Materials and method

The present study was conducted in the Department of Microbiology, Assam University, Silchar, Assam, India. The duration of study was from May 2013 to April 2015. The bacterial strains were collected from Silchar Medical College and Hospital, Silchar, Assam and different community health centers on Silchar town. This tertiary referral hospital serves around 38,26,110 number of populations of Assam [including districts like Cachar, Hailakandi, Karimganj, North Cachar Hills (Dima Hasao)] and neighbouring states like North Tripura, Mizoram, Meghalaya and Manipur. Environmental samples were collected from different sites of rivers of southern Assam, water bodies near waste disposal site and food samples were collected from food vendor shops of Silchar town.

3.1 Samples:

3.1.1 Patient population:

The patient who were admitted to different wards and those who attended the outpatient departments from May 2013 to April 2015 were selected for the study. The subjects included in the present study were diagnosed as cases of one of the following; urinary tract infection, meningitis, pyogenic infection, bacterial pneumonia, bacteremia/septicemia and diarrheal diseases. A total of 967 consecutive non-duplicate clinical specimens were studied during the period (Table 5).

3.1.2 Environmental samples:

Samples were collected from river water (Barak river, Ghagra river, Jatinga river, Singla river, logai river) from eleven different sites of each river (n=55). Water samples were also collected from water bodies (n=27) near waste disposal sites,

while food samples (n=48) were collected from food vendor shops. Samples were collected from November 2014 to August 2015.

3.2 Isolates for the study:

A total of 967 consecutive non - duplicate clinical specimens were studied, from which 864 bacterial isolates were obtained, of which 212 (24.54%) were identified as members of Enterobacteriaceae family comprising 80 (37.74%) isolates from Silchar Medical College and Hospital and 132 (62.26%) from different community health centres (Table 5). All the isolates were identified based on Gram staining characteristics, cultural characteristics and biochemical reaction (Collee et al., 1996).

Table 5: Types of clinical specimen obtained for the study

Clinical specimens	**Hospital**	**Community health centres**	**Total**
Urine	238	274	512
Pus	63	15	78
Stool	53	33	86
Ear swab	47	14	61
Tracheal aspirate	10	-	10
Throat swab	32	9	40
Sputum	55	43	98
Blood	56	25	81
Total	554	413	967

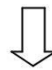
3.3 Isolation and identification of isolates from clinical specimens:

The specimens were streaked onto Blood agar, MacConkey lactose agar and CLED (cystine lysine electrolyte deficient agar) agar (Urine isolates) and incubated at 37^0C overnight. Colony morphology and cultural characteristics were observed microscopically.

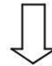
Identification of Gram negative rods was done by Gram staining, motility, and standard biochemical reactions.

3.3.1 Gram staining:

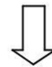
A thin smear of the culture was prepared and allowed to air dry and fixed.



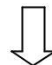
The smear was overlaid with crystal violet for 1 min.



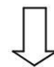
Rinsed with distilled water.



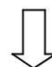
The smear was flooded with Gram's iodine for 1 min.



Rinsed with distilled water.

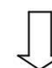


Ethyl alcohol was added drop by drop until no violet color appears in rinse.

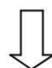


Rinsed with distilled water immediately.

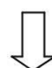
The smear was overlaid with safranin for 30 sec.



Rinsed with distilled water.



Air dried



Observed under oil immersion objective for characteristic Gram stain reaction.

Interpretation:

Gram positive organism stained **violet**.

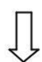
Gram negative organism stained **pink red**.

3.3.2 Motility (hanging drop preparation):

A clean cover slip was taken and paraffin or petroleum jelly 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 centre.



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 test:

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

With the help of the applicator stick a small portion of 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.

Interpretation:

Positive reaction: Production of rapid and continuous gas bubbles.

Negative reaction: Lack of gas bubble formation.

Quality control:

Staphylococcus aureus* ATCC 25923*: Positive

***Streptococcus pyogenes* ATCC 1924 :** Negative

3.3.3.2 Oxidase test:

The 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 dyetetramethyl-*p*-phenylene- diamine.

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

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

Interpretation:

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

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

Quality control:

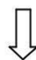
Pseudomonas aeruginosa **(PAO1):** Positive

E.coli **ATCC25922:** Negative

3.3.3.3 Indole test:

This test demonstrates the ability of certain bacteria to decompose the aminoacid tryptophan to indole, which accumulates in the medium.

Culture was inoculated in the medium containing peptone and NaCl, and incubated at 37^0C overnight.



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

Interpretation:

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

Negative reaction: No color development.

Quality control:

E.coli ATCC 25922**:** Positive

K. pneumonia ATCC 700603**:** 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^{0}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.

Interpretation:

Positive reaction: Formation of bright red color.

Negative reaction: Formation of yellow color.

Quality control:

E.coli ATCC 25922**:** Positive

K. pneumonia ATCC 700603**:** 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 glucose phosphate broth (Hi- Media, Mumbai, India) and incubated at 37^0C for overnight.

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

It was mixed properly and reading was taken immediately.

Interpretation:

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

Negative reaction: Yellow color at the surface of the medium.

Quality control:

K. pneumonia ATCC 700603**:** Positive

E.coli ATCC 25922**:** Negative

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

Slant of Simmon's citrate agar (Hi- Media, Mumbai, India) was prepared in the 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 37^0C for overnight.

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).

Quality control:

K. pneumonia ATCC 700603**:** Positive

E.coli ATCC 25922**:** Negative

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

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

Incubated at 35^0C for 18-24 hours.

Interpretation:

Positive reaction: Pink color in the slant.

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

Quality control:

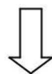
Proteus mirabilis **MTCC 9242:** Positive

E.coli ATCC 25922**:** Negative

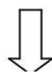
3.3.3.8 Triple sugar iron test: The medium contains protein sources that permit the growth of most bacterial strains. Lactose, sucrose and glucose are present as well as phenol red indicator. Glucose is n concentration of one tenth as

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 test tube was incubated at 37^0C for overnight.

Interpetation:

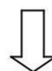
The result was interpreted according to the reaction obtained in the test (Table 6).

Table 6: Summary of TSI reactions

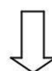
Reaction	Carbohydrate fermented	Typical organisms
Alkaline slant/ acidic butt (K/A reaction)	Glucose is fermented, lactose and sucrose is not fermented. Organism is non-lactose fermenter.	*Shigella* Spp., *Vibrio* spp.
Alkaline slant/ acidic butt/black precipitate of H_2S (K/A H_2Spositivereaction)	Glucose 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 1ml of sterile distilled water



Pure cultures of test organism was inoculated



Incubated at 35^{0}C for 24 hours.

Four to five drops of 10% ferric chloride ($FeCl_3$) was added to the inoculated media

Result was observed

Interpretation:

Positive reaction: Intense green color.

Negative reaction: No color change.

Quality control:

***Proteus mirabilis* MTCC 9242:** Positive

E.coli ATCC 25922**:** Negative

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

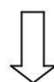
The decarboxylases are enzymes that attack the carboxyl group of specific amino acids, forming amines and carbon dioxide. The amines formed are alkaline, and they alter color of pH indicator. Each decarboxylase is specific for a

particular amino acid. Tests for lysine decarboxylase, ornithine decarboxylase and arginine dehydrolase are generally performed on the enteric bacteria. Lysine decarboxylated to cadaverine; orinthine is decarboxylated to putrescine; arginine undergoes dehydrolase reaction to form citruline, which is then converted to orinthine n 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 at 35^0C for 24 hours

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.

Quality control:

All readings were taken after 24 hours.

Arginine:

Enterobacter cloacae **MTCC 8544*:*** Positive (purple), alkaline

Klebsiella pneumonia ATCC 700603**:** Negative (yellow), acidic

Ornithine:

Enterobacter cloacae* MTCC 8544*: Positive (purple), alkaline

Klebsiella pneumonia ATCC 700603**:** Negative (yellow), acidic

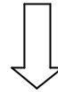
Lysine:

Klebsiella pneumonia ATCC 700603**:** Positive (purple), alkaline

Enterobacter cloacae* MTCC 8544*: Negative (yellow), acidic

3.3.3.11 Sugar fermentation: This test was used to detect an organism's ability to ferment the sugar glucose, lactose, sucrose and mannitol, as well as its ability to convert the end product of glycolysis, pyruvic acid to gaseous byproduct.

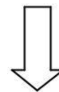
A carbohydrate fermentation broth was prepared at pH7.4 and distributed in four sterile test tubes for each sample.



The broth contains 0.5% of carbohydrae to 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 37^0C for overnight.

Interpretation:

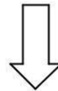
Color of the media changes to pink with gas in Durham's tube: Fermentation with production of gas.

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

Color of the media remains yellow: Not fermented.

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

Inoculum was stabbed in duplicate tubes containing O/F medium



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

The tubes were incubated initially for 24 hours at 37^0C

Yellow coloration of the media was noted

Interpretation:

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

Quality control:

Pseudomonas aeruginosa* PAO1*: Oxidative

E. coli* ATCC25922*: Fermentative

3.3.3.13 Nitrate reduction test: This test detects the ability of organism 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 37^0C 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.

Interpretation:

Positive reaction: Development of brick red color in the media.

Negative reaction: No color development.

Quality control:

E.coli ATCC 25922**:** Positive

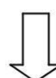
***Acinetobacter baumanii* MTCC9869:** Negative

3.4 Antibiotic susceptibility testing

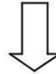
Antibiotic susceptibility was done by Kirby Bauer disc diffusion method and Minimum inhibitory concentration determination.

3.4.1 Disc Diffusion Method:

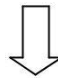
Mueller Hinton Agar plates were prepared with a depth of about 4mm



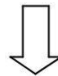
Pure culture was selected for preparation of inoculum. 3-4 similar colonies were selected and transferred into peptone water. Incubated at 35^0C for 2-8 hours, till light to moderate turbidity was achieved. The turbidity of suspension was adjusted to MacFarland's standard 0.5 (1.5x 10^8 CFU/ml)



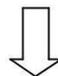
A sterile cotton swab was dipped into the suspension



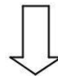
The soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid



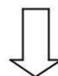
It was streaked evenly onto the entire agar surface of the plate three times, turning the plate 60^0 angle between each streaking



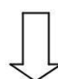
The inoculum is allowed to dry to 5-15 min. with lid in place



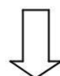
Commercially prepared antibiotic discs (Table 6) were applied using aseptic technique



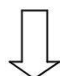
The disc were placed with centres at least 24 mm apart



Incubated immediately at 37^{0}C and examined after 14- 16 hours



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



The zone of inhibition was measured in millimeter and compared with standard chart provided in CLSI guideline (CLSI, 2014)

3.4.1.1 Procedure for the Preparation of a 0.5 MacFarland Standard:

1. Approximately 85 ml of 1% sulfuric acid (H_2SO_4) to a 100 ml volumetric flask was added.
2. Using a volumetric pipette, 0.5 ml of 1.175% anhydrous barium chloride ($BaCl_2$) was added drop wise to the 1% sulfuric acid (H_2SO_4) while constantly swirling the flask.
3. The volume was then brought to 100 ml with 1% H_2SO_4.
4. The solution was stirred for approximately 3 to 5 minutes while examining visually, until the solution appears homogeneous and free of clumps.
5. Optical density was checked according to the QC acceptance norms.
6. Solution was dispensed in glass screw cap labelled tube and sealed with paraffin.
7. The tubes were then stored in a dark room temperature.

Table 7: Antimicrobial agents used for the study

Antimicrobial agents *	Strength(µg)
Nalidixic acid	30
Norfloxacin	10
Ciprofloxacin	5
Ofloxacin	5
Levofloxacin	5
Sparfloxacin	5
Lomefloxacin	5
Gatifloxacin	5
Gemifloxacin	5
Ampicillin	10
Co-trimoxazole	25
Tigecycline	15
Gentamicin	120
Amikacin	30
Imipenem	10
Ceftazidime	30
Cefotaxime	30
Ceftriaxone	30
Polymixin B	300U

***Source: Hi-Media, Mumbai, India**

3.4.2 Minimum inhibitory concentration (MIC) study:

MIC study was done using quinolone antibiotics like: norfloxacin, ciprofloxacin, ofloxacin, levofloxacin and gatifloxacin by agar dilution method and the results were interpreted as per CLSI guidelines (CLSI, 2014).

3.4.2.1 Preparation of stock solution:

A quantity of 10 mg of each antibiotics namely norfloxacin, ciprofloxacin, ofloxacin, gatifloxacin, moxifloxacin and levofloxacin (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.

3.4.2.2 Preparartion of agar dilution plates:

A series of dilution of six quinolone antibiotics ranging from 0.5 µg/ ml to 256 µg/ ml were made in Muller Hinton Agar plates (Table 8).

3.4.2.3 Preparartion of agar plates:

Appropriate dilutions of each antimicrobial solutions were added to the corresponding amount of molten Muller Hinton Agar (Table 8). The agar and antibiotics were mixed thoroughly and poured into Petri dish in a depth of 4 mm. the agar was allowed to solidify at room temperature and stored at 4^0 C.

3.4.2.4 Preparation of inoculum:

Pure culture was selected for preparation of inoculum. A total of 3-4 similar colonies were selected and transferred into peptone water (Hi-Media, Mumbai, India) and incubated at 35^0 C for 2-8 hrs.The turbidity of the suspension was adjusted to MacFarlands standards 0.5 ($1.5x10^8$ CFU/ml).

3.4.2.5 Inoculation of test isolates in antibiotic agar plates:

A quantity of 2 µl of the inoculum was applied to the surface of the agar with the help of the surface micropipette (spot inoculum). A control plate (without any antimicrobials) was inoculated first followed by screen agar plates.

Quality control:

Klebsiella pneumoniae ATCC 700603 and *E.coli* ATCC 25922 were used as positive and negative control.

3.4.2.6 Incubation of antibiotic agar plate: Incubated plates were allowed to stand in room temperature until the moisture in the inoculums spot were absorbed into the agar. The plates were incubated in inverted position at 37^{0} C for 16 hrs.

Interpretation:

The minimum inhibitory concentration was recorded as the lowest concentration.

3.4.2.7 Preparation of the stock solution: An amount of 2 mg/ml stock solution of each antibiotic was prepared.

Table 8: Preparation of plates for MIC

Amount of antibiotic solution added (µl) ¥ (Original stock solution concentration of 2 mg/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
4	19.996	2
2	19.998	1
1	19.999	0.5

[¥]Only a single antibiotic is used in each plate

3.5 Genotypic characterization of *qnr*:

For genotypic characterization of *qnr* determinants, two multiplex PCR was performed by targeting different *qnr* genes namely *qnr*A, *qnr*B, *qnr*C, *qnr*S, *qnr*D and *aac*(6') *Ib-cr* (Table 9).

3.5.1 Multiplex PCR1:

PCR was performed for all isolates which were screened phenotypically positive for the presence of *qnr* determinants namely, *qnr*A,*qnr*B, ,*qnr*S and *qep.* Primers specific for the genes were used (Table 9).

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

3.5.1.2 Boiling centrifugation method:

A quantity of 1 ml of aliquotes of the test organism in Brain Heart Infusion Broth (Hi-Media, Mumbai, India) was centrifuged in 10,00 rpm for 10 minutes. The pellets were resuspended in 100 µl of sterile distilled water, heated to 85^{0}C in dry block for 10 minutes, cooled in ice and centrifuged at 10,000 rpm for 10 mzinutes. These suspensions were used for PCR assay. Previously confirmed isolates harbouring *qnr*A, *qnr*B, *qnr*S was taken as positive control (Source: P.D Hinduja Hospital, Mumbai) and *E. coli* ATCC 25922 was taken as negative control.

3.5.1.3 Preparation of reaction mixture:

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

3.5.1.4 Reaction condition:

Reactions were run under the following conditions: initial denaturation 95^{0} C for 5 min, 32 cycle of 95^{0} C for 20 sec, 54^{0} C for 40 sec, 72^{0} C for 1 min and final extension at 72^{0} C for 7 min.

Table 9: Oligonucleotide used as primers for amplification of different *qnr* genes.

Primer pair	**Target**	**Sequence (5′3′)**	**Product size (bp)**	**Reference**
*qnr*A-1A *qnr*A-1B	*qnr*A	TTCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCAA	628	Wu, et al., 2007
*qnr*B-CS-1A *qnr*B-CS-1B	*qnr*B	CCTGAGCGGCACTGAATTTAT GTTTCTGCTCGCCAGTCGA	546	Wu, et al., 2007
*qnr*S-1A *qnr*S-1B	*qnr*S	CAATCATACATATCGGCACC TCAGGATAAACAACAATAC CC	675	Wu, et al., 2007
*qnr*C-F *qnr*C-R	*qnr*C	GGGTTGTACATTTATTGAATC TCCACTTTACGAGGTTCT	447	Wu, et al., 2007
*qnr*D-F *qnr*D-R	*qnr*D	CGAGATCAATTTACGGGGAAT A AACAAGCTGAAGCGCCTG	582	Cavaco, et al., 2009
aac(6')-Ib-cr-F *aac(6')-Ib-cr*-R	*aac(6')-Ib-cr*	ATGACTGAGCATGACCTTGC TTAGGCATCACTGCGTGTTC	519	Cano, et al., 2009
*qep*A-F *qep*A-R	*qep*A	GCAGGTCCAGCAGCGGGTAG CACGATACTCGGGCAGGAAG	260	Perichon, et al., 2008

3.5.1.5 Gel electrophoresis and analysis of PCR products:

Amplified products of PCR were identified by agarose gel electrophoresis. An amount of 15 µl of each PCR product was run in 1% agarose gel (HiMedia, Mumbai, India) at constant 50 volts for one hour with Tris Borate EDTA (TBE) buffer.

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

Interpretation:

Presence of bands of molecular weight of 628 bp, 548 bp, 675 bp and 260 bp confirmed the presence of *qnr*A, *qnr*B, *qnr*S and *qep,* respectively.

3.5.2 Multiplex PCR2:

PCR was performed for all isolates which were screened phenotypically positive for the presence of *qnr* determinants namely, *qnr*C, *qnr*D and *aac*(6')*Ib-cr*. Previously confirmed isolates harbouring *qnr*D, *qnr*C and *aac*(6')-*Ib-cr* were taken as positive control (Source: INS, BHU, Varanasi) and *E.coli* ATCC 25922 were taken as negative control.

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

3.5.2.2 Preparation of reaction mixture:

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

3.5.2.3 Reaction condition:

Reactions were run under the following conditions: initial denaturation 95^0 C for 5 min, 32 cycle of 95^0 C for 20 sec, 54^0 C for 40 sec, 72^0 C for 1 min and final extension at 72^0 C for 7 min.

3.5.2.4 Gel electrophoresis and analysis of PCR products:

Amplified product of PCR was identified by agarose gel electrophoresis. A quantity of 5 µl of each PCR product was run in 1% agarose gel (HiMedia, Mumbai, India) at constant 50 volts for one hour with Tris Borate EDTA (TBE) buffer.

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

3.5.2.5 Preparation of TBE (10X):

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

Interpretation:

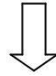
Presence of bands of molecular weight of 447 bp, 582 bp, 519 bp confirmed the presence of *qnr*C, *qnr*D and *aac*(6') *Ib-cr* genes, respectively.

3.6 PCR product purification:

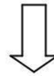
PCR products were purified using gene JET PCR product purification kit (Thermo Scientific, Lithuania). Following steps were used.

volume of 1:1 binding buffer was added to the PCR product

(Volume: Volume)

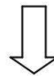


An amount of 800µl of the solution was transferred to the Gene JET purification column and centrifuged it for 60 seconds. The flow- through was discarded.

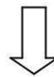


Wash Buffer of 600 µl (diluted with ethanol) was added to the the Gene JET purification

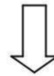
column and centrifuged it for 60 seconds.



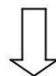
The flow- through was discarded and column placed in same collection tube.



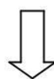
Empty Gene JET purification column was centrifuged for additional 60 seconds to completely remove any residual wash buffer.



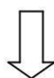
The Gene JET purification column was transferred to a clean 1.5 ml micro centrifuge tube.



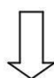
50 µl of Elution Buffer was added to the centre of the Gene JET purification column membrane and centrifuged for 60 seconds.



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 -20^0 C.

3.7 Sequencing of all *qnr* genes:

An amount of 30 µl of purified PCR products were used for sequencing along with *qnr* gene primer each 20 µl (10 picomole each primers). Sequencing results were analyzed using BLAST suite program of NCBI (http://blast.ncbi.nlm.gov/Blast.cgi)

3.8 Transferability of *qnr* genes:

3.8.1 Isolation of plasmid:

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

- ✓ The isolates harboring single *qnr* gene.
- ✓ The isolates harboring multiple *qnr* gene.

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 10).

Table 10: Composition of buffer for isolation of plasmids

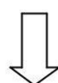
Source: http://kirschner.med.havard.edu/files/protocol/QIAGEN_QIAGEN

Plasmid Purification_EN.pdf

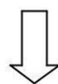
Buffer	**Composition**	**Storage**
Buffer P1 (resuspension buffer)	50mM Tris.Cl,pH 8.0; 10mM EDTA; 100µg/ml Rnase A	2-8^0 C after addition of Rnase A
Buffer P2 (lysis buffer)	200mM NaOH, 1% SDS (w/v)	15- 25^0C
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5	15- 25^0C or 2- 8^0C
Buffer FWB2 (QIA filter wash buffer)	1 M potassium acetate, pH 5.5	15- 25^0C
Buffer QBT (equilibration buffer)	750 mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton®X- 100 (v/v)	15- 25^0C
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v);	15- 25^0C
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris Cl, pH 8.5; 15% isopropanol (v/v)	15- 25^0C
Buffer QN (elution buffer)	1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	15- 25^0C
TE	10 mM Tris Cl, pH 8.0; 1mM EDTA	15- 25^0C
STE	100 mM NaCl; 10 mM Tris Cl, pH 8.0; 1 mM EDTA	15- 25^0C

Procedure:

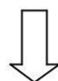
A single colony was picked up from a freshly streaked selective medium plate and inoculated in a starter culture of 2-10 ml LB medium containing (0.25µg/ml) ciprofloxacin and chloramphenicol (0.25µg/ml), and was incubated for 8 hrs. at 37^0 C with vigorous shaking (300rpm)



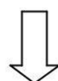
The starter culture was further diluted 1/500 into 3 ml LB medium. Grown at 37^0 C for 16 hrs. with vigorous shaking (300 rpm)



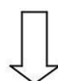
Bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4^0 C



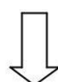
Bacterial pellets were resuspended in 0.3 ml of buffer P1



A quantity of 0.3 ml of buffer P2 was added, mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature (25^0 C) for 5 min



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



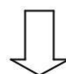
Visualization: Isolated plasmid was run by agarose gel electrophoresis containing 0.8% agarose and size of the plasmid was determined by comparing with DNA ladder.

3.8.2 Transformation of plasmid and their role in antibiotic resistance in recipient:

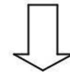
Isolated plasmid was transformed into *E.coli* JM 107 competent cells.

3.8.2.1 Preparation of competent cells:

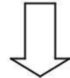
Overnight culture of cells was grown.



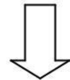
Culture was inoculated by diluting saturated overnight culture 1: 100 into LB media



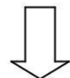
Cells were grown at 37^{0} C until OD600 reaches ~ 0.25



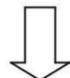
Culture was chilled in ice for 15 min to slow growth



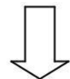
Cells were pelleted by centrifugation at 2500 x g for 15 m in at 4^{0} C



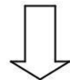
Supernatant was decanted



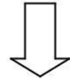
Cells were resuspended in 1/5 volume of original culture of ice-cold 0.1 M $CaCl_2$



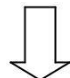
Cells were incubated on ice for 20 min



Cells was pelleted by centrifugation at 2500 x g for 15 m in at 4^{0} C

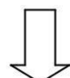


Supernatant was decanted

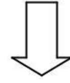


Cells were resuspended in 1/25 volume of original culture of

ice-cold 0.1 M $CaCl_2$, 10% glycerol



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

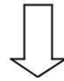


Stored at -80^{0}C

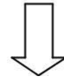
3.8.2.2 Transformation procedure

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

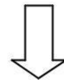
The competent cells were removed from -80^0 C and thawed on ice



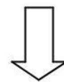
The cells were mixed gently by flicking tubes and a 50µl of cells were taken in sterile micro centrifuge tubes



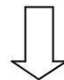
An amount of 10µl of plasmids were added in to cell suspension and the tube was swirled gently for a few seconds to mix



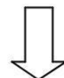
The tubes were incubated on ice for 30 minutes



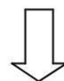
The tubes were placed in water bath at 42^{0}C for 40 seconds without shaking



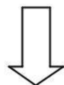
Tubes were removed from water bath and immediately placed on ice for 10 minutes



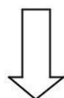
The tubes were diluted by adding the 500µl of SOC medium



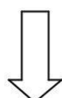
The tubes were kept in shaker incubator at 37^0C and 200 rpm for 2 hours.



The cells were centrifuged for 3 minutes at 5000 rpm



The pellets were spread on LB norfloxacin, LB ciprofloxacin, LB ofloxacin, LB levofloxacin agar medium plates



The plate was incubated for 12- 16 hours in incubator at 37^0 C

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

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

3.8.2.4 Preparation of stock solution

A quantity of 10mg of quinolone (norfloxacin, ciprofloxacin, ofloxacin and levofloxacin) were dissolved in each 5 ml of sterile triple distilled water a vial to make a final volume
2mg/ml.

3.8.2.5 Composition of SOC medium (per litre):

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

3.8.2.6 Preparation of LB norfloxacin, LB ciprofloxacin, LB ofloxacin, LB levofloxacin agar plates :

Antibiotics (norfloxacin, ciprofloxacin, ofloxacin and levofloxacin) at a concentration of 0.25 µg/ml and 0.5 µg/ml were added in to separate molten cool LB agar medium (Hi-Media, Mumbai, India) and was allowed to equilibrate in water bath at 48-50^0 C. It was then mixed thoroughly and poured into petridish. The agar was allowed to solidify at room temperature and stored at 4-8^0 C.

3.8.2.7 Spreading of cells on LB agar plates:

Cells were centrifuged for 3 minutes and pellets were spread on each LB agar medium containing separate 0.25 µg/ml and 0.5 µg/ml of norfloxacin, ciprofloxacin, ofloxacin and levofloxacin.

Interpretation:

Any growth on the LB norfloxacin agar plate, LB ciprofloxacin agar, LB ofloxacin agar or LB levofloxacin agar plates confirmed successful transformation.

3.8.2.8 PCR detection of *qnr* gene in transformed recipient:

Transformed recipient cells harboring plasmid of isolates carrying single and multiple *qnr* genes were subjected to PCR to detect the presence of *qnr* genes (Table 9).

3.8.2.9 Minimum inhibitory concentration of transformants:

MIC study was done using quinolone antibiotics like: norfloxacin, ciprofloxacin, ofloxacin, levofloxacin and gatifloxacin by agar dilution method according to CLSI guidelines before transformation. Further MIC was again determined against the above mentioned antibiotics after transformation. The suspension was adjusted to McFarland standards and incubated on MH agar plates containing 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 µg of antibiotics.

3.8.3 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. A total of 5 multiplex and 3 simplex PCRs were 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 11) (Carattoli et al., 2005).

3.8.3.1 Targeted gene in multiplex PCR:

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

3.8.3.2 Targeted gene in simplex PCR:

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

3.8.3.3 Preparation of DNA template: Plasmid was isolated by plasmid isolation kit [Qiagen mini prep kit (Germany)].

3.8.3.4 Primers: Primers used in the table are described in Table 11 (Carattoli et al., 2005).

3.8.3.5 Preparation of reaction mixture:

Each sngle reaction mixture (25 µl) contained 1.0 µl of template DNA (100ng/ µl), 1 µl of each primer (10 picomole), 12.5 µl of Go green Taq Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.8.3.6 Reaction condition for multiplex PCR:

PCR reactions were run under the following conditions: initial denaturation 95^{0} C for 2 min, 30 cycle of 95^{0} C for 20 Sec, 60^{0} C for 30 Sec, 72^{0} C for 1 min and final extension at 72^{0} C for 5 min.

3.8.3.7 Reaction condition for simplex PCR:

PCR reactions were run under the following conditions: initial denaturation 95^{0} C for 2 min, 30 cycle of 95^{0} C for 20 Sec, 52^{0} C for 30 Sec, 72^{0} C for 1 min and final extension at 72^{0} C for 5 min.

3.8.3.8 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 (HiMedia, Mumbai, India) at constant 50 volts for two hours with Tris Borate EDTA (TBE) buffer.

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

Interpretation:

Presence of bands of different molecular weights confirmed the presence of the different Inc groups in plasmid.

Table 11: Primers used for PCR based replicon typing:

Primer	DNA sequence	Size (bp)
HI1 FW	5'- GGAGCGATGGATTACTTCAGTAC -3'	471
HI1 RV	5'- TGCCGTTTCACCTCGTGAGTA-3'	
HI2 FW	5'- TTTCTCCTGAGTCACCTGTTAACAC-3'	
HI2 RV	5'- GGCTCACTACCGTTGTCATCCT-3'	644
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/B RV	5’- TCTTTCACGAGCCCGCCAAA-3’	
B/O RV	5’- TCTGCGTTCCGCCAAGTTCGA-3’	159

3.8.4 Transfer of resistance determinants through conjugation:

Conjugation was performed with all *qnr* positive isolates. Bacterial conjugation was performed using clinical and environmental isolates harboring *aac*(6’)*Ib-cr* and *qnr* genes as donor strain and streptomycin resistant strain B *E.coli* as recipient strain.

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

3.8.4.2 Donor strain: Genotypically characterized clinical isolates harboring *qnr* genes were selected as donor strain which were phenotypically resistant to either norfloxacin, ciprofloxacin, ofloxacin or levofloxacin.

3.8.4.3 Strain B (Recipient strain): The strain B is devoid of any 'F' factor, carrying streptomycin resistant gene in its chromosome was selected as recipient strain of *E. coli*. Antibiotic was added to the LB media at a concentration of 800µg/ml (Genie, Bangalore).

3.8.4.4 Preparation of antibiotic stock solution:

100mg of ciprofloxacin, norfloxacin, ofloxacin and levofloxacin powder was dissolved in each 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 0.25 µg/ml and 0.5 µg/ml concentration, to inhibit the growth of recipient strain.

3.8.4.5 Preparation of Streptomycin stock: 150 mg of streptomycin was dissolved in 1.5 ml sterile triple distilled water to get a final concentration of 100mg/ml and the vial was covered with aluminium foil and stored at 4^{0} 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, where strain B showed confluent growth at the same concentration.

3.8.4.6 Revival of donor strain and strain B:

1. One set of lyophilized vial of recipient *E. coli* strain B was opened and rehydrated with 0.1ml of sterile LB broth. Also incubated 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 norfloxacin, ciprofloxacin, ofloxacin and levofloxacin with each having concentration of 0.5 µg/ml and the recipient strain on LB supplemented with streptomycin (concentration 800 µg/ml).

3. Remaining 50 µl of the suspension was inoculated in a tube containing 5 ml LB broth with respective antibiotics.

4. Incubated at 37^0 C for 16 to 20 hours.

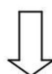
3.8.4.7 Preparation of bacteria: A single colony from each of the donor and the recipient plate was inoculated into 6 ml LB broth containing the respective antibiotic.

3.8.4.8 Procedure of conjugation:

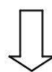
1ml of donor culture was inoculated into each 20 ml of LB broth (in 250 ml conical flask) with norfloxacin, ciprofloxacin, ofloxacin and levofloxacin with each having concentration of 0.5 µg/ml and incubated at 37^0 C in a shaker.

3ml of overnight recipient culture was inoculated into 20ml LB broth (in 250ml

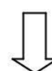
conical flask with streptomycin at a concentration 800µg/ml) and incubated at 37^0 C in a shaker



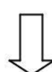
Recipient and donor cultures were incubated till the O.D. of donor culture reaches 0.8- 0.9 at A_{600}



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

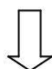


Gently mixed and tube was incubated in an incubator at 37^0 C for 2 hours.

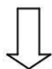


0.2 ml each of donor and recipient cultures were taken out two different test tubes and

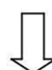
incubated at 37^0 C for 1 hour 30 min.



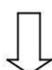
After incubation, 2 ml of sterile LB broth was added to each tube.



Again, both the donor the recipient tubes were incubated at 37^0 C for 2 hours.



0.1 ml each of donor, recipient and the conjugated samples were spread onto each of the antibiotic supplemented plates.



The inoculated plates were incubated overnight at 37^0 C.

3.8.4.9 PCR detection of *qnr* gene in transconjugants:

Multiplex PCR was performed to detect the presence of *qnr* genes in all transconjugants.

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

PCR based replicon typing was performed to detect the presence of different Inc plasmid groups (Table 13) in all transconjugants.

3.8.3.11 Minimum inhibitory concentration of transconjugants:

MIC study was done using quinolone antibiotics like: norfloxacin, ciprofloxacin, ofloxacin, levofloxacin and gatifloxacin by agar dilution method according to CLSI guidelines 2014 before conjugation. Further MIC was again determined against the above mentioned antibiotics after transformation. The suspension was adjusted to McFarland standards and incubated on MH agar plates containing 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 µg of antibiotics.

3.9 DNA fingerprinting of quinolone resistant determinants:

Typing of quinolone resistant organisms was done by Enterobacterial repititive intergenic consensus (ERIC) PCR. ERIC PCR was done for all the members of Enterobacteriacae family that were quinolone resistant.

3.9.1 Enterobacterial repititive intergenic consensus (ERIC) sequences PCR:

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

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

3.9.1.2 Preparation of reaction mixture:

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

3.9.1.3 Reaction condition:

PCR reactions were run under the following conditions: initial denaturation 95^0 C for 2 min, 29 cycle of 95^0 C for 20 sec, 32^0 C for 45 sec, 72^0 C for 4 min and final extension at 72^0 C for 10 min.

3.9.1.4 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

(HiMedia, Mumbai, India) at constant 50 volts for two hours with Tris Borate EDTA (TBE) buffer.

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

Interpretation:

All the isolates were analyzed depending on varying molecular weights.

3.10 Determination of genetic environment:

3.10.1 Detection of variable region of integron among quinolone resistant isolates:

To find out the variable region of integron, PCR was performed using primers 5'-CS and 3'-CS (Levesque et al., 1995) (Table 12) which amplifies 5' conserved and 3' conserved sequences of the integron.

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

3.10.1.2 Primers: Primers used in the experiment are described in table 12:

Table 12: Primers used for detection of variable region of integron:

Primer pairs	Sequence (5' – 3')
5'- CS	GGCATCCAAGCAGCAAG
3'- CS	AAGCAGACTTGACCTGA

3.10.1.3 Preparation of reaction mixture:

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

3.10.1.4 Reaction condition:

PCR reactions were run under the following conditions: initial denaturation 95^0 C for 5 min, 28 cycle of 95^0C for 20 sec, 52^0 C for 1 min, 72^0 C for 2 min and final extension at 72^0 C for 10 min.

3.10.1.5 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 (HiMedia, Mumbai, India) at constant 50 volts for one hours with Tris Borate EDTA (TBE) buffer.

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

Interpretation:

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

3.10.2 Detection of the location of *qnr* and *aac(6')-Ib-cr* gene within integron region:

For detection of gene cassette within the integron region two primers was used namely 5'-CS and 3'-CS (Table 13) (Levesque et al., 1995). Two reactions were performed: in one reaction 5'-CS and reverse primer of *qnr* gene and in other reaction 3'-CS and forward primer of characterized *qnr* gene.

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

3.10.2.2 Primers: Primers used in the experiment is described in table 13:

Table 13: Primers used for the detection of gene cassette within integron region (Levesque et al., 1995)

Primer pairs	Sequence (5' – 3')
5'- CS qnr-A R	GGCATCCAAGCAGCAAG GGCAGCACTATTACTCCCAA
3'- CS qnr-A F	AAGCAGACTTGACCTGA TTCAGCAAGAGGATTTCTCA

5'- CS qnr-B R	GGCATCCAAGCAGCAAG GTTTCTGCTCGCCAGTCGA
3'- CS qnr B F	AAGCAGACTTGACCTGA CCTGAGCGGCACTGAATTTAT
5'- CS qnr- S R	GGCATCCAAGCAGCAAG TCAGGATAAACAACAATACCC
5'- CS qnr –D R	GGCATCCAAGCAGCAAG AACAAGCTGAAGCGCCTG
3'- CS qnr- D F	AAGCAGACTTGACCTGA CGAGATCAATTTACGGGAATA
5'- CS *aac*(*6*')-*Ib-cr*-R	GGCATCCAAGCAGCAAG TTAGGCATCACTGCGTGTTC
3'- CS *aac*(*6*')-*Ib-cr*-F	AAGCAGACTTGACCTGA ATGACTGAGCATGACCTTGC

3.10.2.3 Preparation of reaction mixture:

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

3.10.2.4 Reaction condition:

PCR reactions were run under the following conditions: initial denaturation 95^0 C for 2 min, 28 cycle of 95^0 C for 20 sec, 52^0 C for 1 min, 72^0 C for 2 min and final extension at 72^0 C for 10 min.

3.10.2.5 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

(HiMedia, Mumbai, India) at constant 50 volts for one hours with Tris Borate EDTA (TBE) buffer.

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

Interpretation:

Presence of band of varying molecular weight depending upon the location and arrangement of *qnr* and *aac(6')-Ib-cr* gene in the integron was formed.

3.10.2.6 Gel extraction and purification of amplified PCR product:

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

3.10.2.7 Sequencing:

An amount of 30 µl of purified PCR products were used for sequencing along with different 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.gov/Blast.cgi).

3.11 Association of *qnr* and *aac*(6')*Ib-cr* genes with mobile element:

For detection of association of *qnr* and *aac*(6')*Ib-cr* genes with various insertion sequences like *tnISEcp1*, *ISEcp1, tnpIS26, IS26*; PCR performed using the primers as described in table14:

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

3.11.2 Preparation of reaction mixture:

Each single reaction mixture (50µl) contained 1.5 µl of template DNA (approx100 ng/ µl), 2 µl of primer (20 picomole), 25 µl of Go Taq green Master Mix 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

3.11.3 Reaction condition:

PCR reactions were run under the following conditions: initial denaturation 95^0 C for 2 min, 34 cycle of 95^0 C for 20 sec, 50^0 C for 45 sec, 72^0 C for 2 min and final extension at 72^0 C for 7min.

3.11.4 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 (HiMedia,Mumbai, India) at constant 50 volts for one hours with Tris Borate EDTA (TBE) buffer.

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

Table 14: Primers used for the association of *qnr* genes with mobile element

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

tnpA IS26	AGCGGTAAATCGTGGAGTGA	
IS26	CAAAGTTAGCGATGAGGCAG	

Interpretation:

Presence of bands of varying molecular weight depending upon the location of *qnr* and *aac*(6')*Ib-cr* gene in the integron was formed.

3.11.5 Gel extraction and purification of amplified PCR product:

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

3.11.6 Sequencing:

30 µl of purified PCR products were used for sequencing along with different 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.gov/Blast.cgi).

3.12 Study of mutation in the quinolone resistant determining region by denaturing gradient gel electrophoresis:

PCR was performed for all isolates which were screened phenotypically positive quinolone resistant isolates. *E.coli* ATCC 25922 was used as a positive control.

3.12.1 Preparation of DNA template: DNA was extracted by phenol chloroform DNA extraction method (Barker et al., 1998).

3.12.2 Primers: Primers used are shown in table 15

Table 15: Primers used for the amplification of quinolone resistant determining region:

Primer product	Target	Sequence (5'-3')	Amplified product	Reference
GYRA F GYRA R	Gyr A	CGTCGCGTACTTTAC GCCATGAACG ATACCTTGCCGCGAC CGGTACGG	586	Sorlozano, et al., 2007
PARC F PARC R	Par C	TGTATGCGATGTCTG AACTG CTCAATAGCAGCTCG GAATA	265	Sorlozano, et al., 2007

3.12.3 Preparation of reaction mixture:

Each single reaction mixure (50 µl) contained 5 µl of template DNA (approx.100ng/µl), 3 µl of each primer (30 picomole), 2 µl of dnTP (20m mol), 5 µl of buffer, 1 µl of Pfu (SRL, India), and nuclease free water.

3.12.4 Reaction condition:

Reactions were run under the following conditions: initial denaturation 95^0 C for 2 min, 32 cycle of 95^0 C for 20 sec, 52^0 C for 1 min, 72^0 C for 1.20 min and final extension at 72^0 C for 7 min.

3.12.5 Gel electrophoresis and analysis of PCR products:

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 2 µl of each PCR product was run in 1% agarose gel (HiMedia, Mumbai, India) at constant 50 volts for one hours with Tris Borate EDTA (TBE) buffer.

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

Isolates which were screened phenotypically positive quinolone resistant and deviod of plasmid mediated quinolone resistant determinants were subjected to mutational study in the quinolone resistant determining region by denaturing gradient gel electrophoresis (DGGE).

3.12.6 Denaturing Gradient Gel Electrophoresis (DGGE):

Denaturing Gradient Gel Electrophoresis (DGGE) is an electrophoretic method to identify single base changes in a segment of DNA. Separation techniques on which DGGE is based were first described by Fischer and Lerman. In a denaturing gradient acrylamide gel, double - stranded DNA was subjected to an increasing denaturant environment and will melt in discrete segments called “melting domains”. The melting temperature (Tm) of these domains is sequence specific. When the Tm of the lowest melting domain is reached, the DNA will become partially melted, creating branched molecules. Partial melting of the DNA reduces its mobility in a polyacrylamide gel. Since the Tm of a particular melting domain is sequence-specific, the presence of mutation will encounter mobility shifts at different position in the gel than wild- type. If fragment completely denatures, then migration again becomes a function of size. In parallel DGGE, the denaturing gradient is parallel to the electric field, and the range of denaturant is narrowed to allow better separation of fragments.

3.12.6.1 Reagent preparation

The concentration of denaturant to use varies for the sample being analysed with the Dcode system. Denaturing gradient range used was 40 – 65%. The concentration of acrylamide varied depending on the size of the fragment analysed.

40% Acrylamide/Bis (37.5:1)

Reagent	Amount
Acrylamide	38.93 g
Bis-acrylamide	1.07 g
dH_2O	to 100.0 ml

Filter through a 0.45 µ filter and store at 4^0 C.

50x TAE Buffer

Reagent	Amount	Final Concentration
Tris base	242.0 g	2M
Acetic acid, glacial	57.1 ml	1 M
0.5 M EDTA, pH 8.0	100.0 ml	50 mM
dH_2O	to 1000.0 ml	

Mix. Autoclave for 20-30 minutes. Store at room temperature.

40% denaturing solution

Reagent	Amount
40% acrylamide	3.75 ml
Formamide	4 ml
Urea	4.2 gm

d H_2O	upto 25 ml

65% denaturing solution

Reagent	Amount
40% acrylamide	3.75 ml
50X TAE	0.5 ml
Formamide	6.5 ml
Urea	6.82 gm
d H_2O	upto 25 ml

10% ammonium persulfate

Ammonium persulfate	0.1 gm
dH_2O	1 ml

3.12.6.2 Sample Preparation

1. Optimization of the PCR product was done to minimize unwanted products which may interfere with gel analysis. PCR products were evaluated for purity by agarose gel electrophoresis before being loaded onto a denaturing acrylamide gel.
2. An equal volume of 2X gel loading dye was added to the sample.

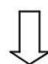
   180- 300 ng of amplified DNA per well (usually 5-10% of a 100µl PCR volume from a 100 ng DNA template) were loaded. A control strain *E.Coli* ATCC 25922 was also loaded in one well.

3.12.6.3 Temperature Controller

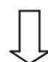
The desired temperature of the buffer in the Dcode system was maintained by the temperature controller.

3.12.6.3.1 Pre-heating the running buffer

Electrophoresis tank was filled with 7L of 1X TAE running buffer.



Temperature control module was placed on the top of the electrophoresis tank. Power cord was attached to the temperature control module and power, pump, heater was turned on.



Temperature controller was set to the desired temperature

Buffer was preheated to set temperature

3.12.6.4 Assembling the Parallel Gradient Gel Sandwich:

1. Gel sandwich was assembled on the clean surface. The large rectangular plate was laid down first, the right and left spacers of equal thickness along with the short edges of the larger rectangular plate was placed.
2. Short glass plate was placed on the top of the spacers.
3. Single screw of each sandwich clamp was loosened and each clamp was placed on the appropriate side with the locating arrows facing up and towards the glass plates.
4. Placing the left and the right clamps onto the long and short plates and placing it in the appropriate notches in the clamp, screws were tightened.

3.12.6.5 Casting parallel Denaturing Gradient Gels:

1. Gray sponge was placed onto the front casting slot. Sandwich assembly was placed on the sponge with the shorter plate facing front. The

sandwich was placed correctly and the handles of the camshaft was turned down to lock the sandwich in place.

2. Tygon tubing was cut into two 15.5 cm lengths and one 9 cm length. The longer pieces of Tygon tubing was used to conduct the gel solution from the syringes into the Y-fitting. The short piece of Tygon tubing conducted the gel solution from the Y-fitting of the gel sandwich. One end of 9 cm Tygon tubing was connected to the Y-fitting which was further connected to luer coupling. The luer fittings were connected to 30ml syringes.
3. The syringes were labeled LO and HI for low density and high density solution respectively. Plunger cap was attached to each syringe plunger head. The syringes were properly aligned with the lever on the gradient delivery system.
4. Cam wheel was rotated counter counterclockwise to vertical or start position.
5. From the stock solution, desired amount of high and low density gel solution was pipetted out into two disposable test tube.0.9% (v/v) each of ammonium persulsulfate and TEMED solutions were added and mixed by inverting several times.
6. Gel solution was drawn into the respective syringes and placed into the gradient delivery system syringe holder. Cam wheel was rotated slowly and steadily to deliver the gel solution.
7. Comb was inserted and the gel solution was left for polymerization.
8. After polymerization, comb was removed slowly and gently.

3.12.6.6 Electrophoresis:

3.12.6.6.1 Assembling the upper buffer chamber:

1. Inner coat was laid on a bench with U- shaped gasket on the inner coat seated properly.

2. The gel sandwich was released from the casting stand by turning the camshafts and the comb was removed.

3. With the short glass plate facing the core, gel sandwich was positioned into the grooves of the sandwich clamps.

4. The gel sandwich was gently pushed onto the core with one simple motion and the upper edge of the short inner glass gets seated against the notches of the U-shaped gasket.

5. Set of glass plates without the glass plates without the spacers were assembled and the screws were tightened to hold the plates in place.

6. 350 ml of running buffer was poured into the upper buffer chamber.

3.12.6.6.2 Sample loading:

1. Clear loading lid was removed. The wells were washed with running buffer to remove any unpolymerized gel material or denaturants in the well.
2. The samples were loaded into the wells using sequencing loading tip.

3. Clear loading lid was placed on top of the temperature control module.

3.12.6.6.3 Running the Gel:

Electrical leads were attatched to a suitable DC power supply.

3.12.6.7 Staining and photographing the gel:

1. After electrophoresis the gel was carefully removed from the glass plate and placed into the dish containing 250 ml of running buffer and 25µl of 10mg/ml ethidium bromide and stained 15 min.

2. After staining, the gel was carefully transferred into the dish containing 250ml of 1X running buffer and distained for 20 min.

3. The gel was placed on a UV transilluminator and the gel was visualized in Gel Doc EZ imager (Bio-Rad).

3.12.6.8 Gel extraction and purification of amplified PCR product:

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

3.12.6.9 Sequencing:

An amount of 30 µl of purified PCR products were used for sequencing along with different 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.gov/Blast.cgi)