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Antibiotic Resistance, Plasmid and RAPD Profiles of Multidrug-resistant Coliform Bacteria Isolated from Sewage Samples of Ghaziabad City, India

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Abstract:

The aim of the present work is to study the antibiotic resistance, plasmid and RAPD profiles of the multidrug resistant (MDR) Coliform bacteria isolated from raw and treated sewage of Ghaziabad city, India. The MDR bacterial population in the raw and treated sewage constituted 7.5% and 19.1% of the total Coliform bacteria respectively. Five MDR Coliform bacteria (2 from raw and 3 from treated sewage) were isolated and identified as *Enterobacter* spp. by morphological and biochemical tests. These MDR strains were resistant to most of the commonly used antibiotics including amikacin. Plasmid isolation studies showed that all MDR strains harboured a single plasmid of approximately 54.4 kb size. Random amplification of polymorphic DNA (RAPD) analysis of genomic DNA produced three RAPD profiles and showed variation between the raw and treated sewage isolates. Further, MDR strain R1 that was resistant to all 16 antibiotics tested showed plasmid-mediated resistance which was confirmed by plasmid curing study.

Keywords: MDR, Coliform bacteria, Antibiotics, Sewage samples, Plasmid, RAPD profile

1.0 Introduction:

Pathogenic bacteria in humans and animals resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. Nowadays, antibiotic resistant bacteria can be found in all different ecological niches. Selective pressure in favour of bacteria possessing these genes has emerged from the abusive use of antibiotics mainly in hospitals, agriculture and animal farming (Kummerer, 2004). Liquid manure of animal as well as human excretions has also led to dissemination of resistant bacteria in the environment (Reinthaler *et al.,* 2003).The genetic flexibility of bacteria has contributed to their survival in altered environments, because of their capacity to acquire and transfer resistant genes. Bacteria have developed resistance to all different classes of antibiotics discovered to date. The most frequent type of resistance is acquired and transmitted horizontally either through conjugation, transformation or transduction of plasmid (Frost *et al*., 2005).

Indiscriminate and inappropriate use of antibiotics in human and animal medication has resulted in the simultaneous development of resistance to several antibiotic classes creating very dangerous multidrug-resistant (MDR) bacterial strains (Alfonso, 2005). The widespread emergence of multidrug resistance among bacterial pathogens has become one of the most serious challenge in clinical therapy (Levy and Marshall, 2004). Some pathogens, such as MDR *Klebsiella pneumoniae* and *Acinetobacter baumannii*, are now virtually untreatable with current antibiotics (McGowan, 2006). The incidence of MDR bacteria has increased during the last few years and has been documented worldwide from both the clinical and environmental samples (Kummerer, 2004; Patwardhan *et al.,* 2008; Li *et al.,* 2010). Studies on occurrence of resistant and MDR bacteria in sewage and sewage receiving aquatic environments have been well studied already in other countries (Reinthaler *et al.,* 2003; Guardabassi *et al.,* 1998; Goni-Urriza *et al.,* 2000; Zhang *et al.,* 2009), but such studies in India are limited (Chitnis *et al.,* 2000). The present study was aimed to analyse the occurrence of MDR bacteria in the raw and treated sewage and its antibiotic susceptibility, plasmid and RAPD profiles.

2.0 Materials and Methods:

2.1 Isolation and Identification of MDR Coliform Bacteria from Sewage Samples:

The raw and treated sewage samples were collected from 0.5 km up- and down-stream, respectively, from Dundehla sewage treatment plant of Ghaziabad city, India during the month of July, 2011. Samples were taken in sterilized BOD bottles, kept in ice boxes and transported to laboratory for microbiological studies. For isolation of Gram-negative bacteria, 100 µl of raw and treated samples were spread on MacConkey agar plates for total Coliform counts and on same medium was supplemented with 20 µg/ml of penicillin, tetracycline and erythromycin for MDR bacteria counts. After incubation at 37°C for 24 h, colonies were counted and expressed in CFU/ml for each plate. MDR Coliform bacteria were isolated antibiotics supplemented MacConkey agar plates and identified as *Enterobacter* spp. by standard biochemical methods (Barrow and Feltham, 1993).

2.2 Antibiotic Susceptibility Testing:

Antibiotic susceptibility testing was performed by using the Kirby-Bauer disk diffusion method (Benson, 1998) on Mueller-Hinton agar using 16 antibiotics containing discs (Hi-Media Mumbai, India) as shown in Table 2. Briefly, organisms were grown overnight at 37°C in 5 ml Luria broth. The cultures were adjusted to match a McFarland 0.5 standard and spread on Mueller-Hinton agar plates using sterile swabs. The plates were dried at room temperature for 30 min before placing the antibiotic discs at equidistance. The plates were incubated at 37°C for 18-20 h, and the zone of inhibition was measured in terms of diameter (mm). Organisms were classified as sensitive, intermediate or resistant by comparing the diameter of inhibition zone with zone interpretative chart provided by manufacturer's along with discs.

2.3 Plasmid Isolation:

Plasmid DNA of MDR bacteria was extracted by the alkaline lysis method (Birnboim and Doly, 1979), analysed by electrophoresis through 0.8% agarose gel, visualised under UV transilluminator, and photographed and recorded using the Gel Documentation system (Model G:BOX, Syngene). To estimate the molecular weight of plasmids, *Escherichia coli* V517 (MTCC 131) was used as a source of standard plasmid marker, which harboured eight different plasmids with known molecular weight viz., 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4 MDa (Macrina *et al.,* 1978).

2.4 Random Amplification of Polymorphic DNA (RAPD) Analysis:

The genomic DNA was extracted by UltraClean™ Microbial DNA isolation Kit (MO BIO Lab Inc, USA) as per manufacturer's instruction. Out of the 10 primer (OPERON Techniques Inc, USA) screened, only 1 amplified primer was selected for further analysis. PCR reaction was prepared in a total volume of 25 µl per tube, containing 12.5 µl of ready to use 2X Taq Master Mix (GenScript, USA Inc.), 2 µl of 25 pmol primer, 2 µl DNA template (20-30 ng) and 8.5 µl of nuclease-free water. Amplification was performed on a TC-512 thermal cycler (Techne, UK) with the following temperature program: one cycle denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, a final extension step at 72°C for 10 min followed by cooling at 10°C. The DNA fragments produced were separated by electrophoresis on 1.4% agarose gel stained with ethidium bromide along with 1.0 kb (Bangalore Genei, India) and 100 bp (Biogene, USA) DNA markers. The molecular weight of amplified bands was approximated by extrapolation on semi-log graph of molecular weight of marker against the distance (mm) migrated by the respective band.

2.5 Plasmid Curing:

The plasmid curing study was performed for highly resistant isolate by physical method (treating cells at 45°C), as described by Fortina and Silva (1996). The isolate was inoculated in Luria broth (Hi-Media) in duplicate. One flask was incubated at 37°C while the other at elevated temperature (45°C) overnight for plasmid curing. The curing was confirmed by loss of plasmid and antibiotic susceptibility testing using antibiotics to which organisms were resistant.

3.0 Results and Discussion:

The mean of total Coliform bacteria counts on MacConkey agar plates for raw and treated sewage were $40x10^3$ CFU/ml and $35x10^3$ CFU/ml, respectively after 24 h of incubation, revealing a 12.5% decrease in bacterial counts in treated sewage (Figure 1). This is in agreement with finding of Andersen *et al* (1993), as they reported a decrease in Coliform population in treated sewage. Further, the MDR bacterial counts for raw and treated sewage sample were $3.0x10^3$ CFU/ml and $6.7x10^3$ CFU/ml, respectively, constituting 7.5% and 19.1% of the

total Coliform bacteria. Our results suggest that treated sewage contained 55.2% more MDR bacteria than the raw sewage. Similar to this result, many reports have shown high percentage of resistant bacteria in treated sewage or its receiving water body (Goni-Urriza *et al.,* 2000; Iwane *et al.,* 2001; Schwartz *et al.,* 2003; Da Silva *et al.,* 2007). The high microbial density and diversity of biofilms and activated sludge may facilitate genetic exchange in wastewater treatment plant (Schluter *et al.,* 2007) and antimicrobial agents are present in wastewater (Kummerer, 2004).These conditions may lead to a selection of antibiotic resistant bacteria in wastewater treatment plant.

The selected 5 MDR strains, namely 2 from the raw (R1 and R2) and 3 from the treated (T1-T3) sewage were identified as *Enterobacter* spp. by morphological and biochemical tests (Table 1). *Enterobacter* spp. have been associated with nosocomial infections and a variety of opportunistic infections involving the urinary and respiratory tracts, and cutaneous wounds, cause significant morbidity and mortality (Peters *et al.,* 2000). In addition, in recent years, they are the increasing cause of community-acquired infections as well (Schwartz *et al.,* 2003). According to the European recommendation for antimicrobial resistance surveillance, *Enterobacter* spp., particularly *E. cloacae* is one among several bacteria which has to be monitored in healthcare centres because infection caused by these organisms is largely associated with decreased immunity (Cornaglia *et al.,* 2004).

The antibiotic resistance pattern of the MDR strains were determined against 16 most commonly used antibiotics. These MDR bacteria were resistant to penicillin, kanamycin, streptomycin, amikacin, neomycin, erythromycin and vancomycin, except R2 which was sensitive to vancomycin (Table 2). However, with exception of MDR isolate R1 and R2, they were sensitive to ampicillin, amoxicillin, norfloxacin, levofloxacin, and ciprofloxacin, respectively. The resistance pattern of MDR bacteria with the number of antibiotics ranged from 7-16 antibiotics and maximum resistance was shown to the aminoglycoside class of antibiotics such as kanamycin, streptomycin, neomycin and amikacin.

Furthermore, with the exception of MDR isolate R1, all were sensitive to newer quinolones such as norfloxacin, ciprofloxacin and levofloxacin. There were no clear trends observed in resistance pattern between bacteria isolated from the raw and treated sewage, as both increase and decrease in resistance pattern was observed. The observed resistance agrees with literature reports that *Enterobacter* species are resistant to most of the antibiotics. The resistance of this species to β-lactam antibiotics, chloranphenicol, quinolones and tetracycline is well documented (Thiolas *et al.,* 2005). There are numerous reports in literature on the increasing resistance of *Enterobacter* species to penicillins and all generations of cephalosporins and their emergence in clinical specimens (Charrel *et al.,* 1996).

The relatively high level of resistance to antibiotics is a reflection of misuse or abuse of these antibiotics during treatment of bacterial infections. Reinthaler *et al* (2003) also reported *E. coli* strains from sewage treatment plants were less resistant against quinolones, while Namboodiri *et al.,* (2011) have reported quinolones-resistant *E. coli* from the faecal flora of Accra residents, Ghana. Hsu *et al* (1992) pointed out that the differences in the extent of bacterial resistance to various antibiotics might reflect the history of antibiotic applications and allow bacterial drug resistance to be used as an indicator of antibiotic application. β-Lactamases are the major defense systems of *Enterobacter* species (Charrel *et al.,* 1996). However, efflux pump-mediated resistance to β-lactam antibiotics, quinolones, tetracycline, and chloramphenicol has been reported (Thiolas *et al.,* 2005).

Plasmid is one of the known most important mediators in facilitating the fast spreading of antibiotic resistance among bacteria (Dale and Park, 2004). Plasmid isolation study revealed that all MDR isolates harboured a single plasmid of 54.4 kb which is equivalent to 35.8 MDa (1 MDa=1.52 kb) of known molecular weight of plasmid in *E. coli* V517 (Figure 2). The result of this study is agreement with finding of those reported by Shahid *et al* (2003) and Oppegaard *et al* (2001), as they have isolated single plasmid of 48.5 kb and 65 kb in MDR isolates of *Pseudomonas aeruginosa* and lactose-fermenting Coliform, respectively.

Table 1: Morpological and biochemical characteristics of MDR bacteria isolated from raw and treated sewage samples

Test	R1	R2	T1	T2	T3
Cell shape	Rod	Rod	Rod	Rod	Rod
Colony colour on EMB	Pink	Pink	Pink	Pink	Pink
Motility	-	-	-	-	-
Urease	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Indole	-	-	-	-	-
MR	-	-	-	-	-
VP	+	+	+	+	+
Citrate	+	+	+	+	+
ONPG	+	+	+	+	+
Glucose	+	+	+	+	+
Sucrose	-	+	+	+	+
Mannitol	-	+	+	+	+
Lactose	+	+	+	+	+

$^{+}$=positive, $^{-}$=negative,

Table 2: Resistance pattern of MDR bacteria isolated from raw and treated sewage samples

Class	Antibiotic/ (conc.)*	R1	R2	T1	T2	T3
β-lactams	Ampicillin (25)	R	I	S	S	S
	Amoxicillin (10)	R	I	S	S	S
	Penicillin (10)	R	R	R	R	R
Quinolones	Nalidixic acid (30)	R	S	R	R	I
	Norfloxacin (10)	R	S	S	I	S
	Levofloxacin (5)	R	S	S	S	S
	Ciprofloxacin (30)	R	S	S	S	S
Aminoglycoside	Kanamycin (30)	R	R	R	R	R
	Streptomycin (25)	R	R	R	R	R
	Gentamicin (30)	R	R	R	S	S
	Amikacin (30)	R	R	R	R	R
	Neomycin (30)	R	R	R	R	R
Macrolides	Erythromycin (15)	R	R	R	R	R
Tetracyclines	Tetracycline (30)	R	S	R	I	I
Glycopeptides	Vancomycin (30)	R	S	R	R	R
Others	Chloramphenicol (25)	R	S	S	I	I

R=resistant, I=intermediate, S=sensitive

*Drug concentration in µg/disc mentioned in parentheses

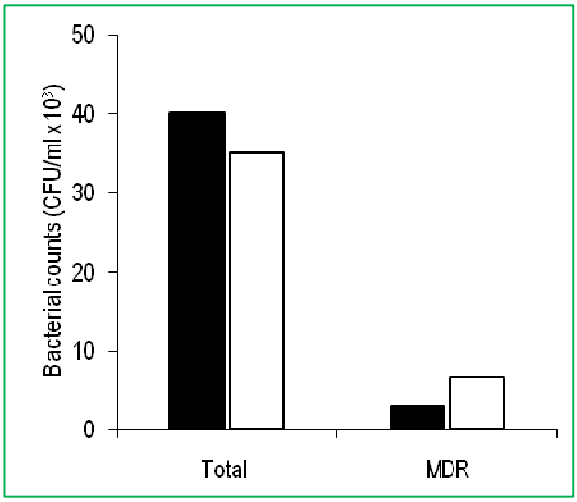

Figure 1: Mean of Total and MDR bacterial counts of raw (■) and treated (□)sewage samples.

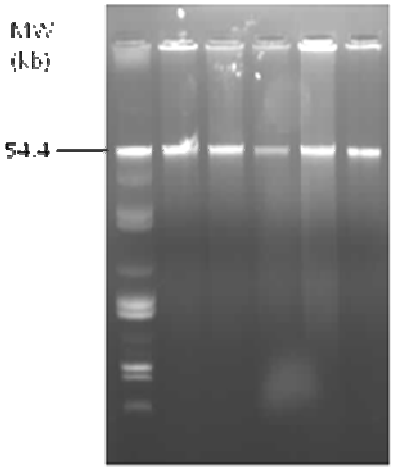

Figure 2: Agarose gel photograph of plasmid DNA of the MDR bacteria. Lane 1: Standard plasmid markers of *E. coli* V517; lanes 2 to 6: plasmid DNA of MDR bacteria (R1, R2, T1, T2 and T3), respectively.

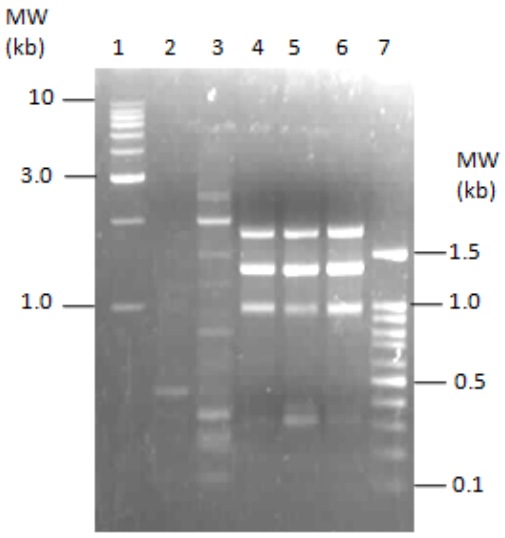

Figure 3: Agarose gel photograph of RAPD profiles of MDR bacteria. Lane 1: 1.0 kb (1.0-10) marker and lane 7: 100 bp (0.1-1.5 kb) marker; lanes 2 to 6: MDR bacteria (R1, R2, T1, T2 and T3), respectively.

RAPD is simple and widely used method for strain differentiation, since it does not require any specific knowledge of the DNA sequences in the target organism. Though RAPD technique has certain limitations, still it is being used as a molecular typing method due to its simplicity, sensitivity, flexibility and relatively low cost (Abou-dobara *et al.,* 2010; Intrakamhaeng and Komutarin, 2012). In this work RAPD-PCR analysis was done using primer OPG- 10 (5'AGGGCCGTCT3') which produced 3 RAPD profiles (Figure 3). Isolate R1 (lane 2) showed 1 RAPD profile (0.5 kb) which was quite different from others. The isolate R2 (lane 3) showed 9 RAPD profiles (0.12-2.5 kb) and was the highest number among the isolates. On the other hand, treated sewage isolates T1-T3 (lanes 4-6) showed a similar RAPD profile (0.3-1.13 kb). The result of RAPD profile clearly indicated a significant variation between the raw sewage isolates and similarity among the treated sewage isolates. Haryani *et al* (2008) found that 4 RAPD profiles among seven studied *Enterobacter cloacae* demonstrated the high discriminatory power of RAPD. In addition, by using RAPD, Trautmann *et al* (2006) showed isolates of *P. aeruginosa* had a similar distribution of genotypes.

To determine the possible linkage of multidrug resistance with plasmid DNA in the isolate, plasmid

curing study was conducted for isolate R1, as this isolate was found to be resistant to 16 antibiotics. Curing of plasmid at elevated temperature (45°C) had resulted in loss of 54.4 kb plasmid (result not shown). Using physical method, Fortina and Silva (1996) obtained curing of 14.3 kb plasmid in *Lactobacillus helveticus* strain ILC 54 at 45°C. The plasmid cured cells became sensitive to all previously resistant antibiotics (data not given), which revealed that antibiotic resistance marker genes were located in plasmid. However, further study such as transfer of plasmid into another suitable host is necessary to confirm the plasmid mediated antibiotic resistance.

4.0 Conclusion:

The present study showed the occurrence of MDR bacteria in raw and treated sewage samples of Ghaziabad city, India. The antibiotic resistance pattern of these MDR bacteria ranged from 7 to 16 antibiotics including most of the antibiotics used presently for treating human infections. One MDR isolate R1 was resistant to 16 antibiotics and showed plasmid-mediated resistance. These MDR bacteria could pose a serious health hazardous if given a chance to infect the community. In that case, the antibiotics to which organisms are resistant will be futile against these isolates. The data of this study are consistent with limited number of samples and bacteria, and thus similar studies need to be carried out to determine multidrug resistance in other pathogenic bacteria causing serious infections.

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References:

1) Abou-dobara, M.I., Deyab, M.A., Elsawy, E.M. and Mohamed, H.H. (2010): Antibiotic Susceptibility and Genotype Patterns of *Escherichia coli*, *Klebsiella pnemoniae* and *Pseudomonas aeruginosa* Isolated from Urinary Tract Infected Patients. *Pol. J. Microbiol*., 59: 207-212.
2) Alfonso, J. A. (2005): Resistance to Antibiotics: are We in the Post-antibiotic Era?. *Arch. Med. Res.,* 36: 697-705.
3) Andersen, S.R. (1993): Effect of Wastewater Treatment on the Species Composition and Antibiotic Resistance of Coliform Bacteria. *Curr Microbial.,* 26: 97-103.
4) Barrow, G.I. and Feltham, R.K.A. (1993): Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd Ed., Cambridge University Press
5) Benson, H.J. (1998): Antimicrobial Sensitivity Testing: the Kirby-Bauer Method. In: Microbiological Applications: Laboratory Manual in General Microbiology, 7th Ed., McGraw Hill, Boston, Massachusetts. 139-141.
6) Birnboim, H.C. and Doly, J.A. (1979): A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. *Nucleic acids Res*., 7: 1513-1523.
7) Charrel, R.N., Pages, J-M., Micco, P. De. and Mallea, M. (1996): Prevalence of Outer Membrane Porin Alteration in beta-lactam-antibiotic-resistant *Enterobacter aerogenes. Antimicrob. Agents Chemother*., 40: 2854–2858.
8) Chitnis, V., Chitnis, D., Patil, S. and Kant, R. (2000): Hospital Effluent: A Source of Multiple Drug Resistant Bacteria. *Curr. Sci*., 79: 989-991.
9) Cornaglia, G., Hryniewicz, W., Jarlier, V., Kahlmeter, G., Mittermayer, H., Stratchounski, L. and Baquero, F. (2004): European Recommendations for Antimicrobial Resistance Surveillance. *J. Clin. Microbiol. Infect*., 10: 349-383.
10) Dale, J.W. and Park, S. (2004): Molecular Genetics of Bacteria. 4th Ed., John Wiley & Sons Inc., Chichester, UK.
11) Fortina, M.G. and Silva, M. (1996): Mapping of Two Plasmids from *Lactobacillus helveticus* ILC54, Plasmid Curing and Preliminary Studies on Their Involvement in Lactose Metabolism and Peptidase Activity. *Biotechnol. Lett*., 18: 1003-1006.
12) Frost, L.S., Leplae, R., Summers, A.O. and Toussaint, A. (2005): Mobile Genetic Elements: the Agents of Open Source Evolution. *Nat. Rev. Microbiol*., 3: 722-732.
13) Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P. and Quentin, C. (2000): Impact of an Urban Effluent on Antibiotic Resistance of Riverine *Enterobacteriaceae* and *Aeromonas* spp. *Appl. Environ. Microbiol*., 66: 125-132.
14) Guardabassi, L., Petersen, A., Olsen, J.E. and Dalsgaard, A. (1998): Antibiotic Resistance in *Acinetobacter* spp. Isolated from Sewers Receiving Waste Effluent from a Hospital and a

Pharmaceutical Plant. *Appl. Environ. Microbiol.,* 64: 3499-3502.

15) Haryani, Y., Tunung, R., Chai, L.C., Lee, H.Y., Tang, S.Y. and Son, R. (2008): Characterisation of *Enterobacter cloacae* Isolated from Street Foods. *Asean Food J*., 15: 57-64.
16) Hsu, C.H., Hwang, S.C. and Liu, J.K. (1992): Succession of Bacterial Drug Resistance as an Indicator of Antibiotic Application in Aquaculture. *J. Fish. Soc. Taiwan*., 19: 55-64.
17) Intrakamhaeng, M. and Komutarin, T. (2012): Antibiotics Resistance and RAPD-PCR Typing of Multidrug Resistant MRSA Isolated from Bovine Mastitis Cases in Thailand. *Sci. Asia.,* 38: 30-35.
18) Iwane, T., Urase, T. and Yamamoto, K. (2001): Possible Impact of Treated Wastewater Discharge on Incidence of Antibiotic Resistant Bacteria in River Water.*Water Sci. Technol.,* 43: 91–109.
19) Kummerer, K. (2004): Resistance in the Environment: A Review. *J. Antimicrob. Chemother*., 54: 311-320.
20) Levy, S.B. and Marshall, B. (2004): Antibacterial Resistance Worldwide: Causes, Challenges and Responses. A Review. *Nat. Med.,* 10: S122–S129.
21) Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M. and Qi, R. (2010): Antibiotic Resistance Characteristics of Environmental Bacteria from an Oxytetracycline Production Wastewater Treatment Plant and the Receiving River. *Appl. Environ. Microbiol.,* 76:3444-3451.
22) Macrina, F.L., Kopeck, D.J., Jones, K.R., Ayers, D.J. and McCowin, S.M. (1978): A Multiple Plasmid-containing *Escherichia coli* Strain: Convenient Source of Size Reference Plasmids Molecules. *Plasmid.,* 1: 417-420
23) McGowan, J.E. Jr.(2006): Resistance in Non-fermenting Gram-negative Bacteria: Multiple Drug Resistance to the Maximum. *Am. J. Med.,* 119(6 suppl 1): S29-S37.
24) Namboodiri, S.S., Opintan, J.A., Lijek, R.S., Newman, M.J. and Okeke, I.N. (2011): Quinolone Resistance in *Escherichia coli* from Accra, Ghana. *BMC Microbiol.,* 11: 44.
25) Oppegaard, H., Steinum, T.M. and Wasteson, Y. (2001): Horizontal Transfer of a Multi-drug Resistance Plasmid Between Coliform Bacteria of Human and Bovine Origin in a Farm Environment. *Appl. Environ. Microbiol.,* 67: 3732-3734.
26) Patwardhan, R.B., Dhakephalkar, P.K., Niphadkar, K.B. and Chopade, B.A. (2008): A Study on Nosocomial Pathogens in ICU with Special Reference to Multiresistant *Acinetobacter baumannii* Harbouring Multiple Plasmids. *Indian J. Med. Res.,* 128: 178-187.
27) Peters, S.M., Bryan, J. and Cole, M.F.(2000): *Enterobacterial* Repetitive Intergenic Consensus Polymerase Chain Reaction Typing of Isolates of *Enterobacter cloacae* from an Outbreak of Infection in a Neonatal Intensive Care Unit. *Am. J. Infect. Cont.,* 28: 123-129.
28) Reinthaler, F.F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F. and Marth, E. (2003): Antibiotic Resistance of *E. coli* in Sewage and Sludge. *Water Res.,* 37: 1685-1690.
29) Schluter, A., Szczepanowski, R., Puhler, A. and Top, E.M. (2007): Genomics of IncP-1 Antibiotic Resistance Plasmids Isolated from Wastewater Treatment Plants Provides Evidence for a Widely Accessible Drug Resistance Gene Pool. *FEMS Microbiol. Rev.,* 31: 449-477.
30) Schwartz, T., Kohnen, W., Jansen, B. and Obst, U. (2003): Detection of Antibiotic-resistant Bacteria and Their Resistance Genes in Wastewater, Surface Water, and Drinking Water Biofilms. *FEMS Microbiol. Ecol.,* 43: 325-335.
31) Shahid M., Malik, A. and Sheeba (2003): Multidurg-resistant *Pseudomonas aeruginosa* Strains Harbouring R-plasmid and AmpC β-lactamases Isolated from Hospitalised Burn Patients in a Tertiary Care Hospital of North India. *FEMS Microbiol. Lett.,* 228:181-186
32) Da Silva, M.F., Vaz-Moreira, L., Gonzalez-Pajuelo, M., Nunnes, O.C. and Manaia, C.M. (2007): Antimicrobial Resistance Patterns in *Enterobacteriaceae* Isolated from an Urban Wastewater Treatment Plant. *FEMS Microbiol. Ecol.,* 60:166-176.
33) Thiolas, A., Bollet, C., La Scola, B., Raoult, D. and Pages J.M. (2005): Successive Emergence of *Enterobacter aerogenes* Strains Resistant to Imipenem and Colistin in a Patient. *Antimicrob Agents Chemother.,* 49:1354-1358.
34) Trautmann M., Bauer, C., Schumann, C., Hahn, P., Hoher, M., Haller, M. and Lepper, P.M. (2006): Common RAPD Pattern of *Pseudomonas aeruginosa* from Patients and Tap Water in A Medical Intensive Care Unit. *Int. J. Hyg. Environ. Health*., 209: 325-331.
35) Zhang, Y., Marrs, C.F., Simon, C. and Xi, C. (2009): Wastewater Treatment Contributes to Selective Increase of Antibiotic Resistance Among *Acinetobacter* spp. *Sci. Total. Environ.,* 407: 3702–3706.