

Prevalence of Plasmid Borne Ampc Gene in Multi- DrugResistant Freshwater and Soil Bacteria

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Abstract: Ampicillin resistant *E.coli*, *Klebsiella*, *Salmonella*, *Proteus*, *Bacillus*, *Chromobacterium*, *Vibrio* and *Pseudomonas* species were isolated from water and soil samples from Kothamangalam area. All of the isolates were multidrug resistant showing resistance against 3 to 9 of the antibiotics tested. Vancomycin resistance was most common with beta lactam resistance. *Klebsiella* was the most abundant isolate showing maximum resistance. The presence of *ampC* in plasmid was determined by PCR amplification with *ampC* specific primers. It was concluded that all the isolates possessed plasmid mediated ampicillin resistance.

Key Words: Multi-drug resistance, Ampicillin, *Klebsiella*, *ampC* gene, plasmid.

I. Introduction

AmpC β -lactamases are cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β -lactamase inhibitor- β -lactam combinations.^[1] The emission of bacteria into the aquatic environment also favours genetic exchange with previously non-resistant populations, thereby increasing the dispersion of this resistant capacity in the bacteria of the environment.^[2] The natural mortality of pathogenic bacteria is very high in extraenteral environments, their great abundance^[3] and the environmental conditions may be keeping these populations viable for some time.^[4] In the last two decades R-plasmid borne multiple resistant bacteria have been emerging as the cause of hospital borne infections and the gram negative bacteria have surpassed the gram positive pathogens in these infections.^[5,6]

II. Materials And Methods

Collection of sample

Water and soil samples were collected in sterile container under aseptic condition. Air was sampled by open plate technique. Samples collected were Sample 1: Water sample from Kuroorthodu near Baseliushospital, Sample 2: Water sample from Kuroorthodu 5km upstream from Baseliushospital, Sample 3: Water sample from a well, Sample 4: Soil sample near Kuroorthodu River, Sample 5: Soil sample away from water source, Sample 6: Air environment of Kothamangalam.

Selection of ampicillin resistant strains

Samples were serially diluted upto 10^7 dilution and spread plated on to LB agar medium with ampicillin solution. Incubated the plates at 37°C for 24 hours. Growth was observed after incubation. The numbers of colonies were counted.

Identification of bacterial isolates

The selected isolates were identified using Gram's staining^[7], Hanging drop method^[8] and biochemical tests^[9]. Biochemical tests used were sugar fermentation test, oxidation fermentation test, mannitol motility test, indole production test, methyl red test, vogesproskauer test, citrate utilization test, urease test, nitrate reduction test, triple sugar iron agar test, coagulase test, oxidase and catalase test.

Antibiotic sensitivity testing^[10]

Using a sterile wire loop colonies were picked and emulsified in peptone water and incubated. Muller Hinton agar plates were prepared. A sterile cotton swab was dipped into the bacterial suspension and was rubbed gently over the plates in several directions to obtain different planes, by rotating the plates and antibiotic discs were placed. Antibiotic disc used were ampicillin, amikacin, amoxicillin, penicillin, ciprofloxacin, vancomycin, neomycin, tobramycin, streptomycin. The plates were incubated overnight at 37°C . Diameter of the zone of inhibition obtained around each antibiotic disc was measured and tabulated.

Plasmid isolation by alkaline lysis method^[11]

Each isolate was inoculated into 5ml LB broth and incubated for 16 hours in a shaking incubator at 37°C . Filled a micro centrifuge tube with 1.5ml bacterial culture and centrifuged at 12,000rpm for 2 minutes.

Decanted the supernatant. Repeated step 1 in the same tube. Spin tube in micro centrifuge for 2 minute. Poured off supernatant and drain tube on paper towel. Added 100µl ice-cold Solution 1 to cell pellet and resuspend cells, and kept at room temperature for 5 minutes. Added 200µl Solution 2, and invert 5 times. Kept the tubes on ice for 10 minutes. Added 150µl ice-cold Solution 3, and invert 5 times gently. Incubated tubes on ice for 10 minutes. Centrifuged tubes for 5 minutes. Transfer supernatant to fresh micro centrifuge tube. Added equal volume of tris saturated phenol mixed and centrifuged for 5 minutes at 12,000rpm. Aqueous phase was collected and added equal volume of chloroform: isoamyl alcohol. Aqueous phase was collected and added 0.6 volume of chilled isopropanol, kept on precipitation for 5 minutes. Centrifuged tubes for 20 minutes. Poured off supernatant without dumping out the pellet. Drain tube on paper towel. Added 1 ml of ice-cold 70% ethanol. Centrifuged at 12,000rpm for 10 minutes. Poured off supernatant and drain tube. Air dried the pellet and dissolved in TE buffer.

Agarose gel electrophoresis of plasmid DNA

The isolated plasmid DNA was determined and analysed on 2% agarose gel .5µl of isolated plasmid DNA and 1µl of 6x gel loading dye was mixed And loaded in to the well. Electrophoresis was done at 4V/cm and was started by switching on the power pack to 50 volt and then to 100volt. after the dye migrated to a sufficient distance, the gel was taken out and visualized under UV Tran – illuminator.

Polymerase chain reaction

PCR was carried out in a total of 20µl reaction mixture containing 1µl each of ampC F and ampC R, 200µM each of dNTPs, distilled water, 10x buffer, 50µg DNA sample and 1 unit of taq polymerase. The cycling condition consisted of an initial denaturation of 2 minutes at 95°C for 30 seconds. 58°C for 30 seconds and 72°C for 30 seconds. A final extension was given at 72°C for 10 minutes. The amplification was carried out in eppendorf master cycler personal with heated lid.

Visualization of PCR products

PCR products were subjected to agarose gel electrophoresis. Aliquots of 5µl of PCR products and 1µl of 6x gel loading dye was mixed well and allowed to run in 2% agarose gel at 100v. A 100bp DNA ladder was loaded to estimate the size of the PCR product. The gel was taken out and visualized under UV trans-illuminator.

Transformation of *E.coli* JM107 using plasmid DNA

Preparation of competent cells

Inoculate bacterial strain *E.coli* JM107 into 3ml LB broth and incubated in a rotary shaker at 37°C 16 hours at 220 rpm. 120 µl of the above culture was added to 12ml freshly prepared LB broth and incubated at 37°C for 150 minutes at 220 rpm. The turbid cultures were subjected to centrifugation at 4000 rpm for 10 minutes at 4°C. Pellet was taken and 12ml chilled 5mM calcium chloride was added by keeping the tubes in ice. Incubated the mixture for 20 minutes in ice. It was then centrifuged to collect pellet for 5 minutes at 4000 rpm at 4°C to the collected pellet added 1.2ml of chilled calcium chloride and 180µl of glycerol was added for further storage.

Transformation of plasmid DNA into competent cells

150µl from the above mixture was incubated along with 1µl of plasmid .It was then subjected to heat shock at 42c for 1 minute and then kept on ice to provide cold shock. Both DNA and cells were incubated for 1 hour in a rotary shaker at 370 c. After 1 hour incubation 150µl of the above mixture was spread plated on 8to LB agar plates with appropriate amounts of Ampicillin. The inoculated plates are incubated at 370c for 16 hours for the detection of transformed colonies.

Detection of transformation

The detection was based on the production of colonies on the LB agar plates containing antibiotic Ampicillin .Non transformed cells fails to produce colonies since they are Ampicillin sensitive.

Confirmation of transformation

The transformation was once again confirmed by isolation of plasmid DNA from formed colonies on LB agar plates. The colony morphology was analyzed. Transformation was done using chilled calcium chloride solution by incubating *E.coli* JM107 strains with plasmid DNA. Transformation was done to confirm the plasmid mediated inheritance of antibiotic resistance.

III. Results

Selection of ampicillin resistant isolates

The numbers of colonies in the water samples (1 to 3) were found to be 50×10^4 , 76×10^5 , 52×10^5 and in soil samples (4 & 5) were 33×10^3 , 132×10^5 respectively. The air samples showed no growth in any of the LB ampicillin plates (Table 1). Out of this 33 ampicillin resistant strains were selected for the study.

Table 1: Isolation of ampicillin resistant bacteria

Sample	Number of colonies on LB with ampicillin	Dilution factor	Volume of sample plated	CFUs
1	50	10^3	0.1	50×10^4
2	76	10^4	0.1	76×10^5
3	52	10^4	0.1	52×10^5
4	33	10^2	0.1	33×10^3 CFU/g
5	132	10^4	0.1	132×10^5
6	0	0	0	0

Identification of bacterial isolates

Isolates were identified as *E.coli* (EC1-EC4), *Klebsiellasp* (KL1-KL18), *Salmonella sp*(SAL1-2), *Proteus sp*(PR1), *Bacillus sp* (BA1-2), *Chromobacteriumsp*(CH1-4), *Vibrio sp* (VB1) and *Pseudomonas sp*(PS1). Results of tests are given in Table 2a and 2b.

Table 2a: Gram staining & Biochemical tests for identification of isolates

Isolate code	Motility	Gram staining staining	Indole	Methyl red	VP	Citrate	Fermentation			OF	Urease	NO ₃ reduction	TSI	Catalase	Oxidase
							G	L	S						
1	M	NR	+	+	-	-	+	+	+	+	-	+	AG/A	+	-
2	NM	NR	-	-	+	+	+	+	+	+	+	+	AG/A	+	-
3	M	NR	-	+	-	-	+	-	-	+	-	+	A/AK	+	-
4	M	NR	+	+	-	+	+	-	+	+	+	+	AG/A	+	-
5	M	NR	-	-	-	-	+	+	+	+	-	-	AK/AK	-	-
6	M	NR	+	+	+	-	+	+	+	+	-	+	AK/AK	+	-
7	M	NR	-	-	-	+	+	+	+	+	-	+	AK/AK	-	-
8	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
9	M	NR	+	+	-	-	+	+	+	+	-	+	AG/A	+	-
10	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
11	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
12	M	NR	+	+	-	-	+	+	+	+	-	+	AG/A	+	-
13	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
14	M	NR	-	-	-	-	+	+	+	+	-	-	AK/AK	-	-
15	M	NR	-	+	-	-	+	-	-	+	-	+	A/AK	+	-
16	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
17	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
18	M	NR	-	-	-	+	+	+	+	+	-	-	AK/AK	-	-
19	M	NR	-	-	-	+	+	+	+	+	-	-	AK/AK	-	-
20	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
21	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
22	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
23	M	PR	+	-	+	+	+	+	+	-	+	+	AK/AK	+	-
24	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
25	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
26	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-

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27	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
28	M	NR	+	+	-	+	+	+	+	+	-	+	AG/A	+	-
29	NM	NR	-	-	+	-	+	+	+	+	+	+	A/AK	+	-
30	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
31	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
32	NM	NR	-	-	+	+	+	+	+	+	+	+	A/AK	+	-
33	M	PR	+	-	+	+	+	+	+	-	+	+	AK/AK	+	-

AG – Acid with gas: A – Acid: AK – Alkaline: OF – Oxidation fermentation G – Glucose: L – Lactose: S – Sucrose: TSI – Triple sugar iron agar test: M-Motile: NM-Non motile: NR-Negative rod: PR-positive rod.

Table 2b: Isolates selected for study

Sample number	Bacterial Isolates	Isolate code
1	<i>Escherichia coli</i>	EC1
	<i>Klebsiella</i> sp.	KL1
	<i>Salmonella</i> sp.	SAL1
	<i>Proteus</i> sp.	PR1
	<i>Chromobacterium</i> sp.	CH1
	<i>Vibrio</i> sp.	VB1
	<i>Pseudomonas</i> sp.	PS1
2	<i>Klebsiella</i> sp.	KL2
	<i>Escherichia coli</i>	EC2
	<i>Klebsiella</i> sp.	KL3
	<i>Klebsiella</i> sp.	KL4
	<i>Escherichia coli</i>	EC3
	<i>Klebsiella</i> sp.	KL5
	<i>Chromobacterium</i> sp.	CH2
	<i>Salmonella</i> sp.	SAL2
	<i>Klebsiella</i> sp.	KL6
	<i>Klebsiella</i> sp.	KL7
	<i>Chromobacterium</i> sp.	CH3
	<i>Chromobacterium</i> sp.	CH4
3	<i>Klebsiella</i> sp.	KL8
	<i>Klebsiella</i> sp.	KL9
	<i>Klebsiella</i> sp.	KL10
	<i>Bacillus</i> sp.	BA1
4	<i>Klebsiella</i> sp.	KL11
	<i>Klebsiella</i> sp.	KL12
	<i>Klebsiella</i> sp.	KL13
	<i>Klebsiella</i> sp.	KL14
5	<i>Escherichia coli</i>	EC4
	<i>Klebsiella</i> sp.	KL15
	<i>Klebsiella</i> sp.	KL16
	<i>Klebsiella</i> sp.	KL17
	<i>Klebsiella</i> sp.	KL18
	<i>Bacillus</i> sp.	BA2

Antibiotic sensitivity test

All the 33 isolates were shown to have antibiotic resistance against at least 2 antibiotics, among the 10 antibiotic tested. Only one isolate of *Klebsiella* sp (KL1) isolated from kuroorthodu river water near Baseliou hospital was

resistant to 9 antibiotics. All the isolates except two (CH2 & KL16) were resistant to all the 3 beta lactam antibiotics. Resistance towards aminoglycosides, macrolide, glycopeptides and quinolones were variable. Only one isolate was found to be (KL1) resistant to ciprofloxacin while all others were sensitive. Results are shown in Table 3a, 3b, 3c and Figure 1 to 5.

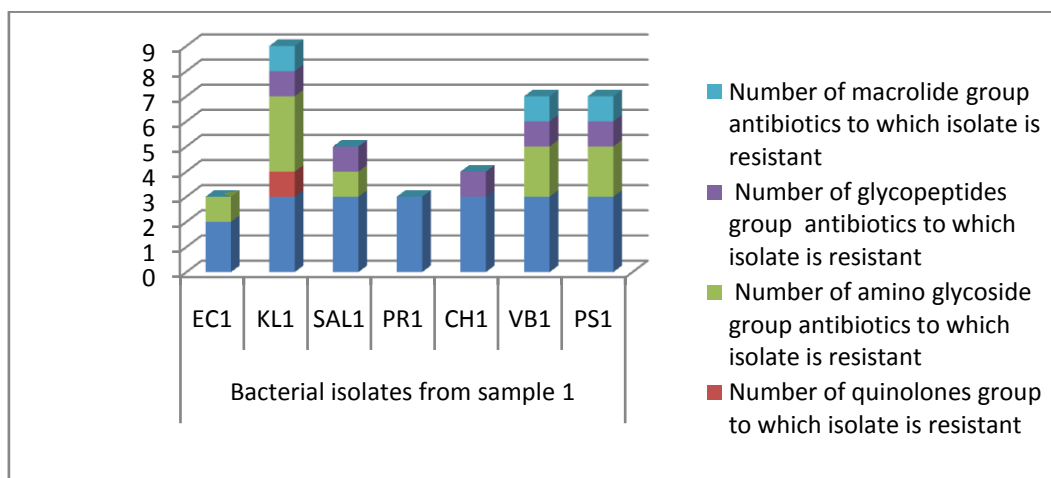


Figure 1 :Antibiotic resistance shown by isolates from sample 1

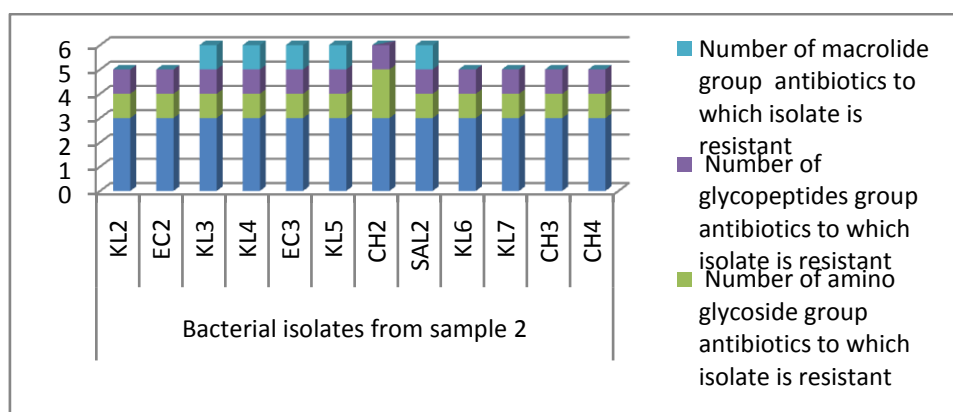


Figure 2 :Antibiotic resistance shown by isolates from sample 2

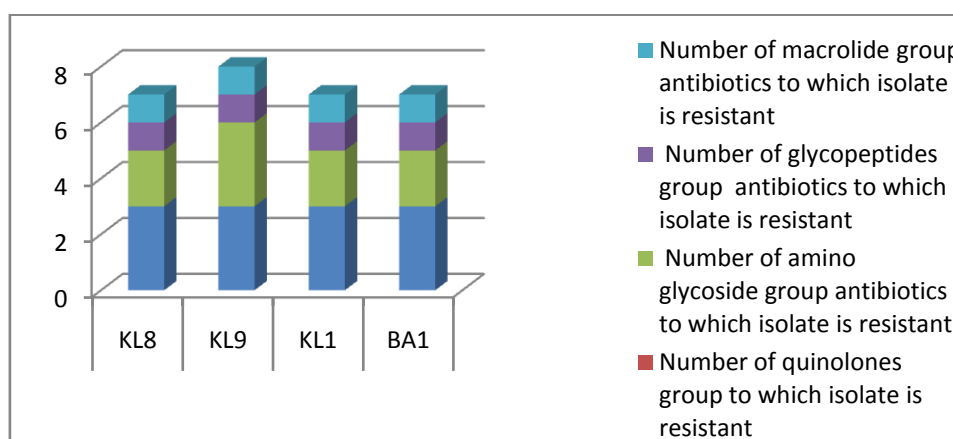


Figure 3 :Antibiotic resistance shown by isolates from sample 3

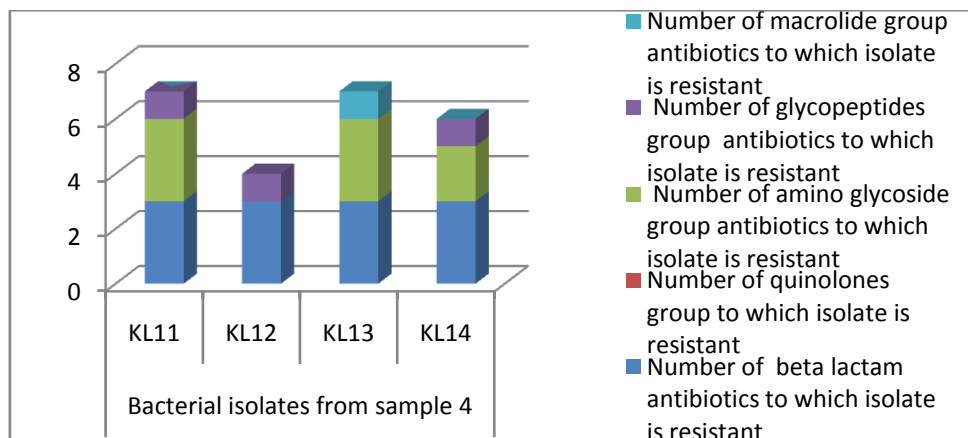


Figure 4 :Antibiotic resistance shown by isolates from sample 4

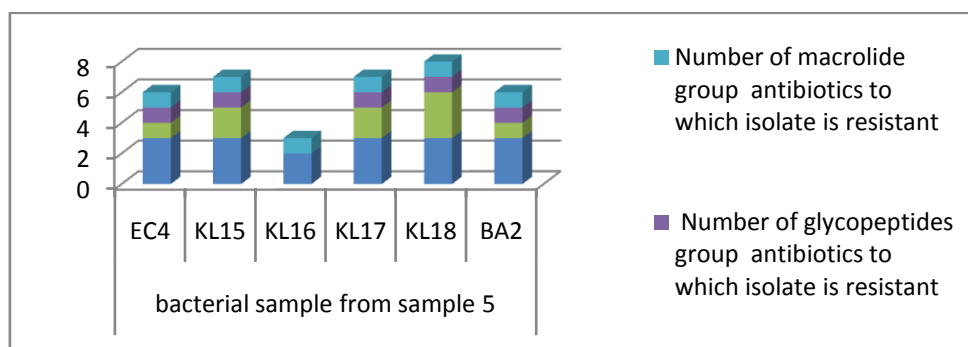


Figure 5 :Antibiotic resistance shown by isolates from sample 5

Isolation of plasmid

All 33 ampicillin resistant bacterial strains contained plasmid and the isolated plasmids were analyzed by agarose gel electrophoresis. Clear bands were obtained.

Quantification of plasmid

Plasmids isolated from the 33 ampicillin resistant strains were quantified using UV spectrophotometer. Relatively high concentrations of plasmid were obtained. OD 260 to OD 280 ratio was near the optimal value of 2 for all the samples which indicated its purity

PCR amplification of ampCgene

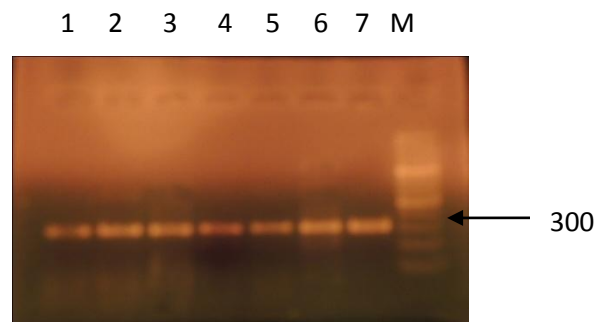
PCR amplification of plasmid DNA samples were carried out with ampC specific primers and the resultant PCR products were electrophoresed in 2% agarose gel. Bands were seen for all isolates which indicating the positive PCR amplification .The 300bp amplicons were confirmed by the comparison with 100bp DNA ladder .This confirmed the presence of ampC gene in the plasmids isolated for all the isolates.

Confirmation of plasmid mediated ampicillin resistance transfer by transformation

All 33 plasmids were transformed into *E.coli*JM107 competent cell. White coloured colonies were obtained on LB ampicillin agar plates indicating successful transformation of *E.coli* using plasmid DNA. Clear bands were obtained by isolating plasmid from transformed colonies.

Figure.6 : PCR products of the plasmid samples

Lane1:EC1,Lane2:KL1,Lane3:SAL1.Lane4:PR1.Lane5:CHI:Lane6:VB1.Lane7:PS1, M:Marker



IV. Discussion

The number of ampicillin resistant bacterial colonies isolated from Kuroorthodu river 5km upstream from the Baseliou hospital was found to be more than that from near hospital. Majority of the ampicillin resistant isolates from upstream were *Klebsiella* along with *Chromobacterium* and *E.coli*, where as in the hospital region variety of antibiotic resistant isolates were more including *Klebsiella*, *E.coli*, *Pseudomonas*, *Vibrio*, *Proteus*, *Salmonella* and *Chromobacterium*. In well sample the number of ampicillin resistant bacteria was less than that of river, *Klebsiella* was dominant. *Klebsiella* was predominated in the soil sample similar to water sample. Air samples failed to develop resistant bacterial isolates..

Klebsiella species from near hospital was the only isolate showed resistance to all the antibiotic including Ciprofloxacin and intermediate resistance to Neomycin. *Klebsiella* isolated from upstream showed resistance to 5 to 6 antibiotics. Whereas that from well showed resistance to 7 to 8 antibiotics, only one organism was resistant to streptomycin. *Klebsiella* from soil near water source showed resistance to 4 to 7 antibiotics, whereas away from water showed resistance to 2 to 8 antibiotics.

E.coli from near hospital showed resistance to only 3 antibiotics, and that from upstream showed resistance to 6 antibiotics. *E.coli* from soil away from water showed resistance to 6 antibiotics. *Chromobacterium* from near hospital was resistance to 5 antibiotics and which is upstream from hospital showed resistance to 5 to 6 antibiotics. *Salmonella* sp and *Proteus* sp from near hospital showed resistance only to 4 antibiotics. *Vibrio* sp and *Pseudomonas* sp from near hospital showed resistance to 7 antibiotics. *Bacillus* sp isolated from soil showed resistance to 5 antibiotics whereas from well showed resistant to 7 antibiotics. The most resistant isolates of *Klebsiella* sp, *Vibrio* sp, and *pseudomonas* sp were in river near hospital, whereas that of *E.coli* and *Chromobacterium* was from upstream water. *Bacillus* sp showed higher resistance in well water than soil.

The incidence of resistant strains among isolates varied significantly among the water sample, without obvious connection with the water source or the level of pollution. The average frequency of multiple resistances was not always high in the same samples in which the overall resistance was high. The species composition varied considerably in different water samples. A significant correlation was observed between the relative frequency of *klebsiella* sp and the incidence of ampicillin resistance in water sample.^[12]

Of the five groups of antibiotics checked Quinolones group alone was able to inhibit the growth of these isolates. Only single bacteria from the near hospital developed resistance to this antibiotic group. It is an alarming situation that all the other 4 antibiotic families will be ineffective in controlling the resistant strains from the environmental samples. The isolates possessed resistance towards all the beta lactam antibiotic, 29 of the isolates were resistant to amino glycoside group and glycopeptides group antibiotics. 22 isolates were resistant to macrolide group antibiotic.

This study points the fact that though the abundance of resistant isolates are more in soil, the isolates from fresh water samples showed resistance to more number of antibiotics. These fresh water sources act as more effective place for multiplication of these bacteria and hence the resistance genes like ampC genes. AmpC genes carrying plasmid was isolated from *E.coli*, *Klebsiella*, *Chromobacterium*, *Proteus*, *Salmonella*, *Pseudomonas*, *Vibrio* and *Bacillus*.

Transfer of R plasmids has been shown to occur under natural environmental conditions^[2,13,14]. Since all the 33 Isolates showed plasmids carrying ampC genes, we can conclude that they would be involved in transmission of resistance to other bacteria in those environments.

V. Conclusion

Resistant isolates were abundant in fresh water and soil samples, which was resistant to large number of antibiotics. Different aquatic compartments and soil within one municipal area were analyzed for resistant bacteria and their ampC genes. Amp resistant bacteria were widespread in the local ecosystem and *Klebsiella* spp. was found to be the most prevalent among the isolates. AmpC resistant genes were carried by plasmids in all the selected isolates.

Frequent use of antibiotics as medicine in treatment of humans and in food of animals results in the prevalence of antibiotics resistant bacteria.^[15] The importance of the different sources of resistance found in the environment, i.e. the presence of antibiotics in the environment and the importance of resistant bacteria result from the use of antibiotics in various fields.^[16]

The antibiotics released into environment due to human activity, especially into the aquatic environment may be the reason for the development and maintenance of the ampC genes by the bacteria. Thus antibiotic resistance helps the bacteria to survive in the presence of antibiotics in nature. The presence of the resistance genes on plasmids makes the exchange of genes easier between the environmental bacteria. This may explain the widespread occurrence of ampC genes in both soil & water samples irrespective of the fact whether the source is near a hospital or not. Ciprofloxacin resistance was seen only in one isolate which was taken from water sample

near the hospital while all other isolates from soil & water were sensitive. This indicates a possible relation between hospital effluent and multi-drug resistance development.

In conclusion it was found that plasmid mediated antibiotic resistance was prevalent in environment. The rapid detection of antibiotic resistance mechanism by PCR could have a significant impact on patient management in terms of appropriate antibiotic prescription and ultimately treatment outcome. The emission of antibiotics into the environment should be reduced as an important part of the risk management

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