was stored at -20°C.

3.9 Sequencing of all ESBL genes:

30 µL of purified PCR products were used for sequencing along with bla_{ESBL} gene primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.10 Cloning of whole CTX-M gene:

For amplification of whole *bla*$_{\text{CTX-M}}$ gene, primer pair was designed for amplification of whole genome of CTX-M gene and PCR was carried out with the designed primers of CTX-M forward 5'-CATTGCAGCAAAGATGAAATC-3' and reverse 5'-CAGCGCTTTTGCCGTCTAAGG-3'gene respectively.

3.10.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method.

3.10.2 Preparation of reaction mixture:

Each single reaction mixture (50µl) contained 1.5 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 25µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.10.3 Reaction condition: Reactions were run under the following conditions: initial denaturation 95°C for 2 min, 35 cycles of 95 °C for 20 Sec, 52°C for 45 Sec, 72°C for 1.30 Sec and final extension at 72°C for 5 min.

3.10.4 Gel electrophoresis and analysis of PCR products:

The DNA fragments of whole CTX-M gene amplified by PCR were identified by agarose gel electrophoresis. 5 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 Kb DNA Hyper ladder (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.10.5 Interpretation:

Presence of 1212 bp confirms the CTX-M whole gene along with promoter region.

3.10.6 Purification of PCR Products:

PCR products were purified by Gene Jet PCR purification kit.

3.10.7 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 Composition of the ligation mixture:

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

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

3.10.9 Transformation of ligated products into *E. coli* JM 107 competent cells:

3.10.9.1 Preparation of competent cells:

Overnight culture of cells were grown with appropriate drug selection

↓

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

↓

Cells were grown at 37°C until OD600 reaches ~0.25

↓

Culture was chilled in ice for 15 min to slow growth

↓

Cells was pelleted by centrifugation at 2500 x g for 15 m in at 4°C

↓

Decant the supernatant

↓

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

↓

Cells were incubated on ice for 20 min

↓

Cells was pelleted by centrifugation at 2500 x g for 15 m in at 4°C

↓

Supernatant was decanted

↓

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

↓

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

↓

Stored at -80°C

3.10.10 Transformation:

Transformation was carried out by heat shock method.

3.10.11 Transformation procedure:

Transformation was carried out by heat shock method which involves following steps-

The competent cells were removed from -80°C and thaw on ice

The cells were mixed gently by flicking tubes and a 50 µl of cells were taken in sterile micro centrifuge tubes

10 µl of plasmids were added in to cell suspension and swirl the tube gently for a few seconds to mix

Incubate the tubes on ice for 30 minutes

The tubes were placed in water bath at 42°C for 40 seconds without shaking

Tubes were removedfrom water bath and immediately placed on ice for 10 minutes

The tubes were diluted by adding the 500 µl of SOC medium

The tubes were kept in shaker incubator at 37°C and 200 rpm for 2 hours.

The cells were centrifuged for 3 minutes at 5000 rpm

The pellets were spread on LB cefotaxime agar medium

The plate was incubated for 12-16 hours in incubator at 37°C

3.10.12 Component of Luria Bertani agar (LB agar) (Per litre):

Peptone	-10g
Yeast extracts	-5g
NaCl	-10g
Agar	-15g

3.10.13 Preparation of stock solution:

10 mg/ml of cefotaxime were dissolved in 5 ml of sterile triple distilled water in a vial to make a final volume 10 ml.

3.10.14 Composition of SOC medium (per litre):

Tryptone	-2g
Yeast extracts	-0.5g
Glucose	-0.4g
NaCl	-0.584 g
KCl	-0.186 g
$MgCl_2$	-0.952 g
$MgSO_4$	-2.408 g

3.10.15 Preparation of screening LB agar medium:

LB agar medium was prepared by mixing ampicillin, X-gal (Chromogen), and IPTG (Bioline, UK).

Ampicillin	-100 mg/ml
X-gal	- 100 mg/ml
IPTG	-240 mg/ml
Cefotaxime	-0.5mg/ml

0.5 µg/ml of cefotaxime was added in to molten cool LB agar medium (Hi-Media, Mumbai, India) which was allowed to equilibrate in water bath at 48–50°C. The agar and antibiotic were mixed thoroughly and poured into Petri dish. The agar was allowed to solidify at room temperature and stored at 4–8°C.

3.10.16 Spreading of cells on LB agar plates:

Cells were centrifuged for 3 minutes and pellets were spread on LB agar medium containing, X-gal, IPTG, and cefotaxime.

3.10.17 Interpretation:

The recombinant transformants were screen by Blue-White Screening process. White bacterial colony on the LB cefotaxime agar plate confirms the successful transformation.

3.10.18 PCR detection of ESBL gene in transformed recombinant:

Transformed recipient cells harbouring $bla_{\text{CTX-M}}$ was subjected to PCR using CTX-M F' and CTX-M R'primer (Table 8).

3.10.19 Minimum inhibitory concentration of recomibinant plasmid:

MIC was done using broad spectrum cephalosporin like: cefotaxime, ceftazidime, ceftriaxone, cefepime and aztreonam by agar dilution method according to CLSI guidelines before transformation. Further MIC's was again performed against the above

mentioned antibiotics after transformation. The suspension was adjusted to 0.5 McFarland standards and incubated on MH agar plates containing, 4, 8, 16, 32, 64, 128, 256 µg of antibiotics.

3.11 Determination of genetic variants of CTX-M and OXA-10:

As CTX-M and OXA-10 types were more predominant among characterized ESBL types, their presence of any mutation/variants among these two types were detected by Denaturing gradient gel electrophoresis (DGGE).

PCR amplified products of CTX-M and OXA-10 were subjected to mutation study. DGGE was performed with a DCode^{TM} Universal Mutation Detection System (Bio-Rad, Richmond, CA). The PCR products were loaded onto a polyacrylamide gel (8% [w/v] acrylamide in 0.5X Tris acetate EDTA (TAE) buffer) with 20–30% to 40–50% denaturant gradients (100% denaturant gradient was 7M urea and 40% deionized formamide). Electrophoresis was carried out using 0.5X TAE buffer and run at 30V for 40 min followed by 4 hours at 130V and a temperature of 60°C. After electrophoresis, the gel was stained for 10 min within ethidium bromide (10mg/ml solution) and then washed in distilled water for 20 min. Gel image was acquired using a Gel Doc^{TM} EZ imager (Bio-Rad).

3.11.1 Interpretation:

Presence of bands depending upon the molecular weight of particular gene. Variation of band pattern is indicative of mutation.

3.12 DNA fingerprinting of ESBL producers:

Typing of ESBL producing organisms were done by Pulsed field gel electrophoresis (PFGE), Enterobacterial repetitive intergenic consensus (ERIC) and Repetitive extragenic palindromic (REP) PCR. PFGE was done for all the CTX-M producing isolates which was found to be the predominant ESBL type. ERIC PCR was done for all the members of *Enterobacteriacae* family that were found to be ESBL producers while REP PCR was performed for non fermenting rods like *Pseudomonas* and *Acinetobacter*.

3.12.1 Pulsed field gel electrophoresis (PFGE):

PFGE is a technique for resolving large chromosomal DNA fragments. Macro restricted linear bacterial DNA fragments from 100 bp to 10 Mbp can be separated. PFGE was performed with digestion of bacterial agarose plugs with *Xba*I restriction enzyme.

3.12.1.1 Preparation of buffers and restriction enzyme (*Xba*I) master mix:

TE buffer:

1M Tris, pH 8.0	-10ml
0.5M EDTA, pH 8.0	-2.0ml
Clinical Laboratory Reagent Water (CLRW)	-1000ml

Cell Suspension Buffer:

1M Tris, pH 8.0	-100ml
0.5M EDTA, pH 8.0	-200ml
Clinical Laboratory Reagent Water (CLRW)	-1000ml
Stock solution of Protinase K:	-20mg/ml

Cell Lysis Buffer:

1M Tris, pH 8.0	-50ml
0.5M EDTA, pH 8.0	-100ml
10% N-Lauroyl sarcosine, Sodium Salt (Sarcosyl)	-100ml
Clinical Laboratory Reagent Water (CLRW)	-1000ml

3.12.1.2 Pre-Restriction Incubation Buffer (for pre-incubation with 10X restriction buffer):

10X Restriction Buffer	-300µl
Clinical Laboratory Reagent Water (CLRW)	-2700µl
Total Volume	-3000µl
Bovine Serum Albumin (BSA) stock:	10mg/ml

Restriction Enzyme Master Mix:

Clinical Laboratory Reagent Water (CLRW)	-2595µl
10X Restriction Buffer	-300µl
BSA (10mg/ml)	-30µl
*Xba*I (10u/µl)	-75µl
Total Volume	-3000µl

0.5X TBE Buffer:

10X TBE Stock	-100 ml
Clinical Laboratory Reagent Water (CLRW)	-1900 ml
Total volume	-2000 ml

Preparation of 10X TBE Buffer:

Tris Base	-10.8 gm
Boric Acid	-5.5 gm
0.5M EDTA	-4 ml

Clinical Laboratory Reagent Water (CLRW) -200 ml

Preparation of Agarose plugs:

Agarose (PFGE grade) -1g

TE Buffer -100 ml

3.12.1.3 Procedure of PEGE:

3.12.1.3.1 Preparation of Plugs:

1% Agarose was prepared in TE buffer

↓

Equilibrated in water bath for 15 minutes at 55-60ºC

↓

Falcon tubes were labelled with culture numbers

↓

Cell suspension buffer was transferred in to Falcon tubes.

↓

Bacterial cells were resuspended in to cell suspension buffer in Falcon Tubes and kept on ice

↓

Cell suspension was diluted by adding extra sterile cell suspension buffer

3.12.1.3.2 Casting of plugs:

PFGE plug molds were labelled with culture number

↓

400µl cell suspensions were transferred in micro centrifuge tubes

↓

20µl of proteinase K was added in cell suspensions in micro centrifuge

↓

Added 400µl of 1% melted agarose in to micro centrifuge tubes containing cell suspension buffer and proteinase K and mixed gently by pipette without any bubble formation

3.12.1.3.3 Cell lysis in agarose plugs:

Falcon tubes were labelled with culture numbers

↓

5ml cell lysis buffer and 25µl of proteinase K was added into Falcon tubes

↓

Agarose plugs was trimmed with scalpel and transferred into the falcon tubes containing cell lysis buffer and proteinase K

↓

Falcon tubes were placed in shaker incubator at 55ºC for 2 hours and 175rpm

3.12.1.3.4 Washing of agarose plugs after cell lysis:

Falcon tubes were removed from shaker incubator

↓

Agaorse plugs were washed with 15 ml pre heated (at 55ºC) sterile clinical laboratory reagent water (CLRW) two times

↓

TE buffer was pre heated in water bath at 55ºC

↓

Agarose plugs were again washed with 15 ml sterile TE buffer four times

↓

TE buffer was poured off from falcon tubes and 10 ml sterile TE buffer was added in each falcon tubes

3.12.1.3.5 Restriction digestion of DNA in Agarose plugs:

1.5 ml micro centrifuge tubes was taken and labelled with culture number

↓

Pre-restriction incubation buffer was prepared and 200µl of buffer was added into the 1.5ml micro centrifuge tubes

↓

Agarose plugs were removed from falcon tubes and placed into the 1.5ml micro centrifuge tubes containing pre-incubation buffer

↓

Plugs were incubated in water bath at 37ºC for 15 minutes

↓

After 15 minutes pre-incubation buffer were discarded from micro centrifuge tubes

↓

Restriction enzyme master mix were prepared and 200µl was added into micro centrifuge containing agarose plugs

↓

Plugs were incubated in water bath at 37ºC for 10 hours

↓

After 10 hours plugs were removed from water bath and 200µl of 0.5X sterile TBE buffer was added and incubate for 5 minutes at room temperature and After 5 minutes tubes were kept in refrigerator at 4°C

3.12.1.3.6 Casting an Agarose gel:

1% agarose gel was prepared in 0.5X TBE buffer

↓

Gel was poured in the casting tray and placed the comb holder into it.

↓

Comb was removed after solidifying the gel

3.12.1.3.7 Loading of agarose plugs in to the wells:

Plugs were placed on teeth of comb

↓

And by the help of needle the plugs were loaded in to the appropriate wells

↓

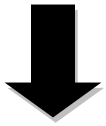
After loading of the plugs remaining wells were filled or sealed by melted and cooled 1% agarose gel

3.12.1.3.8 Electrophoresis conditions:

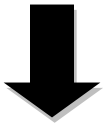
Initial switch time	-2.2 seconds
Final switch time	-63.8 seconds
Voltage	-6V
Included Angle	-120ºC
Run time	-10 hours

3.12.1.3.9 Staining and documentation of an agarose gel:

When electrophoresis was over and stained the gel with ethidium bromide in to sterile Clinical Laboratory Reagent Water (CLRW) and left the gel for 20 minute into the tray



After 20 minutes the CLRW removed from the tray and washed the gel into the sterile Clinical Laboratory Reagent Water (CLRW) for 25 minutes.



After washing gel image was documented in gel doc imager

3.12.2 Enterobacterial repetitive intergenic consensus (ERIC) sequences PCR:

All the ESBL producers were typed by ERIC PCR using ERIC-F 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC-R 5'-AAGTAAGTGACTGGGGTGAGCG-3' primers (Versalovic et al., 1991).

3.12.2.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.12.2.2 Preparation of reaction mixture

Each single reaction mixture (50 µl) was contained 1.5 µl of template DNA (100 ng/µl), 3 µl of each primer (30 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.12.2.3 Reaction condition:

Reactions was run under following condition: initial denaturation 95°C for 3 min, 30 cycles of 95 °C for 20 Sec., 46°C for 40 Sec, 72°C for 3 min and final extension at 72°C for 10 min.

3.12.2.4 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR and were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in GelDoc EZ imager (Bio-Rad).

3.12.2.5 Interpretation:

All the isolates were analysed depending on varying molecular weights.

3.12.3 DNA fingerprinting of ESBL producing non fermenting gram negative rods by Repetitive extragenic palindromic (REP) PCR:

Typing of all ESBL producing *Pseudomonas* Spp. (n=144) and *Acinetobactaer baumanii* (n=8) isolates were done by Rep PCR, using primers: Rep-F 5'- ICGICTTATCIGGCCTAC-3' and Rep-R 5'- NCGNCTTATCNGGCCTAC -3' as described by Versalovic et al., 1994.

3.12.3.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.12.3.2 Preparation of reaction mixture

Each single reaction mixture (50 µl) contained 1.5 µl of template DNA (100 ng/µl), 3 µl of each primer (30 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.12.3.3 Reaction condition:

Reactions was run under following condition: initial denaturation 95°C for 7 min, 30 cycles of 95 °C for 1 min., 40°C for 1 min, 72°C for 8 min and final extension at 72°C for 16 min.

3.12.3.4 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR and were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.12.3.5 Interpretation:

All the isolates were analysed depending on varying molecular weights.

3.13 Determination of genetic environment:

3.13.1 Characterization of Integron by integrase gene PCR:

All the ESBL positive isolates were tested for detection and characterization of integron by integrase gene PCR representing class 1 and class 2 integrons respectively with the target of class 1 (160 bp) and class 2 (288 bp) fragments (Table 9) (Koeleman et al., 2001).

3.13.1.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.13.1.2 Primers: Primers used in the experiment are described in table 9.

3.13.1.3 Preparation of reaction mixture:

Each single reaction mixture (25 µl) contained 1.5 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.13.1.4 Reaction condition:

Reactions were run under the following condition: initial denaturation 95°C for 3 min, 34 cycles of 95 °C for 1min, 54°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 5 min.

Table 9: Primer sequence for integrase gene PCR

Primer	Type of PCR	Nucleotide sequence (5' to 3')	Product size (bp)
Int1F	Detection and characterization of class 1 and class 2 integrons by integrase gene PCR	CAG TGG ACA TAA GCC TGT TC	160
Int1R		CCC GAG GCA TAG ACT GTA	
Int2F		TTG CGA GTA TCC ATA ACC TG	288
Int2R		TTA CCT GCA CTG GAT TAA GC	

3.13.1.5 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR and were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

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

3.13.1.6 Interpretation: Presence of band of molecular weights of 160 bp and 288 bp confirms the presence of class 1 and class 2 integron genes.

3.14 Determination of genetic context/ genetic environment of ESBL genes:

3.14.1 Association of *bla*$_{\text{ESBLs}}$ with gene cassette

For detection of association of gene cassette with *bla*$_{\text{ESBL}}$ gene, 59 base element (be) cassette PCR was performed using HS 286 and HS 287 primers (Table 10). Two PCR reactions were done, one reaction with HS 287 and reverse primer of ESBL gene, another reaction with HS 286 and forward primer of ESBL gene.

3.14.1.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.14.1.2 Primers: Primers used in the experiment are described in table 10.

Table 10: Primer used for detection of association of gene cassettes

Primer	Nucleotide sequence (5' to 3')	Reference
HS 286	GGGATCCTCSGCTKGARCGAMTTGTTAGVC	Stokes et al., 2001
HS 287	GGGATCCGCSGCTKANCTCVRRCGTTAGSC	

3.14.1.3 Preparation of reaction mixture:

Each single reaction mixture (30 µl) consist of1.5 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 15 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.14.1.4 Reaction Condition:

PCR reactions were run under the following conditions: initial denaturation 95°C for 5 min, 28 cycles of 95 °C for 1min, 54°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

3.14.1.5 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.14.1.6 Interpretation:

Presence of band of varying molecular weight depending upon the location of ESBL gene in the integron was formed.

3.14.1.7 Gel extraction and purification of amplified PCR products:

For purification of DNA from gel; Gel extraction was performed using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania). The following steps were used:

Gel slice was excised containing the DNA fragment by using a clean scalpel and placed it into a pre weighted sterile micro centrifuge tube

Weight of the gel slice containing DNA was recorded

1:1 volume of Binding Buffer was added into the tubes containing the gel slice (volume: weight)

The gel mixture was incubated at 60°C for 10 minutes until the gel slice dissolved completely

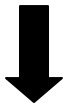
800 µl of the gel slice was transferred in to the gene Jet purification column

Centrifuged the column for 1 minutes at 1200xg and Flow through was discarded after that column placed back in to the same collection tube

700 µl of Wash Solution was added in to the column and centrifuged it for 1 minutes

Flow through was discarded and column placed in the same tubes

The Column was centrifuged for additional 1 minute to completely remove residual buffer



The column was transferred in to a new clean 1.5 ml micro centrifuge tube

50 µl of Elution Buffer was added to the centre of the purification column membrane

The column was centrifuged for 1 minute and flow through containing purified DNA was collected in the new micro centrifuge tube

Purified DNA was stored at -20°C

3.14.1.8 Sequencing:

30 µL of purified PCR products were used for sequencing along with HS 286 and HS 287 primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.14.2 Detection of variable region of integron among ESBL producing isolates:

To find out the variable region of integron, PCR was performed using primers 5'–CS and 3'–CS (Levesque et al., 1995) (Table 11) which amplifies 5' conserved and 3' conserved sequences of the integron.

3.14.2.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.14.2.2 Primers: Primers used in the experiment are described in table 11.

Table 11: Primers used for detection of variable region of integron

Primer pairs	Sequence(5' -3')
5' – CS	GGC ATC CAA GCA GCA AG
3' – CS	AAG CAG ACT TGA CCT GA

3.14.2.3 Preparation of reaction mixture:

Each single reaction mixture (50 µl) contained 1.5 µl of template DNA (100 ng/µl), 2 µl of each primer (10 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.14.2.4 Reaction Condition:

PCR reactions were run under the following conditions: initial denaturation 95°C for 5 min, 28 cycles of 95 °C for 1min, 52°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

3.14.2.5 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.14.2.6 Interpretation:

Presence of bands of varying molecular weight depending upon the location of ESBL gene and arrangements of other genetic determinants within the region of the 5'-CS and 3'-CS.

3.14.3 Detection of the location of ESBL gene within integron region:

For detection of gene cassette within the integron region two primers was used namely 5'-CS and 3'-CS (Table 12) (Livesque et al., 1995). Two reactions were performed: in one reaction 5'-CS and reverse primer of β-lactamase gene and in other reaction 3'-CS and forward primer of characterized β-lactamase gene.

3.14.3.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et al., 2005).

3.14.3.2 Primers: Primers used in the experiment is described in table 12.

Table 12: Primers used for detection of gene cassette within integron region

Primer pairs	Sequence(5’ -3’)
5’ – CS CTX-M R’	GGC ATC CAA GCA GCA AG ACCGCGATATCGTTGGT
3’ – CS CTX-M F’	AAG CAG ACT TGA CCT GA CGCTTTGCGATGTGCAG
5CS OXA-10 R’	GGC ATC CAA GCA GCA AG TCCCACACCAGAAAAACCAG
3CS OXA-10 F’	AAG CAG ACT TGA CCT GA TCAACAAATCGCCAGAGAAG
5CS SHV R’	GGC ATC CAA GCA GCA AG ATTTGCTGATTTCGCTCG
3CS SHV F’	AAG CAG ACT TGA CCT GA AGGATTGACTGCCTTTTTG
5CS PER F’	GGC ATC CAA GCA GCA AG AAT TTG GGCTTA GGG CAG AA
3CS PER R’	AAG CAG ACT TGA CCT GA ATG AAT GTC ATT ATA AAA GC
5CS VEB R’	GGC ATC CAA GCA GCA AG CGAAGTTTCTTTGGACTCTG
3CS VEB F’	AAG CAG ACT TGA CCT GA CATTTCCCGATGCAAAGCGT
5CS GES R’	GGC ATC CAA GCA GCA AG TTTGTCCGTGCTCAGGAT
3CS GES F’	AAG CAG ACT TGA CCT GA AGTCGGCTAGACCGGAAAG

3.14.3.3 Preparation of reaction mixture:

Each single reaction mixture (50 µl) contained 1.5 µl of template DNA (100 ng/µl), 2 µl of each primer (10 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.14.3.4 Reaction Condition:

PCR reactions were run under the following conditions: initial denaturation 95°C for 5 min, 28 cycles of 95 °C for 1min, 52°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

3.14.3.5 Gel electrophoresis and Visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.14.3.6 Interpretation:

Presence of band of varying molecular weight depending upon the location and arrangement of ESBL genes.

3.14.3.7 Gel extraction and purification of amplified PCR products:

For purification of DNA from gel; Gel extraction was performed using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania).

3.14.3.8 Sequencing:

30 µL of purified PCR products were used for sequencing along with 5CS and 3 CS primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.14.4 Detection and characterization of whole integron gene cassette within the integron:

For amplification and detection of whole gene cassette present within the integrons were amplified by using two primers namely: HS 458 and HS 459 (Table 13). Two reactions were performed simultaneously one with HS 458 and reverse primer of *bla* gene another with HS 459 and forward primer of *bla* genes (Table 8 and 13).

3.14.4.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.14.4.2 Primers: Primers used in the experiment is described in table 8 and 13.

Table 13: Primers used for detection of whole gene cassette present within the integrons

Primer	Sequence	Reference
HS 458	GTTTGATGTTATGGAGCAGCAACG	Marquez et al., 2008
HS 459	GCAAAAAGGCAGCAATTATGAGCC	

3.14.4.3 Preparation of reaction mixture:

Each single reaction mixture (50 µl) was contained 1.5 µl of template DNA (100 ng/µl), 2 µl of each primer (10 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.14.4.4 Reaction Condition:

PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 30 cycles of 95 °C for 20 Sec, 48°C for 1 min, 72°C for 3 min and final extension at 72°C for 10 min.

3.14.4.5 Electrophoresis and visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.14.4.6 Interpretation:

Presence of band of varying molecular weight depending upon the location of ESBL gene in the integron was formed.

3.14.4.7 Gel extraction and purification of amplified PCR products:

For purification of DNA from gel; Gel extraction was performed using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania).

3.14.4.8 Sequencing:

30 µL of purified PCR products were used for sequencing along with HS 458 and HS 459 primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.15 Association of ESBL genes with mobile genetic elements:

For detection of association of bla_{ESBL} genes with various insertion sequences like *tnpISEcp1*, *ISEcp1*, *tnpIS26*, *IS26*; PCR was performed using the primers as described in table 14.

3.15.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.15.2 Primers: Primers used in the experiment is described in table 14.

3.15.3 Preparation of reaction mixture:

Each single reaction mixture (50 µl) was contained 1.5 µl of template DNA (100 ng/µl), 2 µl of each primer (20 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.15.4 Reaction Condition:

PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 34 cycles of 95 °C for 20 Sec, 50°C for 45 Sec, 72°C for 2 min and final extension at 72°C for 7 min.

3.15.5 Electrophoresis and visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

Table 14: Primers used for detection of association of ESBL genes with mobile gene elements

Name	Primer sequence (5'-3')	Reference
tnpA ISEcp1	AATACTACCTTGCTTTCTGA	Eckert et al., 2006
ISEcp1	TTCAAAAAGCATAATCAAAGCC	
ISEcp1 reverse	CAACCACCTTTCAATCATTTTT	
tnpA IS26	AGCGGTAAATCGTGGAGTGA	
IS26	CAAAGTTAGCGATGAGGCAG	

3.15.6 Interpretation:

Presence of band of varying molecular weight depending upon the location of ESBL gene in the integron was formed.

3.15.7 Gel extraction and purification of amplified PCR products:

For purification of DNA from gel; Gel extraction was performed using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania).

3.15.8 Sequencing:

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

3.16 Inverse PCR:

Inverse PCR is used to amplify the flanking DNA sequences with only known DNA sequences. This PCR was done by using as primers oligonucleotides complementary to the 5'-ends of the segment. The orients of the free 3'-OH of the primers outward of the sequence as a result the newly synthesized chain grows from the borders of the concerned segment. In inverse PCR target DNA is cut with restriction enzyme by the process restriction digestion and after restriction digestion the DNA fragments is allowed to circularize by ligation and resulting in a looped fragment that can be primed for PCR from a single section of known sequence.

3.16.1 Procedure of Inverse PCR:

Inverse PCR was performed for detection/presence of the Novel insertion sequence within the more predominant type of ESBL gene i.e. CTX-M-15 producing isolates. A set of primer was designed CTX-M INV F-5'-GAGCGCTTTGCGATGTGCAGC-3' and CTX-M INV R-5'-TCAGCGTGAACTGGCGCAGTG-3' (Figure 15).

3.16.2 Preparation of DNA template: Genomic DNA was isolated by phenol-chloroform method (Sambrook and Russel, 2001).

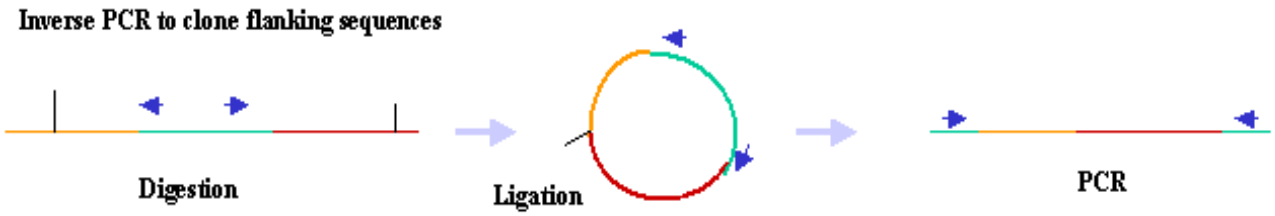


Figure 15: Overview of inverse PCR

[**Source:** http://www.changbioscience.com/primo/primoinv.html]

3.16.3 Procedure of DNA isolation:

Pure bacterial culture was inoculated in to Luria Bertani broth (Hi-Media, Mumbai, India).

↓

Cultures were incubated at 37°C for 18 hours

↓

1.5 ml of freshly grown culture was centrifuged at 1000 rpm for 10 min.

↓

The cell pellets were re-suspended in 567µl of TE buffer (The suspension was vortexed)

↓

30µl of SDS (10%) was added

↓

3µl of protinase K of 20µg/ml concentration (final 100µg/ml) was added and was mixed gently by inversion and incubated at 37°C for 1 hour

↓

100µl of 5 M NaCl was added and vortexed for 15 second

↓

80µl of 10% CTAB was added and mixed well and incubated for 15 min

↓

Equal volume of choloroform and Iso-amyl-alcohol (24:1) was added and mixed by vortexing for 15 second

↓

Centrifuge at 10,000 rpm for 10 min

↓

Aqueous phase was collected

↓

Equal volume of phenol: IAA (25:24:1) was added and vortexed for 15 sec.

↓

Centrifuged at 10,000 rpm for 10 min

↓

The aqueous phase was collected

↓

2µl of RNase (30µg/ml final concentration) was added

↓

Incubated for 30 min

↓

Equal volume of iso-propanol was added and kept in room temperature for 5 min

↓

Above mixture was centrifuged at 10,000 rpm for 10 min

↓

The pellet was washed by 200µl 70% ethanol

↓

The above solution was centrifuge at 10,000 rpm for 10 min

↓

Pellets were dried at 37°C

↓

Pellets were re-dissolved in TE buffer.

3.16.4 Digestion of genomic DNA by restriction enzyme *Sac*II (CCGC↓GG):

2µl of genomic DNA was digested by *Sac*II **(CCGC↓GG)** with digestion buffer.

3.16.5 Composition of digestion Mixture:

Nuclease free water	-15.8µl
Buffer	-2 µl
Restriction enzyme (*Sac*II)	-0.2µl
Template	-2 µl
Total volume	-20µl

3.16.6.Inactivation of the restriction enzyme:

Restriction enzyme *Sac*II **(CCGC↓GG)** was inactivated by keeping the digestion mixtures tubes in dry bath at 54°C for 20 minutes.

3.16.7 Purification of digested DNA:

Digested DNA was purified by Gene Jet PCR purification kit.

3.16.8 Ligation of purified digested DNA:

Nuclease free water	-168 µl
10X buffer	-20µl
Ligase	-2 µl
Template	-10 µl
Total volume	-200 µl

3.16.9 Preparation of PCR reaction mixture:

Each single reaction mixture (30 µl) contained 5 µl of template DNA (20 ng/µl), 1 µl of each primer (10 picomole), 15 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.16.10 Reaction condition

Reactions was run under following condition: initial denaturation 95°C for 2 min, 35 cycles of 95°C for 20 Sec., 56°C for 1min, 72°C for 1.25 min and final extension at 72°C for 5 min.

3.16.11 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by Agarose gel electrophoresis. 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 Kb DNA Hyper ladder (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.16.12 Interpretation:

Presence of band of varying molecular weight depending upon the location of ESBL gene in the integron was formed.

3.16.13 Gel extraction and purification of amplified PCR products:

Gel extraction was performed for purification of DNA from gel using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania).

3.16.14 Sequencing:

50 µL of purified PCR products were used for sequencing along with Inverse primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.17 Genetic location and transferability of ESBL genes:

3.17.1 Isolation of plasmid

Plasmid was isolated by using QIAGEN® Plasmid Mini kit (Germany) for each isolates on the basis of:

- The isolates harbouring single β-lactamase gene
- The isolates harbouring multiple β-lactamase gene
- The isolates showing multiple drug resistance (showing resistance to more than one type of antibiotics)

QIAGEN plasmid purification protocol was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in high salt buffer and then concentrated and desalted by isopropanol precipitation (Table 15).

Table 15: Composition of buffers for isolation of plasmids

Buffer	Composition	Storage
Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A	2–8°C, after addition of RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)	15–25°C
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5	15–25°C or 2–8°C
Buffer FWB2 (QIAfilter wash buffer)	1 M potassium acetate pH 5.0	15–25°C
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)	15–25°C
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	15–25°C
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)	15–25°C
Buffer QN (elution buffer)	1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	15–25°C
TE	10 mM Tris·Cl, pH 8.0; 1 mM EDTA	15–25°C
STE	100 mM NaCl; 10 mM Tris·Cl, pH 8.0; 1 mM EDTA	15–25°C

Source:

http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAGENPlasmidPurification_EN.pdf

3.17.1.1 Procedure:

A single colony was picked from a freshly streaked selective plate and inoculated in a starter culture of 2-10 ml LB medium containing cefotaxime (0.5µg/ml) and chloroamphenicol incubated for 8 hrs. at 37°C with vigorous shaking (300rpm)

The starter culture was further diluted 1/500 into 3 ml LB medium. Grown at 37°C for 16 hrs. with vigorous shaking (300rpm)

Bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C

Bacterial pellets were resuspended in 0.3 ml of buffer P1

0.3 ml of buffer P2 was added, mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature (25°C) for 5 min

0.3 ml of chilled buffer P3 was added; mixed immediately and thoroughly by vigorously inverting 4-6 times, and incubated on ice for 5 min

Centrifuged at 20000 rpm in micro centrifuge for 10 min. Supernatant was collected promptly, containing plasmid DNA

QIAGEN-tip 20 equilibrated by applying 1 ml Buffer QBT, and the column was allowed to empty by gravity flow

The supernatant from step 7 was applied to QIAGEN-tip 20 and allowed to enter the resin by gravity flow

The QIAGEN-tip 20 was washed with 2×2 ml Buffer QC

DNA was eluted with 0.8 ml Buffer QF

DNA was precipitated by adding 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room temperature isopropanol to the eluted DNA. Mixed and centrifuged immediately at 10,000 rpm for 30 min in a micro centrifuge.

Supernatant was decanted

DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 min. and Supernatant was decanted without disturbing the pellet

The pellet was air dried for 5-10 min, and redissolved in TE buffer

3.17.1.2 Visualization: Isolated plasmid was run by agarose gel electrophoresis containing 1% agarose and size of the plasmid was determined by comparing with molecular marker.

3.17.2 Transformation of plasmid and their expression of antibiotic resistance in a recipient host:

Isolated plasmids were transformed in to *E. Coli* JM 107 competent cells.

3.17.2.1 Preparation of competent cells:

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 at 37°C until OD600 reaches ~0.25

↓

Culture was chilled in ice for 15 min to slow growth

↓

Cells was pelleted by centrifugation at 2500 x g for 15 m in at 4°C

↓

Decant the supernatant

↓

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

↓

Cells were incubated on ice for 20 min

↓

Cells was pelleted by centrifugation at 2500 x g for 15 m in at $4^{o}C$

Supernatant was decanted

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

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

Stored at -80°C

3.17.2.2 Transformation:

Transformation was carried out by heat shock method.

3.17.2.3 Transformation procedure:

Transformation was carried out by heat shock method which involves following steps-

The competent cells were removed from -80°C and thaw on ice

The cells were mixed gently by flicking tubes and a 50 µl of cells were taken in sterile micro centrifuge tubes

10 µl of plasmids were added in to cell suspension and swirl the tube gently for a few seconds to mix

Incubate the tubes on ice for 30 minutes

The tubes were placed in water bath at 42°C for 40 seconds without shaking

Tubes were removed from water bath and immediately placed on ice for 10 minutes

The tubes were diluted by adding the 500 µl of SOC medium

The tubes were kept in shaker incubator at 37°C and 200 rpm for 2 hours.

The cells were centrifuged for 3 minutes at 5000 rpm

The pellets were spread on LB cefotaxime agar medium

The plate was incubated for 12-16 hours in incubator at 37°C

3.17.2.4 Component of Luria Bertani agar (LB agar) (Per litre):

Peptone	-10g
Yeast extracts	-5g
NaCl	-10g

Agar	-15g

3.17.2.5 Preparation of stock solution:

10 mg/ml of cefotaxime were dissolved in 5 ml of sterile triple distilled water in a vial to make a final volume 10 ml.

3.17.2.6 Composition of SOC medium (per litre):

Tryptone	-2g
Yeast extracts	-0.5g
Glucose	-0.4g
NaCl	-0.584 g
KCl	-0.186 g
$MgCl_2$	-0.952 g
$MgSO_4$	-2.408 g

3.17.2.7 Preparation of LB cefotaxime agar plates:

0.5 µg/ml of cefotaxime was added in to molten cool LB agar medium (Hi-Media, Mumbai, India) which was allowed to equilibrate in water bath at 48 – 50°C. The agar and antibiotic were mixed thoroughly and poured into Petri dish. The agar was allowed to solidify at room temperature and stored at 4–8°C.

3.17.2.8 Spreading of cells on LB agar plates:

Cells were centrifuged for 3 minutes and pellets were spread on LB agar medium containg 0.5 µg/ml of cefotaxime.

3.17.2.9 Interpretation:

Any growth on the LB cefotaxime agar plate confirms the successful transformation.

3.17.2.10 PCR detection of ESBL gene and integron in transformed recipient:

Transformed recipient cells harbouring plasmid of isolates carrying single and multiple ESBL genes subjected to PCR to detect the presence of ESBL genes (Table 8) and integrons in plasmid (Table 9).

3.17.3 PCR detection of Incompatibility group (Inc) in transformants:

For detection of incompatibility group of plasmids, PCR based replicon typing was performed targeting 18 different replicon types. 5 multiplex and 3 simplex PCRs was performed 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 16) (Carattoli et al., 2005).

3.17.3.1 Targeted gene in multiplex PCR:

In multiplex PCR 15 genes were targeted to perform 5 multiplex PCR: FIA, FIIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, X and Y.

3.17.3.2 Targeted gene in simplex PCR:

In simplex PCR three genes were targeted to perform three simplex PCR: F, K and B/O.

3.17.3.3 Preparation of DNA template: Plasmid was isolated by plasmid isolation kit.

3.17.3.4 Primers: Primers used in the experiment is described in table 16.

3.17.3.5 Preparation of reaction mixture:

Each single reaction mixture (25µl) consist of 1.0 µl of template DNA, 1 µl of each primer (10 picomole), 12.5µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.17.3.6 Reaction Condition for multiplex PCR:

PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 30 cycles of 95 °C for 20 Sec, 60°C for 30 Sec, 72°C for 1 min and final extension at 72°C for 5 min.

3.17.3.7 Reaction condition for simplex PCR:

PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 30 cycles of 95 °C for 20 Sec, 52°C for 30 Sec, 72°C for 1 min and final extension at 72°C for 5 min.

3.17.3.8 Visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

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

3.17.3.9 Interpretation:

Presence of different molecular weights of bands confirms the presence of the different Inc groups in plasmids.

3.17.3.10 PCR Purification of amplified products:

Amplified PCR products were purified by PCR purification kit.

Table 16: Primers used for detection of Inc group in plasmids

Primer	DNA sequence	Size (bp)
HI1 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

3.17.3.11 Sequencing:

30 µL of purified PCR products were used for sequencing along with different Inc groups primers 20 µL each (10 picomole each primers). Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.17.3.12 Antibiogram study of transformants (Co-resistance study)

Co resistance study was performed to see the presence of the other drug resistant genes in the plasmid along with ESBL genes. An initial antibiogram was performed on *E. coli* JM107 against Cefopodoxime (10µg), Amikacin (30µg), Gentamicin (10µg), Ciprofloxacin (30µg), Trimithoprim/Sulphamethoxazole (1.25/23.75µg), Tigecycline (15µg), Cefepime (30µg), Imipenem (10µg), Meropenem (10µg), Ceftriaxone (30µg), Aztreonam (30µg) and Cefoxitin (30µg) before transformation. Furthersusceptibility

testing was again performed against the above mentioned antibiotics after performing transformation for all the recipient cells. The test was performed by Kirby bauer disc diffusion method.

3.17.3.13 Minimum inhibitory concentration of transformants:

MIC was done using broad spectrum cephalosporin like: cefotaxime, ceftazidime, ceftriaxone, cefepime and Carbapenems like imipenem, meropenem, Ertapenem and aztreonam by agar dilution method according to CLSI guidelines before transformation. Further MIC's was again performed against the above mentioned antibiotics after transformation. The suspension will be adjusted to 0.5 McFarland standards and incubated on MH agar plates containing, 4, 8, 16, 32, 64, 128, 256 µg of antibiotics.

3.17.4 Transfer of resistance determinant through conjugation:

Conjugation was performed with all ESBL positive isolates. Bacterial conjugation was performed by using Genei^{TM} bacterial conjugation teaching kit 21730.

3.17.4.1 Strain description: The two strains included in the study were-

3.17.4.2 Donor strain: Genotypically characterized *bla*$_{ESBL}$ harbouring clinical isolates were selected as donor strain which were phenotypically resistant to cefotaxime.

3.17.4.3 Strain B (Recipient strain): The strain B supplied in the Genei^{TM} conjugation kit is devoid of any 'F' factor, carrying streptomycin resistant gene in its chromosome was selected as recipient strain of *E. coli*. Added the antibiotic to LB media at a concentration of 800 µg/ml.

3.17.4.4 Preparation of antibiotic stock solution: 100 mg of cefotaxime powder was dissolved in 10 ml of sterile triple distilled water to get a final concentration of 10 mg/ml. The antibiotic solution was added to LB media at 0.5 µg/ml concentration, to inhibit the growth of recipient strain.

3.17.4.5 Preparation of Streptomycin stock: 150 mg of streptomycin (Genei^{TM} conjugation kit) was dissolved in 1.5ml sterile triple distilled water to get a final

concentration of 100mg/ml and covered the vial with aluminium foil and store at 4°C. Streptomycin was added to LB media at a concentration 800 µg/ml. The concentration of antibiotic was standardized based on the inhibitory concentration of wild type donor strains against streptomycin. All donor strains were inhibited at 800 µg/ml of streptomycin where strain B showed confluent growth at the same concentration.

3.17.4.6 Revival of donor strain and strain B:

1. One set of lyophilized vial of recipient *E. coli* strain B was opened and rehydrated with 0.1 ml of sterile LB broth. Also inoculated the single colony of donor strain into 0.1 ml of LB broth.
2. In duplicates 25 µg of suspension of the donor strain was streaked onto LB plates with cefotaxime (concentration 0.5 µg/ml) and the recipient strain on LB supplemented with streptomycin (concentration 800 µg/ml)
3. Remaining 50 µl of suspension was inoculated in a tube containing 5 ml LB broth with respective antibiotics.
4. Incubated at 37°C for 16 to 20 hours.

3.17.4.7 Preparation of Bacteria: A single colony from each of the donor and the recipient plate was inoculated in to 6 ml LB broth containing the respective antibiotic. The tubes were incubated overnight in a shaker at 35°C.

3.17.4.8 Procedure of Conjugation

1 ml of donor culture was inoculated into 20 ml of LB broth (in 250 ml conical flask) with cefotaxime at concentration 0.5 µg/ml and incubated at 37°C in a shaker

3 ml of overnight recipient culture was inoculated into 20 ml LB broth (in 250 ml conical flask) with streptomycin at a concentration 800 µg/ml and incubated at 37°C in a shaker

Recipient and donor culture were incubated till the O.D. of donor culture reaches 0.8-0.9 at $A_{600.}$

0.2 ml of each of donor and recipient strain was mixed in a sterile cotton plugged test tube for conjugation and labelled it as conjugation sample

Gently mixed and tube was incubated in an incubator at 37°C for 2 hours.

0.2 ml of each of the donor and the recipient cultures were taken into two different test tubes and incubated at 37°C for 1 hour 30 min.

After incubation, 2 ml of sterile LB broth was added into each tube.

Again, both the donor and therecipient tubes were incubated at 37°C for 2 hours.

0.1 ml of each of the donor, recipient and the conjugated sample was spread onto each of the antibiotics supplemented plates

The inoculated plates were incubated overnight at 37°C.

3.17.4.9 PCR detection of β-lactamase genes and integrons in transconjugants:

Multiplex PCR and integrase gene PCR was performed to detect the presence of ESBL genes (Table 8) and integrons (Table 9) in all transconjugants.

3.17.4.10 PCR detection of Incompatibility group (Inc) in transconjugants:

PCR based replicon typing was performed to detect the presence of different Inc plasmid groups (Table 16) in all transconjugants.

3.17.4.11 Antibiogram study of transconjugants (Co-resistance study):

Co-resistance study was performed to see the presence of the other drug resistant genes in the plasmid along with ESBL genes. An initial antibiogram was performed on recipient strain B against Cefopodoxime (10µg), Amikacin (30µg), Gentamicin (10µg), Ciprofloxacin (30µg), Trimithoprim/Sulphamethoxazole (1.25/23.75µg), Tigecycline (15µg), Cefepime (30µg), Imipenem (10µg), Meropenem (10µg), Ceftriaxone (30µg), Aztreonam (30µg) and Cefoxitin (30µg) before conjugation. Further susceptibility testing was again performed against the above mentioned antibiotics after performing conjugation for all the transconjugants. The test was performed by Kirby-Bauer disc diffusion method.

3.17.4.12 Minimum inhibitory concentration of transconjugants:

MIC of transconjugantswere done using broad spectrum cephalosporin like: cefotaxime, ceftazidime, ceftriaxone, cefepime and Carbapenems like imipenem, meropenem, Ertapenem and aztreonam by agar dilution method according to CLSI guidelines before conjugation. Further MIC's was again performed against the above mentioned antibiotics after conjugation. The suspension will be adjusted to 0.5 McFarland standards and incubated on MH agar plates containing, 4, 8, 16, 32, 64, 128, 256 µg of antibiotics.

3.18 Plasmid stability study:

Plasmid stability of all ESBL producers (Wild types) as well as their transformants and transconjugants was analysed by serial passages method for consecutive 115 days at 1:1000 dilutions without antibiotic pressure.

Bacterial cultures were inoculated in LB broth sterile medium and incubate at 37°C for 12 hours in shaker incubator at 160 rpm. After every 12 hours fresh LB broth was prepared and cultures were transferred old LB broth culture to freshly prepared sterile LB broth at 1:1000 dilutions.

3.18.1 PCR detection of β-lactamase genes and integrons after every passage:

Multiplex PCR assay and integrase gene PCR was carried out for the presence of *bla*$_{\text{ESBLs}}$ (Table 8) and integrons (Table 9) in all the wild types, tansformants as well as transconjugants after each passage.

3.19 PCR mapping of multiple ESBL genes arranged within integron:

For PCR mapping of carrying multiple ESBL genes within integron, **long PCR** was performed with 5'CS and 3'CS as well as HS 458 and HS 459 primers (Table 11 and 13). Further these amplified products were purified and PCR was done for presence of ESBL genes (Table 8) using purified products as a template.

After PCR confirmation the gene cassettes were amplified by one **short range of PCR I** using 5'CS and reverse primer of *bla*$_{\text{ESBL}}$ gene and in other reaction 3'CS with forward primer of *bla*$_{\text{ESBL}}$ gene (Table 8). PCR products were purified and again confirmed by PCR for presence of *bla*$_{\text{ESBL}}$ genes (Table 8).

Another **short range of PCR II** was performed using 5'CS with Forward primer of *bla*$_{\text{ESBL}}$ genes and 3'CS and Reverse primer of *bla*$_{\text{ESBL}}$ genes was used to detect arrangement of *bla* gene in the reverse orientation (Table 8 and 11).

Further these cassettes were again amplified by **short range of PCR III** using HS 286 and forward primer of *bla* genes as well as HS 287 with reverse primer of *bla* genes (Table 8 and 10). PCR products were purified and PCR was performed for further confirmation of ESBL genes (Table 8) in amplified gene cassettes using purified product as a template.

Further, location and arrangement of multiple ESBL genes were determined using forward primer of one ESBL type and reverse primer of another (Figure 16).

Figure 16: Overview of PCR mapping

3.19.1 Preparation of DNA template: DNA was extracted by boiling centrifugation method (Freschi et. al. 2005).

3.19.2 Primers: Primers used in the experiment is as described in table 8, 10, 11, and 13.

3.19.3 Preparation of reaction mixture:

Each single reaction mixture (50 µl) contained 1.5 µl of template DNA (100 ng/µl), 2 µl of each primer (10 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.19.4 Reaction Condition:

Long PCR: PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 32 cycles of 95 °C for 20 Sec, 52°C for 1 min, 72°C for 10 min and final extension at 72°C for 15 min.

Short range PCR I: PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 34 cycles of 95 °C for 20 Sec, 48°C for 1 min, 72°C for 3 min and final extension at 72°C for 7 min.

Short range of PCR II: PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 34 cycles of 95 °C for 20 Sec, 50°C for 1 min, 72°C for 3 min and final extension at 72°C for 10 min.

Short range of PCR III: PCR reactions were run under the following conditions: initial denaturation 95°C for 2 min, 32 cycles of 95 °C for 20Sec, 52°C for 1 min, 72°C for 3 min and final extension at 72°C for 10 min.

3.19.5 Visualization of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 15 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 50 volts for two hours with TBE buffer.

Molecular marker of 10 kb hyper ladder I (Bioline, UK) was run concurrently. The gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.19.6 Interpretation:

Presence of band of varying molecular weight depending upon the location of ESBL gene in the integron gene cassettes.

3.19.7 Gel extraction and purification of amplified PCR products:

For purification of DNA from gel; Gel extraction was performed using Gene Jet Gel Extraction kit (Thermo Scientific, Lithuania).

3.19.8 Cloning of purified products in to pGEM-T vector:

Purified PCR products were ligated on pGEM-T vector.

3.19.9 Ligation and selection:

Purified PCR products were ligated in pGEM-T vector (Promega, Madison, USA). 20 µl per tube of ligation mixture was prepared. The ligated products were transformed in *E. coli* JM107 competent cells. And the recombinant transformants were screen by Blue-White screening method.

3.19.10 PCR detection of β-lactamase genes and integrons in recombinants:

Multiplex PCR assay and integrase gene PCR was carried out for the presence of *bla*$_{ESBLs}$ and integrons in all recomibinants.

3.19.11 Isolation of Plasmids from recombinants:

The recombinants strains were grown in LB broth medium containing 0.5 µg/ml cefotaxime. The recombinant plasmids were purified by plasmid purification kit.

3.19.12 Sequencing of recombinant plasmids:

50 µL of purified plasmids were used for sequencing along with 20 µL each (10 picomole each primer) primers. Sequence results were analysed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.20 Detection of transcription level of ESBL genes under inducing and non inducing conditions

3.20.1 Culture of isolates under non inducing conditions:

Isolates harbouring multiple ESBL genes were inoculated in LB broth medium without any antibiotic pressure and were incubated in shaker incubator (160rpm) at 37°C for two hours until O.D600nm reached 0.5-0.7.

3.20.2 Induction of ESBL producing isolates harbouring multiple ESBL genes with antibiotic pressure

Isolates harbouring multiple ESBL genes were inoculated in LB broth medium in inducing condition by adding cefotaxime, ceftazidime and ceftriaxone at 0.5 µg/ml individually and were incubated in shaker incubator (160rpm) at 37°C for two hours until O.D600nm reached 0.5-0.7.

3.20.3 Isolation of mRNA from bacterial cells

mRNA was purified with Qiagen RNeasy Mini Kit (Germany) using following procedure

3.20.3.1 Procedure:

- ✓ The incubated culture was centrifuged for 10 minutes at 8000 rpm at 4°C.
- ✓ The supernatant was decanted and residual supernatant was removed by gently dabbing the inverted tube once on a paper towel.
- ✓ 700 µl RLT buffer was added and vortexed vigorously for direct lysis of bacterial cells.
- ✓ Lysate was centrifuged for 3 minutes at 8000 rpm and supernatant was collected in to new tubes.
- ✓ 1 volume of 70% ethanol was added to the lyaste and mixed well by pipetting.
- ✓ 700 µl lysate was added to an RNeasy Mini spin column placed in a 2 ml collection tube and was centrifuged for 30 seconds at 8000 rpm.
- ✓ Flow through was discarded and step 1 was repeated.
- ✓ 700 µl RW1 buffer was added, the tube was centrifuged for 30 seconds at 8000 rpm and the flow through was discarded.
- ✓ 500 µl RPE buffer was added and the tube was centrifuged for 2 minutes at 8000 rpm. Flow through was discarded.
- ✓ The column was placed in to the new 1.5 ml collection tube and was centrifuged for 1 minute at 8000 rpm.
- ✓ 80 µl RNase free water was added to elute the RNA by centrifugation for 1 minute at 8000 rpm.
- ✓ mRNAs were quantified by Picodrop (Pico 200, Cambridge, UK).
- ✓ The tube was stored in -20°C mini cooler and immediately proceeds for cDNA preparation.

3.20.4 cDNA synthesis of mRNA

cDNA was prepared using QuantiTect® Reverse Transcription kit (Qiagen, Germany) with following procedure

3.20.4.1 Procedure:

- ✓ Template RNA, gDNA wipeout buffer, Quantiscript® reverse transcriptase, and Quantiscript RT buffer was collected separtately.
- ✓ The genomic DNA elimination reaction was prepared on ice.

gDNA wipeout buffer, 7X	-2µl
Template RNA	-12µl
Total reaction volume	-14µl

- ✓ Total Genomic DNA elimination reaction was incubated for 2 minutes at 42°C and after that immediately placed on ice.
- ✓ The reverse transcriptase master mix was prepared on ice accordingly:

Reverse transcriptase master mix	-1µl
Quantiscript RT buffer, 5X	-4µl
RT primer mix	-1µl
Template RNA (Entire genomic elimination reaction)	-14µl
Total reaction volume	- 20µl

- ✓ Tubes containing total reverse transcriptase master mix were incubated for 15 minutes at 42°C.
- ✓ Additionally tubes containing total reverse transcriptase master mix were incubated for 3 minutes at 95°C to inactivate the Quantiscript reverse transcriptase.
- ✓ The reverse transcription reactions were stored in -20°C for further use.

3.20.5 Real Time PCR

Relative quantification of gene expression allows quantifying differences in the expression level of a specific gene between different isolates. Output data is expressed as a fold-change or a fold difference of expression levels. Real Time PCR was performed targeting ESBL genes in the isolates harbouring multiple ESBL genes.

3.20.5.1 Procedure

3.20.5.1.1 Primers: For Real Time PCR, internal primers (Table 17) specific for the genes namely, CTX-M, SHV, OXA-2, OXA-10, and PER were used for reactions with cDNA as template. RNA polymerase gene rpsl was used as internal control.

3.20.5.1.2 Preparation of DNA template: Prepared cDNA was used as template for the reaction.

3.20.5.1.3 Preparation of reaction mixture: Each single reaction mixture (20 µl) contained 2.0 µl of template cDNA (80ng/µl), 1 µl of each primer (10picomole), 10 µl of Power SYBER® green PCR master mix (Applied Bio system, Warrington WA1 4SR, UK.) and nuclease free water.

Table 17: Internal primers used for expression of multiple ESBL genes

Name of target genes	Sequence (5'-3')
SHV In F'	AGCTGCTGCAGTGGATGGTG
SHV In R'	GTCTTATCGGCGATAAACCA
CTX-M In F'	TTGAGATCAAAAAATCTGAC
CTX-M In R'	CTAAGCTCAGCCAGTGACAT
OXA-10 In F'	ATGGCAACCAGAATATCAGT
OXA-10 In R'	TAGAAACTCCACTTGATTTAA

OXA-2 In F’	ATGAGCTATTTGCAAAGGAA
OXA-2 In R’	CGCCATTACTTGTCGAAGGA
PER In F’	CAGGATCTCTGCAGCACCTT
PER In R’	CGATGATCAGGTGCAGTATC
rpsl F’	GCAAAAACGTGGCGTATGTACTC
rpsl R’	TTCGAAACCGTTAGTCAGACGAA

3.20.5.1.4 Reaction condition: Reactions were run under the following conditions: initial denaturation 95°C for 2 min, 40 cycles of 95 °C for 20 sec, 52°C for 40 sec, 72°C for 30 sec.

3.20.5.1.5 Interpretation:

Relative quantification (RQ) values of each ESBL genes were compared with the reference samples in basal label and by induction with antibiotic pressure RQ versus log10.

3.20.5.1.6 Statistical analysis:

All the data of Real Time PCR were analysed by one way ANOVA and turkey’s multiple comparison test using Graph Prism 3 software.