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PREVALENCE OF MULTIDRUG RESISTANCE IN *E. COLI* & *K. PNEUMONIA* STRAINS ISOLATED FROM RIVER YAMUNA IN NEW DELHI INDIA

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ABSTRACT

The objective of this study is to reveal the present condition of pollution level of the water of Yamuna River in Delhi due to direct injection of untreated domestic drains and industrial effluents. Very expensive water treatment technologies are not capable for the treatment of polluted Yamuna river water. Generally untreated wastewater contains high level of organic and inorganic materials, numerous pathogenic microorganisms, nutrients and many other toxic compounds. Delhi has been suffering the problems increasing amount of sewage water and industrial effluents into Yamuna river for the last few decades due to horizontal and vertical expansion of the city. Yamuna river is main source of water to well qualified, high populated Delhi peoples and Agra canal originated from Okhla barrage. The water quality was good in the sites where the river enters into the Delhi stretch before the Wazirabad barrage while the river becomes highly polluted after the discharge of various drains into it. In the present study the impact of urban runoff on the water quality of Yamuna River at Delhi has been investigated which supplies into Agra canal for irrigation purposes. Water samples were collected from the different ecologically important seven different locations and analyzed for various parameters (Isolation of *E. coli* & *K. pneumoniae*, Biochemical test, Molecular identification, Antimicrobial susceptibility

test, Phenotypic detection of ESBL) & Molecular identification of ESBL & PMQR in (Delhi).

KEYWORDS: ESBL, PMQR, *E. coli*, *K. pneumonia* and Yamuna River.

INTRODUCTION

We are currently observing the spread of a rising number of anthropogenic antibiotic resistant bacteria of *E. coli* & *K. pneumonia* from water sample. Antibiotics are the biologically active compounds of natural or synthetic origin which are widely used to prevent or treat infections in humans, animals and food producing insects and plants.[1] However the emergence and spread of antibiotic resistance has a emerged as a issue of major concern worldwide[2] as the development resistance for antibiotics in bacteria will make the use of these antibiotics ineffective. A wide range of biochemical and Physiological mechanism may be responsible for resistance. The abuse of antibiotics in human medicines, animal treatment and agriculture combined with inadequate wastewater treatment has led to the presence of antibiotics and antibiotic resistant bacteria in the environment particularly in the surface waters.[3][4]

Subsequently it has led to the development of multiple drug resistance in many bacterial species.[5][6]

Antibiotic resistant gram negative bacilli (e.g. Enterobacteriaceae, Pseudomonadales) are favored, as many species are native inhabitants of water environments and they are capable of high transspecies genetic exchanges.[7][8]

So today surface waters may not only serve as reservoirs for resistance genes but also as a "market place" where susceptible strains (especially in the presence of antibiotics from waste water) can acquire new resistances.[9][10][11][12]

Occurrence and prevalence of antibiotics and multidrug resistant *E. coli* and *K. pneumonia* from various sources has been investigated in India by a few researchers.[13][14][15][16] However Resistance status of *E. coli* and *K. pneumonia* in the River Yamuna in Delhi Stretch is inadequately studied so far. The present was aimed to determine the antibiotic resistance patterns of the *E. coli* and *K. pneumonia* isolated from the River Yamuna, and to determine the existence of multidrug resistant strains.

MATERIAL AND METHODS

In all 114 Gram negative cultured isolates from Water samples taken from Yamuna River were collected over a period spanning 7 months i.e from June 2016 to December 2016. All the isolates were subjected to identification of *E. coli* & *K. pneumonia* and these were analyzed for resistance to β-lactam antibiotics.

Only one colony per plate was collected to avoid duplicates, unless isolates showed different resistance profiles.

Isolation and identification of Pathogens

Gram-negative samples from water samples in sterile vial then inoculated in plates containing Luria agar, MacConkey agar and EMB agar consecutively, by standard culture techniques. A calibrated loop was used to inoculate in MacConkey agar. The plates were incubated at 35 °C to 37 °C for 24 hrs before inspecting for growth of organisms.

Selected pink colonies were cultured on EMB agar. Isolates were further identified by a panel of biochemical test (IMViC test). Final identification was done by 16S rRNA gene analysis.

Biochemical test

Apart from colony morphology the routine biochemical tests (IMViC test) were performed to confirm the ability to produce Indole, mixed acid fermentation of glucose, the presence of acetylmethylcarbinol (acetoin) and citrate utilization.

Molecular identification

Genomic DNA was prepared by using methods of PCI & Boiling method and PCR amplification of 16S rRNA gene was carried out using the following primers 5'- ACT CCT ACG GCA GGC AGC-3' and 5'- CCG TCA ATT CAT TTG AGT TT-3'. PCR conditions for this gene comprised a thermal temperature of 94°C for 5 min, followed by 30 cycles of 94 °C for 1 min, annealing at 70°C for 30 sec. and extension at 72 °C for 1 min, followed by final extension for 10 min at 72°C.

Antibiotic susceptibility testing

Antimicrobial susceptibility was performed by disk diffusion method according to Clinical Laboratory Standard Institute (CLSI). The antibiotics used were as follows: Ceftazidime(30mcg).

Cefpodoxim(10mcg), Cefotaxime(30mcg), Ampicillin(10mcg), Ciprofloxacin(5mcg), Amikacin(30mcg), Metronidazole(4mcg), Meropenem(10mcg), Azithromycin(15mcg), and Ceftriaxone(30mcg).

Phenotypic detection of ESBL

The organism was spread on Muller-Hinton agar (Hi Media). Disk-diffusion method for ESBL screening was performed using cephalosporin alone (cefotaxime(30mcg) alone and with Clavulanic acid(10mcg), ceftazidime(30mcg) alone and with clavulanic acid (10mcg), Cefepime(30mcg) alone and with Clavulanic acid (10mcg), and cefepirome(30mcg) alone and combination with clavulanic acid (10mcg) according to CLSI guidelines.

Combination Disc Test (CDT)

While conducting antibiotic susceptibility test disc of ceftazidime (30 mcg), ceftazidime plus clavulanic acid (30/10 mcg),cefotaxime (30mcg), cefotaxime plus clavulanic acid (30/10 mcg), Cefepime (30mcg), Cefepime plus clavulanic acid (30/10mcg), Cefepirome (30mcg), cefepirome plus clavulanic acid (30/10mcg) were placed on Muller-Hinton agar (Hi Media) and incubated at 37 °C. Organism was considered as ESBL producer if there was a ≥5mm increase in zone diameter between these two combinations.

Molecular identification of ESBL (TEM gene)

Plasmid DNA was prepared by using methods of Alkaline lysis method and PCR amplification of TEM gene was carried out using the following primers 5'- TCC TGT TTT TGC TCA CCC AG- 3' and 5'- CCT ATC TCA GCG ATC TGT CTA -3'. PCR conditions for this gene comprised a thermal temperature of 95°C for 5 min, followed by 30 cycles of 94°C for 30sec, annealing at 64°C for 30 sec. and extension at 72 °C for 45sec, followed by final extension for 10 min at 72°C.

Molecular identification of ESBL (CTX-M gene)

Plasmid DNA was prepared by using methods of Birnboim and Doly Alkaline lysis method and PCR amplification of CTX-M gene was carried out using the following primers 5'ATG TGC AGY ACC AGT AAR GT- 3' and 5'- GCT GCC GGT YTT ATC MCC -3'. PCR conditions for this gene comprised a thermal temperature of 95°C for 5 min, followed by 30 cycles of 94 °C for 30sec, annealing at 65°C for 40 sec. and extension at 72 °C for 45sec, followed by final extension for 10 min at 72°C.

Molecular identification of PMQR (qnrS gene)

Plasmid DNA was prepared by using methods of Birnboim and Doly Alkaline lysis method and PCR amplification of *qnrS* gene was carried out using the following primers 5' GAC GTG CTA ACT TGC GTG AT- 3' and 5'- GAT CTA AAC CGT CGA GTT CG -3'. PCR conditions for this gene comprised a thermal temperature of 95°C for 5 min, followed by 30 cycles of 94 °C for 30sec, annealing at 55 °C for 30 sec. and extension at 72 °C for 45sec, followed by final extension for 10 min at 72 °C.

Molecular identification of PMQR (aac(6')-lb-cr gene

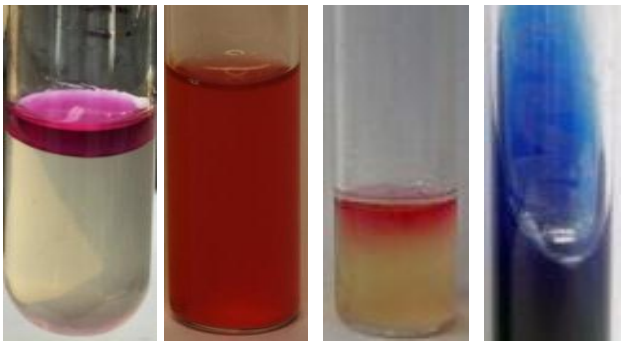
Plasmid DNA was prepared by using methods of Alkaline lysis method and PCR amplification of aac(6')-lb-cr gene was carried out using the following primers 5' TTG CGA TGC TCT ATG AGT GGC TA - 3' and 5'- CTC GAA TGC CTG GCG TGT TT -3'. PCR conditions for this gene comprised a thermal temperature of 95°C for 5 min, followed by 30 cycles of 94 °C for 30sec, annealing at 55 °C for 30 sec. and extension at 72 °C for 45sec, followed by final extension for 10 min at 72 °C.

RESULTS

In total 60 *E. coli* and 54 *K. Pneumoniae* were isolated non selective conditions (according antibiotic resistance). All the selected wild-type *E. coli* & *K. pneumonia* strains were further confirmed by biochemical test and the results of all these tests were similar for all the strains.

Details of strains used for further investigation

In total of 57 strains were selected for further studies. Out of which *E. coli* were 30 & *K. pneumonia* were 27 after confirmation of 16S rRNA gene analysis.



A B C D

a: Indole b: Methyl Red

c: VP d: Citrate

Fig. 1: (A) (a & b) Representative images used for biochemical identification of *E. coli* (B) (c & d) Representative images used for biochemical identification of *K. pneumonia.*

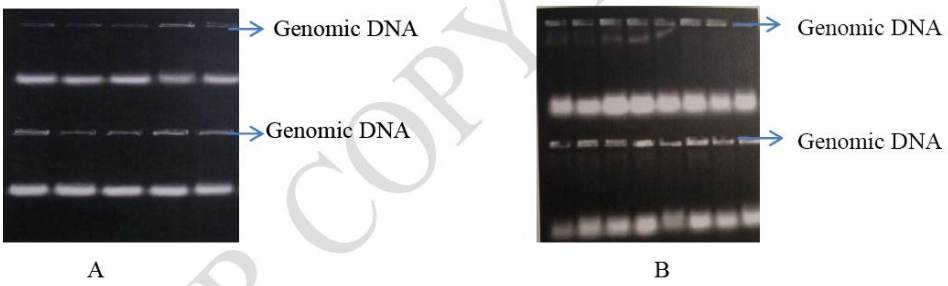


Figure.2: (A) Genomic DNA isolation from *E. coli* by PCI method

(B) Genomic DNA isolation from *K. pneumonia* by PCI method

Fig. 3: (A) Genomic DNA isolation from *E. coli* by Boiling method

(B) Genomic DNA isolation from *K. pneumonia* by Boiling

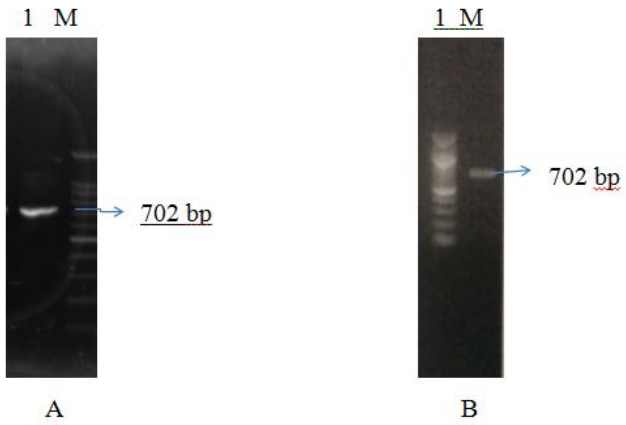


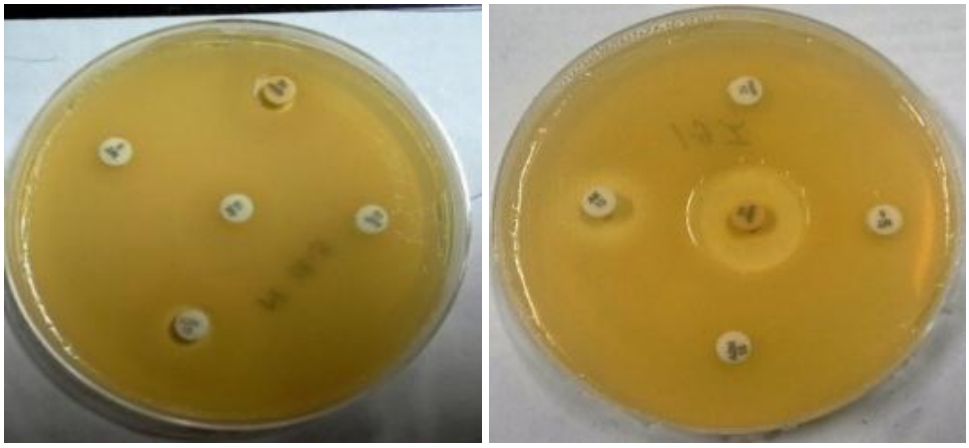
Fig. 4: Amplification products observed in 1% agarose gels.

(a) 702 bp band of amplicon for 16S rRNA from *E. coli* strains

(b) 702 bp band of amplicon for16S rRNA from *K. pneumonia* strains

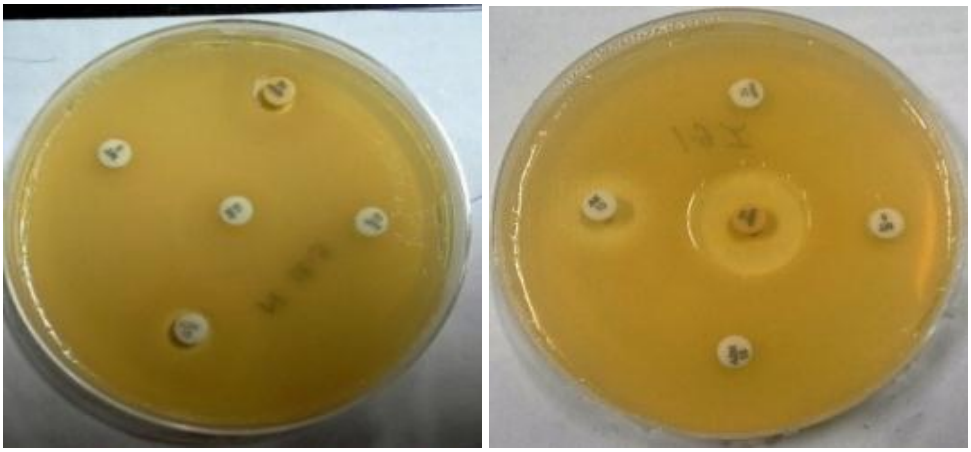
Details of strains used for further investigation

In total of 57 strains were selected for further studies. Out of which *E. coli* were 30 & *K. pneumonia* were 27 after confirmation of 16S rRNA gene analysis.



A **B**

Figure 5: Mueller-Hinton agar plate showing Antibiotic susceptibility testing of *E. coli* strains by disk diffusion test.



A **B**

Figure 6: Mueller-Hinton agar plate showing Antibiotic susceptibility testing of *K. pneumonia* strains by disk diffusion test.

Antimicrobial Susceptibility testing of *E. coli*

Almost all ESBL-producing isolates (80%) were multiresistant (resistant to three or more antibiotic groups). ESBL positive *E. coli* & *K. pneumoniae* isolates showed resistance to Ceftazidime(30mcg) (70%), Cefpodoxim(10mcg) (50%), Cefotaxime(30mcg) (70%), Ampicillin(10mcg) (100%), Ciprofloxacin(5mcg) (100%), Amikacin(30mcg) (10%), Metronidazole(4mcg) (83.33%), Meropenem(10mcg) (30%), Azithromycin(15mcg) (40%) and Ceftriaxone(30mcg) (80%). High levels of resistance to 7 to 8 antimicrobial agents were observed in the presence of multidrug-resistant ESBL & PMQR-producing *E.coli*.

Table 1: Scoring of antibiotic resistance among *E. coli* (n = 30)

S.No.	Antibiotics	No. (%) of resistant strains	No. (%) of susceptible strains
1	Ampicillin	100 %	0 %
2	Cefotaxime	70 %	30 %
3	Ceftriaxone	80 %	20 %
4	Ciprofloxacin	100 %	0 %
5	Cefpodoxim	50 %	50 %
6	Ceftazidime	70 %	30 %
7	Metronidazole	83.33 %	16.67 %
8	Meropenem	30 %	70 %
9	Azithromycin	40 %	60 %
10	Amikacin	10 %	90 %

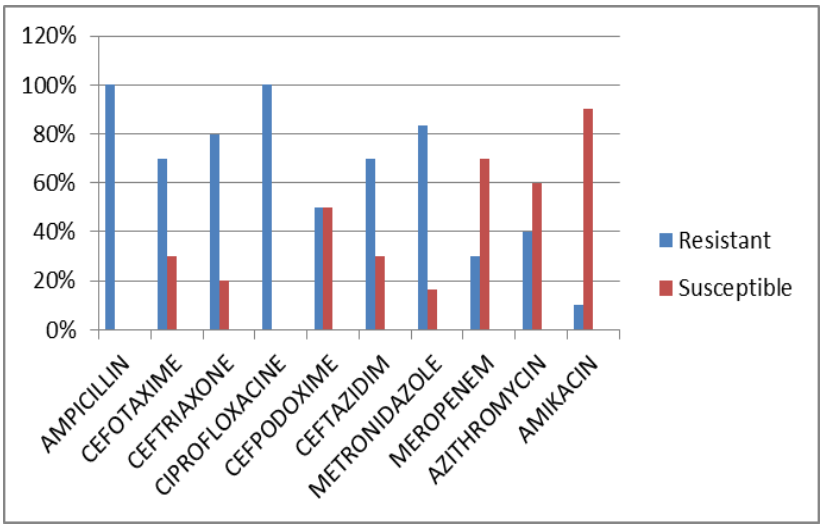


Figure 7: Bar Diagram of antibiotic profile of *E. coli.*

Antimicrobial Susceptibility testing of *K. pneumonia*

Almost all ESBL-producing isolates (82%) were multiresistant (resistant to three or more antibiotic groups). ESBL positive *E. coli* & *K. pneumoniae* isolates showed resistance to Ceftazidime(30mcg) (77.77%), Cefpodoxim(10mcg) (4074%), Cefotaxime(30mcg) (92.59%), Ampicillin(10mcg) (100%), Ciprofloxacin(5mcg) (100%), Amikacin(30mcg) (100%), Metronidazole(4mcg) (100%), Meropenem(10mcg) (40.74%), Azithromycin(15mcg) (40.74%) and Ceftriaxone(30mcg) (81.48%). High levels of resistance to 7 to 8 antimicrobial agents were observed in the presence of multidrug-resistant ESBL & PMQR-producing *K. pneumonia.*

Table 2: Scoring of antibiotic resistance among *K. pneumonia* (n = 27).

S.No.	Antibiotics	No. (%) of resistant strains	No. (%) of susceptible strains
1	Ampicillin	100 %	0 %
2	Cefotaxime	92.59 %	7.41 %
3	Ceftriaxone	81.48 %	18.52 %
4	Ciprofloxacin	100 %	0 %
5	Cefpodoxim	40.74 %	59.26 %
6	Ceftazidime	77.77 %	22.23 %
7	Metronidazole	100 %	0 %
8	Meropenem	40.74 %	59.26 %
9	Azithromycin	40.74 %	59.26 %
10	Amikacin	100 %	0 %

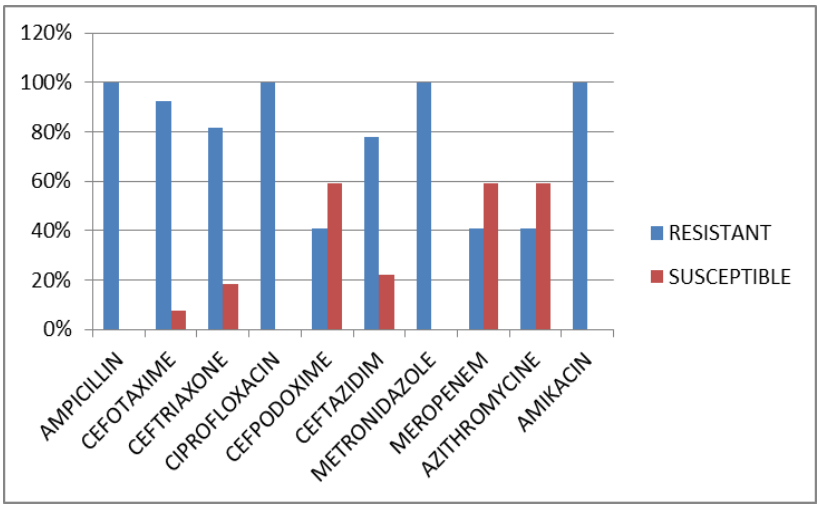
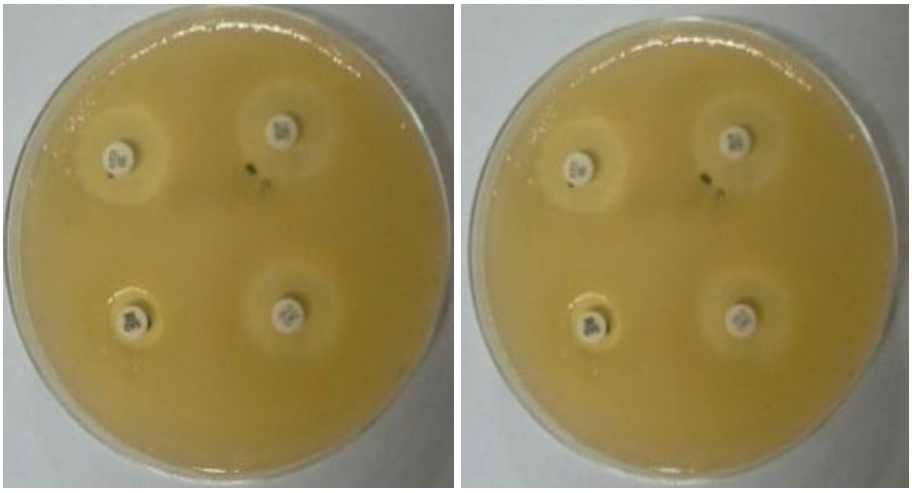


Figure 8: Bar Diagram of antibiotic profile of *K. pneumonia.*

Phenotypically conformation of ESBL in *E. coli*

Phenotypically confirmation of ESBL, almost all 75% were showed ESBL Positive *E. coli* and *Klebsiella Pneumonia*e isolates showed resistance to cephalosporin alone (Cefotaxime(30mcg) alone and with Clavulanic acid(10mcg) 73.33%, (Ceftazidime(30mcg) alone and with clavulanic acid (10mcg) 76.66%, Cefepime(30mcg) alone and with Clavulanic acid (10mcg) (70%), and cefepirome(30mcg) alone and combination with clavulanic acid (10mcg) 83.33%.



A B

Figure 9: Mueller-Hinton agar plate showing ESBL A & B of *E.coli* strains of samples detected by Phenotypic disc confirmatory test.

Table 3: Scoring of ESBL producing resistant strains among *E. coli* (n = 30).

S.No.	Antibiotic Disk	No (%) of ESBL producing strains	No (%) of ESBL Susceptible strains
1	Cefotaxime alone and with Clavulanic acid	73.33 %	26.67 %
2	Ceftazidime alone and with Clavulanic acid	76.66 %	23.34 %
3	Cefepime alone and with Clavulanic acid	70 %	30 %
4	Cefepirome alone and with Clavulanic acid	83.33 %	16.67 %

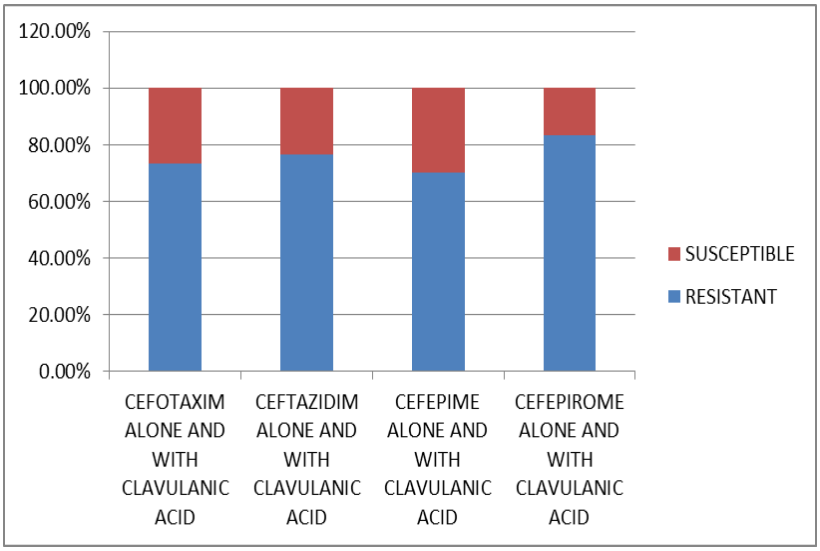
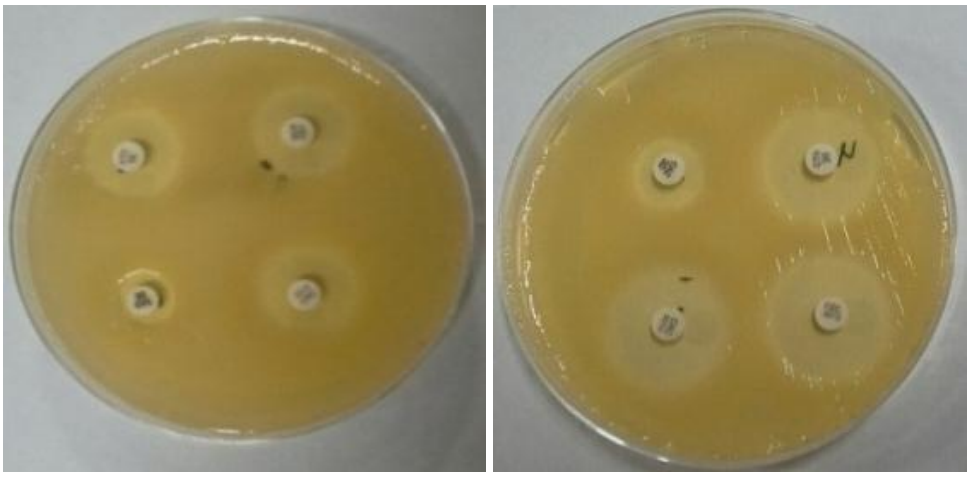


Figure 10: Bar Diagram of ESBL producing strain in *E. coli* of Yamuna River.

Phenotypically conformation of ESBL in *K. pneumonia*

Phenotypically confirmation of ESBL, almost all 70% were showed ESBL Positive *E. coli* and *Klebsiella Pneumonia*e isolates showed resistance to cephalosporin alone (Cefotaxime(30mcg) alone and with Clavulanic acid(10mcg) 66.66%, (Ceftazidime(30mcg) alone and with clavulanic acid (10mcg) 70.37%, Cefepime(30mcg) alone and with Clavulanic acid (10mcg) 66.66%, and cefepirome(30mcg) alone and combination with clavulanic acid (10mcg) 74.07%.



A **B**

Figure 11: Mueller-Hinton agar plate showing ESBL A & B of *k. pneumonia* strains of samples detected by Phenotypic disc confirmatory test.

Table 4: Scoring of ESBL producing resistant strains among *K. pneumonia* (n = 27).

S.No.	Antibiotic Disk	No (%) of ESBL producing Resistant strains	No (%) of ESBL producing Susceptible strains
1	Cefotaxime alone and with Clavulanic acid	66.66 %	33.34 %
2	Ceftazidime alone and with Clavulanic acid	70.37 %	29.63%
3	Cefepime alone and with Clavulanic acid	66.66 %	33.34%
4	Cefepirome alone and with Clavulanic acid	74.07 %	25.93%

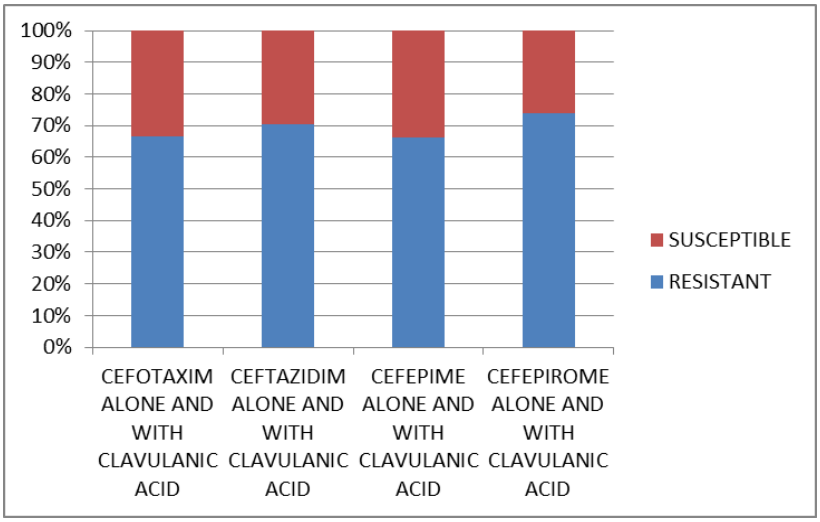


Figure 12: Bar Diagram of ESBL producing strain in *K. pneumonia* of Yamuna River.

Distribution of genes encoding β-lactamases of ESBL in *E. coli* & *K. pneumonia*

Following PCR-based detection, the *bla*TEM, *bla*CTX-M genes were identified TEM in *E. coli* (30/46) 65.21% & *K. pneumonia*(20/30) (66.66 %) and in CTX-M in *E. coli* (16/46) 34.7% & *K. pneumonia* (11/30) 36.66 % strains in that order.

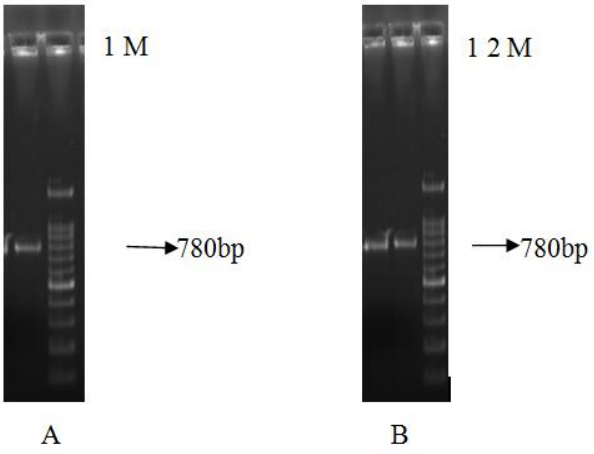


Figure 13: Amplification products observed in 1% agarose gels.

(A) 780 bp band of amplicon for TEM gene from *E. coli* strains

(B) 780 bp band of amplicon for TEM gene from *K. pneumonia* strains

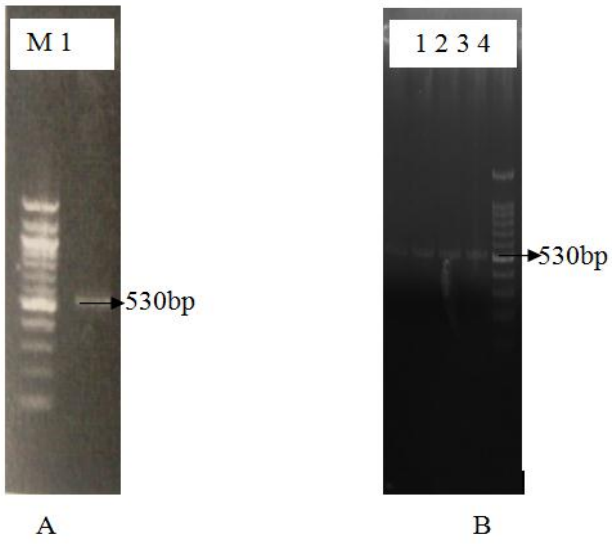


Figure 14: Amplification products observed in 1% agarose gels.

(A) 530 bp band of amplicon for CTX-M gene from *E. coli* strains

(B) 530 bp band of amplicon for CTX-M gene from *K. pneumonia* strains

Distribution of genes encoding PMQR in *E. coli* & *K. pneumonia*

Following PCR-based detection, the *qnrS*, aac-lb-cr genes were identified in *E. coli* (14/46) 30.43% & *K. pneumonia* (12/30) 40% and aac-lb-cr in the *E. coli* (9/46) 19.56% & *K. pneumonia* (7/30) 23.33% strains in that order.

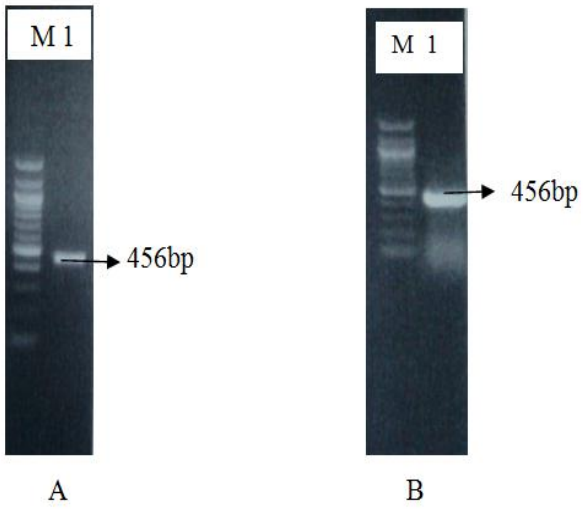


Figure 15: Amplification products observed in 1% agarose gels.

(A) 456 bp band of amplicon for qnrS gene from *E. coli* strains.

(B) 456 bp band of amplicon for qnrS gene from *K. pneumonia* strains.

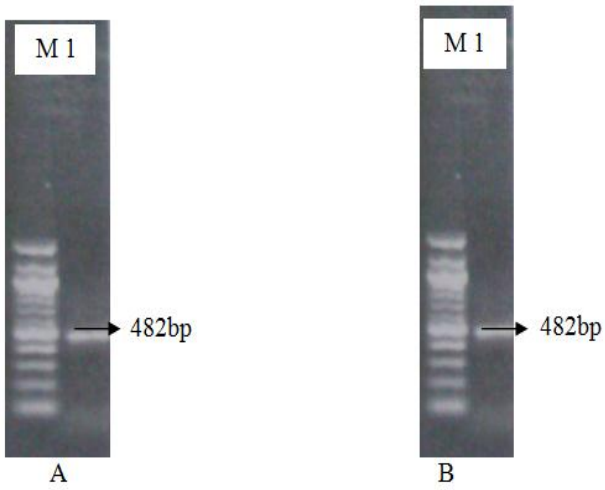


Figure 16: Amplification products observed in 1% agarose gels.

(A) 482 bp band of amplicon for aac-lb-cr gene from *E. coli* strains

(B) 482 bp band of amplicon for aac-lb-cr gene from *K. pneumonia* strains

CONCLUSION

This study clearly demonstrates the presence of acquired antibiotic resistance in Enterobacteri aceae, in Yamuna river caused by human activities. An ominous concerns extensive emergence of multidrug resistance among microbes such as *E. coli* and *K. pneumonia* which inhabit human intestine and readily contaminate the drinking water sources like rivers due to fecal contamination. Results of antimicrobial susceptibility test showed that all the isolated *E. coli* and *K. pneumonia* strains were resistant to most of the tested antibiotics, which may be explained by high and uncontrolled use of these antibiotics in humans, animals pollution from pharmaceutical companies as well as heavy metals or biocides.

Periodic monitoring of antimicrobial susceptibility should be the recommended strategy to counteract as the presence of high number of antibiotic resistant bacteria in river waters as this has serious ecological and public health implications.

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