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"DISCERNMENT OF PLASMID-MEDIATED MULTIDRUG RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM HOSPITAL EFFLUENTS"

Amritha G. Kulkarni[1], Mohith Sen Bhandarkar and A.B. Vedamurthy*

P.G. Department of Studies in Biotechnology and Microbiology Karnatak University, Dharwad – 580 003, INDIA.

***Corresponding Author: A.B. Vedamurthy**
P.G. Department of Studies in Biotechnology and Microbiology Karnatak University, Dharwad – 580 003, INDIA.

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ABSTRACT
Most of the original antibiotics have been rendered obsolete due to antibiotic overuse, abuse, and misuse. Hospital effluents and sewage are an excellent compartment for the selection of Multidrug-resistant(MDR) opportunistic, facultative pathogens such as *Escherichia coli,* which cause many of the nosocomial infections. The present study reveals the resistance profile of multidrug-resistant *Escherichia coli* isolated from hospital wastewater effluents of different hospitals in north Karnataka and the factors conferring multidrug-resistance. Several strains of *Escherichia coli* were isolated from hospital effluents and analyzed by agar disc diffusion to determine their susceptibility patterns to antimicrobial agents commonly used in these hospitals. The strain of *Escherichia coli* S-2 identified as *Escherichia coli*-SUS5EC by 16s-rRNA sequencing was found to be resistant to maximum number of antibiotics such as Ceftriaxone, Cefotaxime, Erythromycin, Penicillin, Amoxicillin, Ampicillin, Azithromycin out of thirteen antibiotics used and hence proven to be a multidrug-resistant mutant of *Escherichia coli*. The Plasmid DNA was isolated and plasmid mediated multidrug-resistance was confirmed by plasmid curing with the above-mentioned antibiotics. This finding substantiates the hypothesis that hospital sewage effluents contribute to the dissemination of transferable multidrug-resistance in bacteria. Thus, public health potential and the possible impact of Multidrug-resistant commensal *Escherichia coli* and other bacteria occurring in hospital effluents can be a major threat to the treatment of disease by commercially available antibiotics and is a major concern which needs further investigation and monitoring and the mechanisms for such diffusion, and methods to block the entry of such strains into the human population and their subsequent spread, need to be defined.

KEYWORDS Hospital Effluents, Antibiotic resistance, Multidrug resistant, *Escherichia coli*, Plasmid, Plasmid Curing.

INTRODUCTION
As said by the Nobel Laureate, Joshua Lederberg: "Antibiotic resistance as a phenomenon is, in itself, not surprising. Nor is it new. It is, however, newly worrying, because it is accumulating and accelerating, while the world's tools for combating it decrease in power and number". Antimicrobial drugs are used extensively in both human and veterinary medicine (Kümmerer., 2004; Senka *et al.,* 2008; Jury *et al.,* 2010; Wright., 2010). One of the main causes of antibiotics drug resistance is antibiotic overuse, abuse and in some cases, misuse, due to incorrect diagnosis. After application many of the compounds used in medicine are only partially metabolized or non-metabolized by patients and are then discharged into the hospital sewage system or directly into municipal waste water if used at home, resulting in the enrichment of antibiotic-resistant bacteria (Senka *et al.,* 2008). The microflora that survive in the hospital wastewaters are mainly saprophytic bacteria from the atmosphere, soil, medical devices and water employed in the hospital practice; the pathogens are mainly released with the patient excreta (Chitnis *et al.,* 2000; Johnson *et al.,* 2007; Wright., 2010; Rashid *et al.,* 2015). These bacteria that are exposed to a wide range of biocides such as pharmaceuticals, radionucleides and solvents, thus providing selective pressure that result in development of antibiotic resistant bacteria and resistance. Acquired resistance to first-line antimicrobial agents may arise by cellular mutation or by the acquisition of genetic elements in the form of plasmids or transposons (Gangle., 2005; Nuñez *et al.,* 2007; Islam *et al*., 2011). Not all strains are pathogenic, but those that are can cause sepsis, urinary-tract infections, meningitis, and gastroenteritis (Gangle., 2005).

These organisms pose a major health threat since Plasmid mediated resistance is widely transmitted between different bacterial species and genera including human pathogens (Davison., 1999; Rashid *et al.,* 2015). Multi-drug resistant strains of *Escherichia coli* are prevalent in both human and animal isolates in different parts of the world (Islam *et al.,* 2008;). Antimicrobial

resistance in *Escherichia coli* has been reported worldwide and increasing rates of resistance among *Escherichia coli* is a growing concern in both developed and developing countries. Acquired resistance to first-line antimicrobial agents increasingly complicates the management of extraintestinal infections due to *Escherichia coli*, which are a major source of illness, death, and increased healthcare costs. (Naeem. et al., 2007) However, in the present situation, not many investigations are being conducted in India to study the occurrence of these resistant strains and their human health implications. Although there are guidelines implemented by the Government of India for the proper disposal of these antimicrobial drugs, they are rarely being followed. The Multiple drug resistant bacteria are frequently occurring in these environments due to the improper hygienic conditions and unethical disposal of antibiotics. Besides health fears, there is growing concern about the potential ecological impacts from both the presence of antibiotics and resistant bacteria in the environment. Therefore our present investigation was focused on to the survey of bio-disposal methods employed in hospitals and to analyze the factors conferring Multiple Drug Resistance in the strains of *Escherichia coli*, which is a common faecal contaminant found in hospital effluents.

MATERIALS AND METHODS

Collection of samples

The hospital effluent samples were collected from the waste water released from different Government hospitals in and around Dharwad. The samples were collected from hospital outlets connected to municipal sewage or septic tank. The samples were serially diluted and directly inoculated onto Eosin-Methylene Blue Agar (Hi-media laboratories, Mumbai) and Nutrient Agar plates and were incubated at 37^0C for 24 hrs under aerobic conditions. Based on the morphological characterization and microscopic observations organisms were suspected to belong to the genera: *Salmonella, Klebsiella, Enterococci, Escherichia coli*.

Isolation and Identification of Multi-Drug resistant Escherichia coli:

With the aim to study the resistance profile of Multi-Drug Resistant coliforms, The colony characteristics of the isolated colonies were studied and the one which matched with the colony characteristics of *Escherichia coli* were selected. Subsequently, the isolates of *Escherichia coli* were subjected to biochemical analysis (Indole test, Methyl red test, Voges-Proskauer test, Urease test, Citrate test) for confirmation of identity of *Escherichia coli.*

Antimicrobial susceptibility analysis

The Bauer Kirby disc diffusion method (Bauer AW.*et al.* 1999) was used to test susceptibility of the isolated and identified organisms to different antibiotics at varying concentrations (Table 1).

Table 1: Different concentrations of Antibiotics used for Analysis of Drug Resistance.

Name of the Antibiotics	*Concentration (µg/ml)*			
Ceftriaxone	**1**	**2**	**3**	**4**
Cefotaxime	**0.4**	**0.6**	**0.8**	**1.0**
Erythromycin	**1**	**2**	**3**	**4**
Gentamicin	**1.0**	**1.5**	**2.0**	**2.5**
Ciprofloxacin	**1**	**2**	**3**	**4**
Norfloxacin	**2**	**4**	**6**	**8**
Ofloxacin	**1**	**2**	**3**	**4**
Penicillin	**0.5**	**1.0**	**1.5**	**2.0**
Roxithromycin	**2**	**4**	**6**	**8**
Cefixime	**1**	**2**	**3**	**4**
Amoxicillin	**0.5**	**1**	**2**	**3**
Ampicillin	**1**	**2**	**3**	**4**
Azithromycin	**1**	**2**	**3**	**4**

Bacterial Growth Curve Analysis

By using light absorbance technique, the growth of bacteria in broth was measured at 590nm in a spectrophotometer. Then the resistant pattern was analyzed by drawing a graph using the chart of absorbance in different concentrations of antibiotics.

Assessment of Plasmid mediated Multi-Drug Resistance

- ***Plasmid isolation***

Plasmid extraction was carried out by alkaline lysis technique (Bonfiglio *et al. 1995)*. The extracted plasmid was then isolated using a horizontal 1% agarose gel electrophoresis technique.

- ***Preparation of the cells***

The pure cultures were transferred to 10 ml screw cap tubes containing 3 ml Luria Bertani (LB) broth with 30 µg/ml of each antibiotic. The broths were then incubated at 37^oC with loose capping and vigorous shaking (>250 rpm) for overnight. Then inoculums were transferred to another 3ml LB broth at a 1:200 ml rate containing same concentration of ciprofloxacin and incubated for 4-6

hours at 37°C with loose capping and vigorous shaking (>250 rpm). After a sufficient growth with slight turbidity the incubation stopped and the cells were prepared for extraction

- ***Plasmid extraction protocol***

The plasmid isolation was done according to the protocol as mentioned in *Islam et al*., 2008**:** 1.0 ml of overnight culture was taken in an Eppendorf's tube (1.5ml) and cells were collected by centrifugation for 7 minutes at 12,000 rpm. The supernatant was discarded. The pellet was thoroughly suspended in 100 µl of solution I and the solution was kept at room temperature (32°C) for 10 min. Then 200 µl of solution II (lysis solution) was added and mixed gently by inverting the tube for a few times. After that 150 µl of ice-cold solution III (neutralizing solution) was added and mixed vigorously by vortexing for a few seconds. The tubes were kept on ice for 5 minutes. The mixture was then centrifuged at 12,000 rpm for 15 minutes to pellet the chromosomal DNA. The clear supernatant was (approximately 400 µl) was taken to fresh Eppendorf's tubes. Then two volumes of cold 95% ethanol (800 µl) were added in each tube and vortexed for a few seconds to mix well. It was then kept in room temperature for about 20 min for DNA precipitation. The precipitated DNA was collected by centrifugation for 15 minutes at 12,000 rpm. The supernatant was discarded. The pellet was dried in a drier at 45oC for 20 minutes. At last the dried DNA was dissolved in 30 µl TE buffer and kept at 4°C.

- ***Separation of plasmid DNA by agarose gel electrophoresis***

Plasmid DNA was separated by horizontal electrophoresis in 1% agarose slab gels in a Tris-Acetate EDTA (TAE) buffer at room temperature at 80 volt (50 mA) for 4 h. Briefly, 30 µl of plasmid DNA solution was mixed with 3 µl of tracking dye and was loaded into the individual well of the gel. The gel (5mm thick) was stained with 0.5 µg/ml of ethydium bromide for 15 min at room temperature and then destained with distilled water for 10 min. DNA bands were visualized and photograph was taken using the apparatus Bio-rad, Wide Mini-Sub Gel GT. The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel. Plasmids were present in *E. coli* isolates.

- ***16s mRNA sequencing:***

The isolated samples were sent for 16s RNA sequencing to identify the strain of the *Escherichia coli* showing the highest Multi-drug Resistance.

- ***Determination of LD50:***

The isolate was subcultured in nutrient broth media (g/l Peptone-10, NaCl-10 and Yeast extract-5) and grown for 18 h. A set of tubes containing 5 ml of the same media were autoclaved followed by addition of acryflavin at 2.5, 5, 10, 25 and 50 µg/ml along with a control tube and a blank. The 18 h old culture was added (0.025 ml) into the tubes containing acryflavin and control but not in the blank. The tubes were incubated for 24 h. The biomass was read at 600 nm against blank. The concentration of acryflavin, which could inhibit the growth of the culture by 50%, was selected for further studies.

- ***Plasmid Curing***

The tube containing 5 ml of autoclaved media was added with LD-50 concentration of acryflavin and 0.025 ml of inoculum followed by incubation for 24 h along with control. The second set of tube with media and acryflavin was inoculated with the culture of day 1 and incubated for 24 more. This was continued for 5 days and every day, the Plasmid was isolated and preserved at -20°C, until further use . The plasmid was isolated using HiPurA Plasmid DNA Miniprep purification spin kit (No. MB508), supplied by HiMedia, Mumbai. Following the isolation of Plasmid DNA samples, the electrophoresis of the samples were done.

RESULTS

A total of 10 Hospital effluent samples were analyzed and analysis of treatment history collected from these hospitals showed that they used mainly Amoxicillin, Ampicillin, Azithromycin, Cefixime, Cefotaxime, Ceftriaxone, Ciprofloxacin, Erythromycin, Gentamicin, Norfloxacin, Ofloxacin, Penicillin, and Roxithromycin.

Five different colonies of drug resistant *Escherichia coli* were isolated from the effluents and the biochemical analysis confirmed their identity. Also, based on the morphological characterization and microscopic observations certain other organisms were suspected to be Salmonella, Klebsiella and Enterococci.

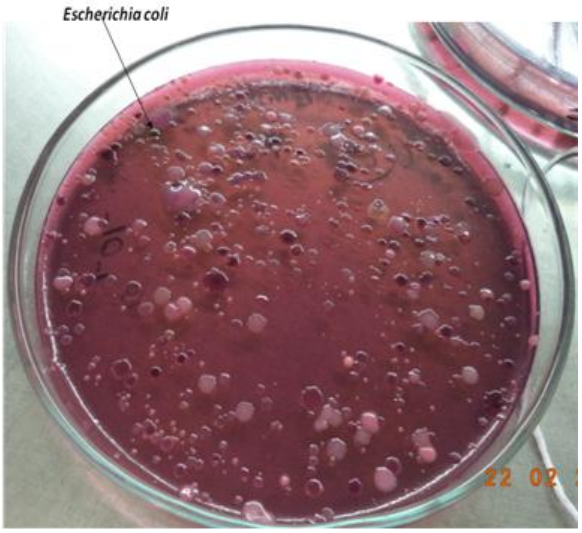


Fig 1: Different Bacterial Colonies isolated from sample S-2 on EMB agar plates.

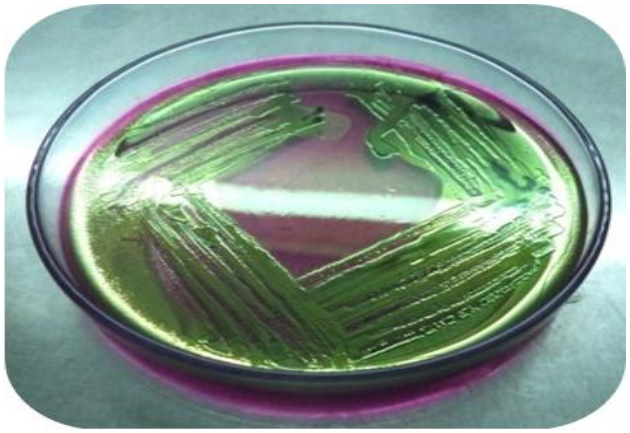
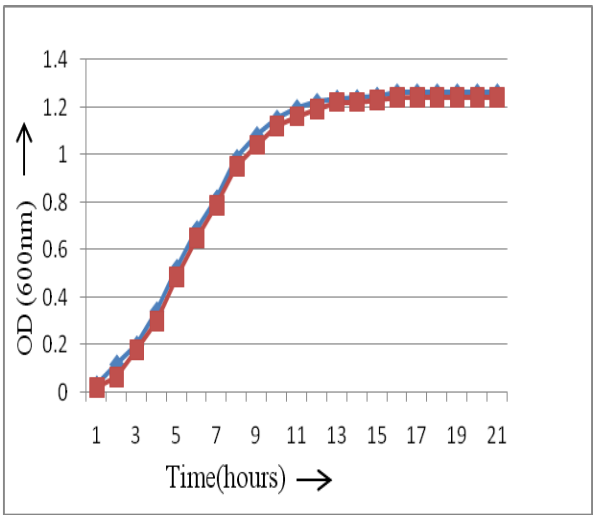


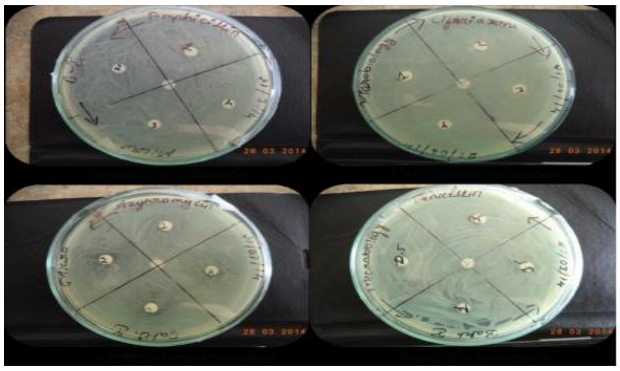
Fig 2: Pure Culture of *Escherichia coli* isolated from Sample S-2.

Table 2: The Biochemical Characterization of the isolated cultures.

Test	S -1	S-2	S-3	S-4	S-5
Indole	+ve	+ve	+ve	+ve	+ve
MR	+ve	+ve	+ve	+ve	+ve
VP	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	-ve	-ve



Graph 1: The bacterial cultures showed normal growth dynamics on the LB Broth Media.



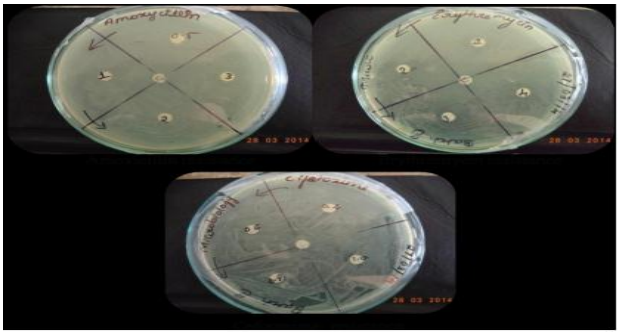


Fig 3: Antibiotic-Resistance of the colonies *Escherichia coli* S-2grown on L.B Agar Media.

The sequence obtained from 16s RNA sequencing the bacterial strain was identified and phylogenetic tree was constructed using NCBI BLAST (Basic Local Alignment Search Tool) and the strain was identified to be *Escherichia coli- SUS5EC* whose sequence is as follows:

G G G G C C G A A A G C A G C T G C T G C T T C
G C T G A C G A G T G G C G G A C G G G T G A G
T A T G T C T G G G A C C T G C C T G A T G G A G
G G G G A T A A C T A C T G G A C C G G T A G C
T A A T A C C G C T T A A C G T C T C C A G A C C
A A G G A G G G G G A C C T T C G G G C C T C T
T G C C A T C G G A T G T G C C C A G A T G G G
A T T A G C T T G T T G G T G G G G G T A A C G G C
T C A C C A A G G C G A C G A T C C C T A G C T
G G T C T T G A C A G G A T G A C C A G C C A C
A C T G G A A C T G A G A C A C G G T C C A G A
C C T C C T A C G G G A G G C A G C A G T G G G
G A A T A T T G C A C A A T G G G C G C A A G C
C T G A T G C A G C C A T G C C G C C G T G T A T
G A A G A A G G C C T T C G G G G T T G T A A A
G T A C T T T C A G C G G G G A G G A A G G G G A
G T A A A G T T A A T T A C C T T T G C T C G T T
G A C G T T A C C C G C A G A A C A A G C A C C
G G C T A A C T C C G T G C C A G C A G C C G C
G G T A A T A C G G A G A G T G C A A G C G T T
A A T C G G A T T T A C T G G G C T T T A A A G C
G C A C T C A G G C G G T T T G T T T A G T C T G
A T G T G A A A T C C C C G G G G G C T C A A C C
T G G G A A C T G C G T C T G A T A C T G G C A
A G C T T G A G

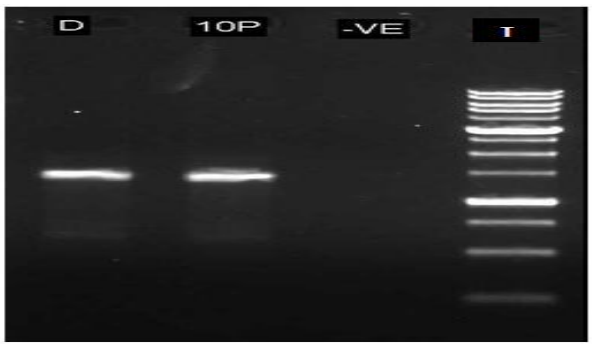


Fig 9: PCR amplified product in Agarose gel D: Amplified sample, 10P: positive marker, -VE: negative marker, I: DNA Ladder

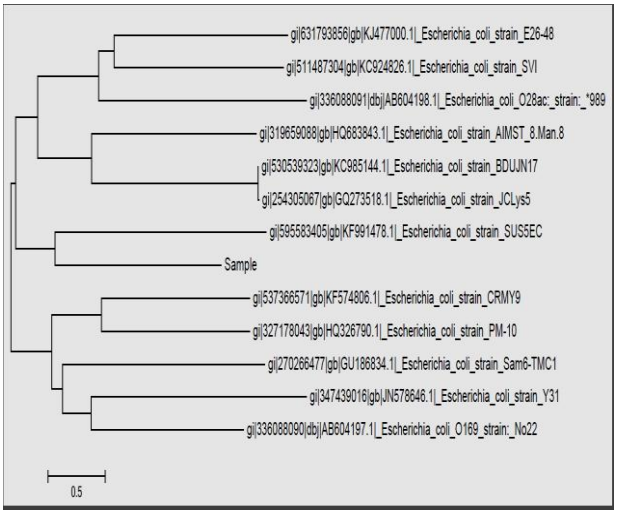


Fig 10: **Phylogenetic tree.**

The phylogenetic tree showed *Escherichia coli- SUS5EC* showed 95% match to the sequence of the sample S-2.

The plasmid curing results showed that, the curing of the plasmid had begun on the first day itself significantly and, the complete curing of the plasmid has occurred by third day (fig:6). The plasmid cured culture (S-2) was again subjected to antibiotic susceptibility test. The strain of Escherichia coli which previously showed resistance to the 7 antibiotics before plasmid curing, was found to be sensitive to the same 7 antibiotics after plasmid curing (fig:7).

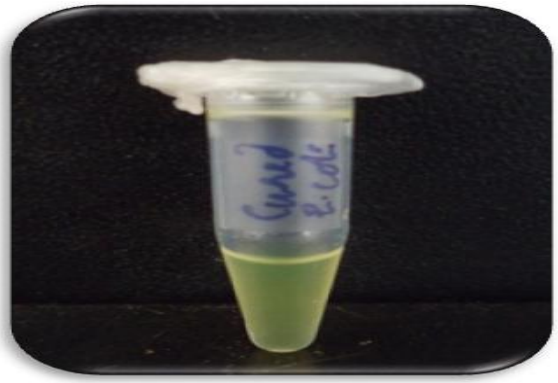
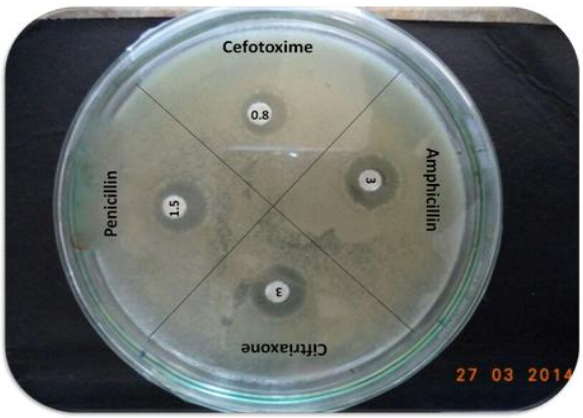


Fig 7: Plasmid cured culture of *Escherichia coli.*



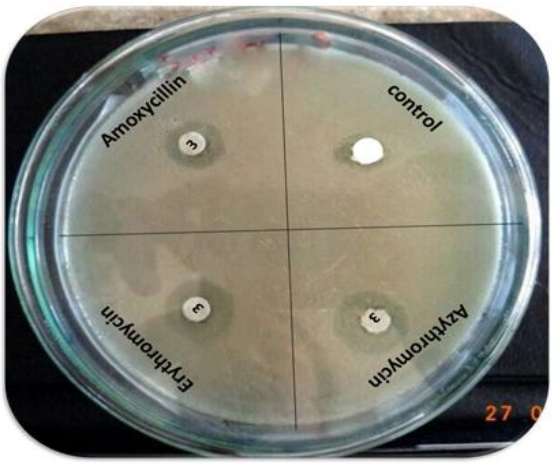


Fig 8: Antibiotic susceptibility test of cured samples S-2.

DISCUSSION

One of the greatest challenges of practicing medicine and surgery in hospitals in the post-antibiotic era is multidrug-resistant nosocomial infections (Kummerer, 2004). Our work showed similar results as previous studies, the release of wastewater from hospitals is directly associated with an increase in the prevalence of antibiotic resistance (Elmanama *et al.*, 2006; Alam *et al*., 2014). Hospital wastewater contained a higher number of single and multiple antibiotic resistances among coliform species. Also, the MDR pattern seen in the bacteria isolated from hospital effluent samples included most of the antibiotics in common use. Some investigators claim that there is danger to humans only if resistant pathogens spread to humans (Phillips, 1999; Phillips, 2002; Phillips *et al.*, 2004). However, considering the extreme versatility of bacteria in adapting to selective pressure, and their ability to regulate their drug resistance genes, the pathogens that are autochthonous to the environment can acquire resistance genes from animal fecal bacteria (Perretin *et al.*, 1997; Teubner., 1999; Chitnis *et al.*, 2000). Increased introduction of antimicrobial agents into the environment via medical therapy, agriculture and animal husbandry has resulted in selective pressures on bacterial populations.

In the present study, a high frequency of resistance among *E. coli* isolates isolated from hospital wastewater to commonly used antibiotics was observed. The present investigation ascertained that there was an increased incidence of both pathogenic and opportunistic pathogenic bacteria in hospital sewage. The resistance patterns were also recorded against these antibiotics in different combinations for one of the MDR isolate. The worst fear apprehended is the transfer of such resistance to bacterial pathogens causing infection in the community, therefore most of the presently available antibiotics will be futile against the infectious organism. The origin of such MDR bacterial strains appears to be the hospital environment and the selective pressure responsible for expanding such bacterial populations in hospitals (Naeem. et al., 2007). Similar studies have also

reported on transferable plasmids encoding resistance to various heavy metals and antibiotics in gram negative bacteria (Alam et al., 2014). Our studies showed that all of the *E. coli* isolates were multidrug resistant, which correlated previous studies (Islam *et al*., 2008; Alam et al., 2014). Our findings closely resemble those of a recent study of Antimicrobial Drug–Resistant Escherichia coli from Humans and Poultry Products, Minnesota and Wisconsin, (Johnson, 2007). The finding of this study presents a potential health problem as the predominant coliform species have increasingly been associated with outbreaks of hospital infections (Alam et al., 2014). The clinical and economic impact of MDR gram-negative bacilli is substantial and greatly worrisome. An international agreement on the definitions of such bacteria could potentially facilitate an orchestrated response against these pathogens.

CONCLUSION

The present investigation ascertained the infectivity of the liquid-waste disposal methods employed in hospitals as there was an increased incidence of Multidrug-resistant coliforms in hospital effluents. This finding substantiates the hypothesis that hospital sewage effluents contribute to the dissemination of Multi-drug resistant bacteria in the environment. This phenomenon is deeply alarming due to possible transfer of drug resistance plasmid. So, continuous monitoring is essential to assess and control the impact of MDR organisms. The significance of this for public health however, is essentil to fully characterize and quantify the input of MDR organisms isolates from hospitals and their potential spread to other sources.

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