

## Survival of antibacterial resistance microbes in hospital-generated recycled wastewater

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### ABSTRACT

Hospital wastewater has the potential to be a threat to the hospital environment as it can contain pathogenic bacteria that may facilitate the resistant nature of organisms within effluent or water treatment plants. The recycling of hospital wastewater should have good quality. This study was carried out to highlight the incidence of antibiotic resistant bacteria in hospital-generated recycled water. This study was conducted in a tertiary care teaching hospital during June 2013–June 2014. One hundred and forty wastewater samples were aseptically collected at different stages in the recycling plant. The samples were processed within 2 hours following standard procedures for identification of bacteria and the pathogenic bacteria were isolated. The mostly identified pathogens were *Staphylococcus aureus* (11.42%), *Pseudomonas aeruginosa* (9.28%), *Enterococcus faecalis* (10%) and *Bacillus subtilis* (8.57%) which were removed by treatment, but *Escherichia coli* (16.42%), *Klebsiella pneumoniae* (8.57%), and *Proteus mirabilis* (11.42%) survived in the final sedimentation tank (lagoon) from where this water will be used for gardening purposes. An antibiogram study showed these pathogens were resistant to first-line antibiotics. Effluent treatment plants in hospitals should be monitored for the fulfillment of the guidelines and quality control of treated water to stop the emergence of multi-drug resistant bacteria in the hospital environment.

**Key words** | effluent treatment plant, hospital effluents, multi-drug resistance organisms, pathogens, recycling

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### INTRODUCTION

The trend in reuse of wastewater for agriculture and aquaculture was put forward because of the growing world population, unrelenting urbanization, increasing scarcity of good quality water resources and rising fertilizer prices. Wastewater can originate from domestic, industrial, hospital, commercial or agricultural activities, surface runoff or storm water. Hospital wastewater should be carefully handled because hospitals discharge considerable amounts of chemicals and microbial agents in the wastewater. Hospitals and healthcare settings are regarded as reservoirs for large numbers of pathogenic bacteria (Mulvey & Simor

2009). The majority of tertiary care hospitals have the effluent treatment plant for recycling of wastewater and utilizing back to hospital. Wastewater in hospital is produced from canteens, laundries, medical waste incinerators, clinical laboratories and wards (Akter *et al.* 2000). Under the Environmental Protection Act 1986, the effluent limits are applicable to those hospitals which are connected to sewers without a terminal sewage treatment plant (CPCB 2009).

Water recycling involves three types of treatment: (1) primary treatment, (2) secondary treatment and (3) tertiary

treatment. Primary treatment involves basic processes to remove suspended solid waste and reduce its biochemical oxygen demand – the amount of oxygen microorganisms must consume to breakdown the organic material present in the wastewater. This, in turn, increases dissolved oxygen, which is good for aquatic organisms and food webs. Secondary treatment uses biological processes to catch the dissolved organic matter missed in the primary treatment. Microbes consume the organic matter as food, converting it to carbon dioxide, water, and energy. The bacterial load in the secondarily treated water is decreased compared to the primary treated sample. However, a significant number of bacteria still remain, especially those that are resistant to antimicrobials (Kim & Aga 2007).

The final level of wastewater treatment is tertiary treatment, which is a process that goes beyond the previous steps and can include using sophisticated technology to further remove contaminants or specific pollutants. Tertiary treatment is typically used to remove phosphorous or nitrogen, which cause eutrophication. Hospital effluents contain pathogenic organisms and various chemical agents which induce a resistance nature and easily escape from water treatment plants. Hospital effluents may constitute the perfect scenario for exchange of resistance genes between clinical and environmental pathogens (Brown *et al.* 2006; Martinez 2009) and also the bacteria in wastewater are exposed to a wide range of biocides that could act as a selective pressure for the development of resistance (Russell 2001). According to the World Health Organization (WHO) (2014) report, when the organisms become resistant to first-line drugs then second-line drugs are tried further which increases the duration of treatment and increases health care costs as well as the economic burden on the country.

Extended Spectrum  $\beta$ -Lactamase (ESBL) producing isolates which disseminate in the environment through hospital wastes. ESBL is an enzyme that allows bacteria to become resistant to a wide variety of penicillins and cephalosporins. If the hospital effluents are not treated properly, concentrated forms of infectious agents and antibiotic resistant microbes are shed into communities resulting in water-borne diseases such as dysentery, gastroenteritis, urinary tract infections and infectious diseases which cannot be treated by antibiotics (Sharma *et al.* 2010). The presence of antibiotic resistant bacteria is one of the greatest health

concerns for humans as they can lead to the development of antibiotic resistant bacteria and possibly the horizontal transfer of resistance factors to human pathogens (Fick *et al.* 2009).

This study was planned at SRM Hospital and Research centre, for a period of 1 year (June 2013–June 2014). The major aim of this study was to identify the presence of drug-resistant pathogens in recycled wastewater from different wastewater collection systems such as the collection tank, equalizer, aeration, sedimentation, filter collection tank, sand filter and lagoon of the recycled water treatment plant.

## METHODS

### Sewage treatment plants

In total, about 45 lakh litres of water is recycled per day by three sewage treatment plants. Wastewater is recycled in a sequential process starting from the entry of wastewater followed by the equalizer tank, aeration tank, sedimentation tank, sand filter, carbon filter and finally discharge to lagoon water for natural sedimentation. The treatment plant was constructed and operated according to Frank's (2008) *Wastewater Treatment Plant Operator Manual*.

### Sewage treatment plant I

This treatment plant is specially used for treating hospital effluents. Apart from the biomedical waste and infectious waste which will be placed in 1% sodium hypochlorite, the hand washes from various hospital departments, canteens in the hospital, hostels, etc. are received by sewage treatment plant I. It takes 8 h for the recycling process and the recycled water is sent to the lagoon, from there it is used for gardening and sanitation purposes.

### Collection of wastewater

The American Public Health Association (APHA) guidelines were followed for the collection of water samples (APHA 1999). One hundred and forty samples were collected in wide mouth sterile glass bottles from seven

different stages of wastewater treatment after completion of treatment before the processed wastewater moves to the next stage at different timings. The temperature and pH of the water samples was recorded which will be used to determine the reduction of organisms during the recycling processes (Mulvey & Simor 2009). The functions of the each stage are shown in Figure 1. The stages include the equalizer tank, aeration tank, clarifier tank, sedimentation tank, filter collection tank, sand filter and lagoon, followed by immediate transfer to the laboratory. The processing of recycled water samples was performed within 24 h of collection.

### Total heterotrophic bacterial count

The samples were mixed with 100 mL phosphate buffered dilution water before processing. From the homogenous solution, 1 mL of sample was taken according to the

guidelines of USEPA (1974) (Sharma *et al.* 2010). The wastewater samples were serially diluted up to  $10^{-4}$  dilution factor using sterile distilled water (APHA 1999) which will be suitable for colony counts and 0.1 mL of sample were inoculated on nutrient agar medium to obtain a pure culture by spread plate technique (APHA 1999). After 24 h of incubation at 37 °C the colonies were counted (CFU/mL = number of bacterial colonies/volume of sample × dilution factor) using a colony counter and recorded. The spread plate method generally yields higher bacterial counts than the pour plate method, although it is limited to a smaller sample volume (APHA 1999).

The colony morphology of different organisms was noted, each colony was picked, Gram stained and sub-cultured in MacConkey agar for identification of lactose fermenting (LF) and non-lactose fermenting (NLF) colonies and in blood agar to note the hemolysis Mackie & MacCartney (1996). NLF colonies were further subcultured in

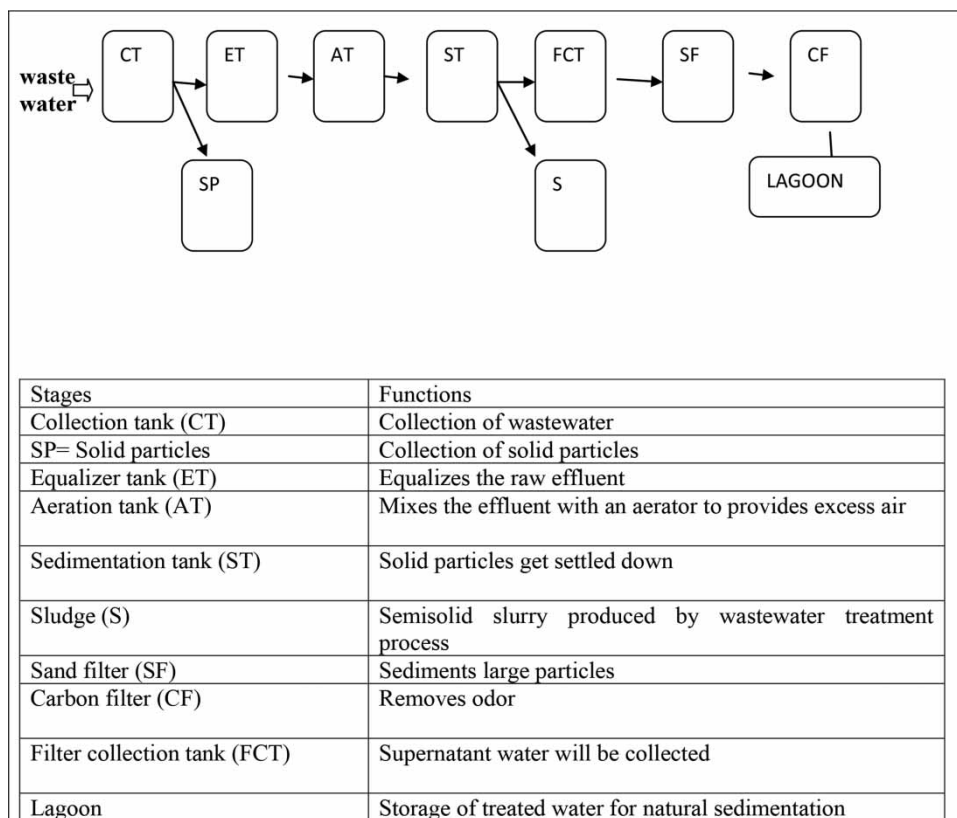


Figure 1 | Hospital effluent treatment plant and its functions.

deoxycholate citrate agar (DCA) and thiosulfate citrate bile salt agar (TCBS) for identification of organisms.

The pure colonies were identified by colony morphology and cultural characteristics of the organism were recorded and further continued by biochemical tests such as the oxidative fermentation test, nitrate reduction test, triple sugar iron agar test, indole, methyl red, Voges Proskauer, citrate, urease, coagulase, mannitol motility medium, lysine iron agar test for identification of water-borne pathogens as described in Bergey's *Manual of Determinative Bacteriology* (Holt et al. 1993). Some of the commensal organisms were also isolated but they were omitted from this study.

### Antimicrobial susceptibility pattern of pathogens

Antimicrobial susceptibility testing was performed for each pathogen by the standard disk diffusion method with reference to the Clinical Laboratory Standards Institute (CLSI 2012) guidelines. Bacterial inoculums were prepared by suspending the freshly grown bacteria in 4–5 mL sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was performed using the antibiotics vancomycin ciprofloxacin, ampicillin, piperacillin, penicillin, gentamycin and ofloxacin, in Muller Hinton agar.

The plates were incubated aerobically at 37 °C for 24 h. The zones of inhibition were measured in millimeter units and compared with CLSI (2012) guidelines to identify the sensitivity and resistance pattern of each pathogen. ATCC strains were used as control. Bacterial strains that were possible producers of ESBL were identified against the antibiotics cefuroxime, ceftazidime plus clavulanic acid (CAC) and ceftazidime (CAZ) according to the criteria established by the CLSI (2012).

## RESULTS

A total of  $n = 140$  waste water samples were collected. One hundred and six (75.68%) organisms were identified. The number of bacterial colonies are shown in Table 1 with colony forming units (CFU).

**Table 1** | Number of bacterial colonies isolated

Stage	No. of bacterial colonies at $10^{-4}$ dilution factor	CFU/ML
Collection tank	205	$205 \times 10^5$
Equalizer tank	185	$185 \times 10^5$
Aeration tank	178	$178 \times 10^5$
Sedimentation tank	152	$152 \times 10^5$
Filter collection tank	93	$93 \times 10^5$
Sand filter	56	$56 \times 10^5$
Lagoon	42	$42 \times 10^5$

Table 1 confirms that the collection tank shows the highest amount of colonies while it gets substantially reduced when passing different stages of the treatment plant.

*Staphylococcus aureus* was observed as Gram-positive cocci in chains, showing growth in Mannitol salt agar and a positive reaction towards the oxidative fermentation test and coagulase test. *Enterococcus faecalis* was observed as Gram-positive cocci showing growth in brain heart infusion agar. *Bacillus subtilis* was observed as Gram-positive bacilli showing growth in Luria Bertani broth and a positive reaction in the Voges Proskauer test and it ferments glucose, sucrose and xylose. *Escherichia coli* was observed as Gram-negative bacilli showing growth in MacConkey agar and a positive reaction in indole, methyl red and urease test. *Klebsiella pneumoniae* was observed as Gram-negative bacilli and a positive reaction in Voges proskauer test. *Proteus mirabilis* was observed as Gram-negative bacilli with swarming motility in blood agar and *Pseudomonas aeruginosa* was observed as Gram-negative bacilli giving a positive result in the oxidase test, producing a greenish-yellow pigment in nutrient agar.

Microbes present at various stages of recycling were noted and most frequently isolated bacterium and their occurrence at different stages are listed in Table 2.

Table 2 shows that *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* were able to survive in all stages of recycling. Organisms showed resistant and sensitive nature for one or more drug. The antimicrobial susceptibility testing results are listed in Table 3.

Out of 106 organisms isolated, three organisms were identified as Gram-positive and four organisms as

**Table 2** | Pathogens identified at various stages of the recycling process

S. no.	Treatment stages	Name of the pathogen isolated
1	Collection tank	<i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterococcus faecalis</i> and <i>Bacillus subtilis</i>
2	Equalizer tank	<i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterococcus faecalis</i> and <i>Bacillus subtilis</i>
3	Aeration tank	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterococcus faecalis</i> and <i>Bacillus subtilis</i>
4	Sedimentation tank	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Pseudomonas aeruginosa</i> and <i>Bacillus subtilis</i>
5	Filter collection tank	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> and <i>Bacillus subtilis</i>
6	Sand filter	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> and <i>Bacillus subtilis</i>
7	Lagoon	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i>

Gram-negative. Sixty-four sensitive organisms (60.37%) for first-line drugs were sub-cultured and 42 resistance organisms (39.62%) were further processed.

Figure 2 shows the percentage of sensitive and resistance nature of seven different microbes isolated from the wastewater treatment plant. The microbes with a resistance nature are subjected to drugs of higher concentration and

are evaluated by the disk diffusion method (CLSI 2012). Tables 4 and 5 shows the organisms with least zone of inhibition.

ESBL producing strains were confirmed using CAZ which is a third generation cephalosporin with ceftazidime – clavulanic acid (CAC) combination. If the zone of inhibition of CAC is greater than 5 mm when compared to CAZ, the organism will be generally referred as an ESBL producer. By antibiotic susceptibility testing we were able to find out that some strains of *Escherichia coli* were ESBL producers.

## DISCUSSION

The hospital wastewater collecting system in our hospital is utilized mainly for the reuse of water after recycling for gardening and sanitary purposes. The report of this study is to create awareness about the water that should be checked for its quality before use.

Wastewater samples were collected from different stages at the treatment plant. Temperature and pH were recorded during the study, and it was found that the growth characteristics of pathogen will be affected by temperature and pH. Example thermophilic organisms will grow at more than 40 °C whereas *Yersinia enterocolitica* will grow at very low temperatures. Fluctuations in the pH cause oxidative stress among organisms, particularly *Escherichia coli* (Maurer et al. 2005).

The colony morphology of different organisms were presented as tiny, large, circular, irregular, mucoid, pigmented colonies. Gram staining was performed and further cultured

**Table 3** | Mostly identified pathogens and the percentage of sensitive and resistance

S. no.	Organisms	Total (percentage) n = 106	Susceptibility pattern	
			Sensitive	Resistance
1	<i>Staphylococcus aureus</i>	16 (11.42%)	7 (43.75%)	9 (56.25%)
2	<i>Enterococcus faecalis</i>	14 (10%)	10 (71.42%)	4 (28.57%)
3	<i>Bacillus subtilis</i>	12 (8.57%)	4 (33.33%)	8 (66.66%)
4	<i>Escherichia coli</i>	23 (16.42%)	17 (73.91%)	6 (26.086%)
5	<i>Klebsiella pneumoniae</i>	12 (8.57%)	5 (41.66%)	7 (58.33%)
6	<i>Proteus mirabilis</i>	16 (11.42%)	12 (75%)	4 (25%)
7	<i>Pseudomonas aeruginosa</i>	13 (9.285)	9 (69.23%)	4 (30.76%)
	Total	106	64	42

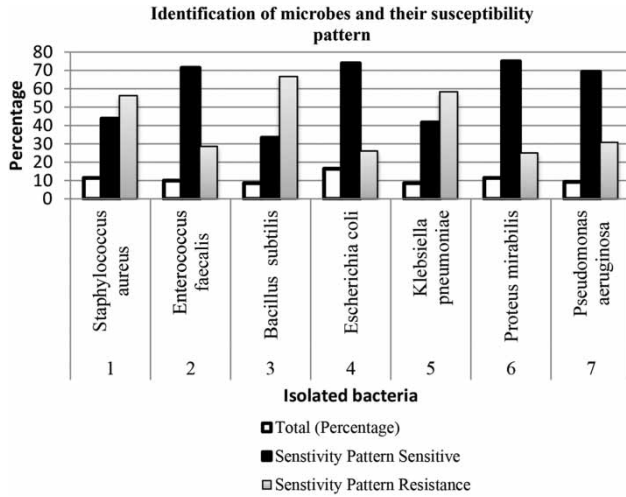


Figure 2 | Graphical representation of microbes and their susceptibility testing.

on MacConkey agar, blood agar, Luria Bertani agar, DCA and TCBS for its morphological observations and then it is finally subjected to biochemical testing for confirmation of organisms.

A study was conducted on identification of bacteria from biochemical waste, the results show that *Bacillus*

*subtilis* (12%), *Staphylococcus aureus* (9%), *Klebsiella pneumoniae* (6%), and *Escherichia coli* (15%) were isolated (Anitha & Indira 2012), which was comparable with our results of *Staphylococcus aureus* (11.42%), *Escherichia coli* (16.42%), *Klebsiella pneumoniae* (8.57%), thus it proves that microbes move towards their resistance nature if not properly managed.

The load of microbes was reduced at different stages of effluent treatment. Chitnis et al. (2004) showed that the number of coliforms reduced while processing through the different stages of effluent treatment. We have observed that the collection tank showed the highest microbial load but it was tremendously reduced when passing through the equalizer tank, aeration tank, sedimentation tank, sand filter and finally to the lagoon, but still due to inadequate filtration the pathogens escape and survive in recycled water. Soda ash and aluminum sulfate (alum) are used in the equalizer tank of the wastewater treatment plant to maintain the pH, temperature fluctuations and to balance the organic concentration. *Staphylococcus* are sensitive to aluminium sulphate and

Table 4 | Antibiotic susceptibility testing of isolated Gram-positive organisms

S. no.	Organism	Antibiotic disc (Disk potency) (Zone of inhibition in mm), Sensitive (S), Intermediate (I) and Resistant (R)								
		AMP (10)	CIP (5)	E (5)	VA (30)	PEN (10)	GEN (10)	TEI (30)	LZ (30)	HLG (120)
1	<i>Staphylococcus aureus</i>	10 I	6 R	8 R	16 I	6 R	8 R	15 S	27 S	25 S
2	<i>Enterococcus faecalis</i>	17 S	6 R	10 I	20 R	12 R	6 R	16 S	26 S	6 R
3	<i>Bacillus subtilis</i>	6 R	12 R	12 I	9 R	12 R	17 I	22 S	20 S	27 S

AMP: ampicillin; CIP: ciprofloxin; E: erythromycin; VA: vancomycin; PEN: penicillin; GEN: gentamycin; TEI: teicoplanin; LZ: linezolid; HLG: high level gentamycin.

Table 5 | Antibiotic susceptibility testing of isolated Gram-negative organisms

S. no	Organism	Antibiotic disc (Disk potency in µg) (Zone of inhibition in mm), Sensitive (S), Intermediate (I) and Resistant (R)											
		AMP (10)	AK (30)	COT (1.25/23.7)	CPM (30)	CFS (30)	CAZ (30)	CAC (30)	CIP (5)	IMP (10)	PTZ (10)	CL (10)	MRP (10)
1	<i>Escherichia coli</i>	6 R	8 R	6 R	16 R	20 I	16 I	27 S	16 I	29 S	22 S	12 I	29 S
2	<i>Klebsiella pneumoniae</i>	6 R	11 I	21 S	22 I	18 I	21 S	26 S	12 I	21 I	19 I	11 I	30 S
3	<i>Proteus mirabilis</i>	6 R	6 R	15 I	17 I	22 S	15 I	6 R	18 I	27 S	25 S	6 R	28 S
4	<i>Pseudomonas aeruginosa</i>	11 I	20 I	6 R	12 I	16 I	6 R	9 R	12 I	17 I	13 R	13 S	11 R

AMP: ampicillin; AK: amikacin; COT: cotrimazole; CPM: cefepime; CFS: ceftazidime; CAC: ceftazidime + clavulanic acid; CAZ: ceftazidime; CIP: ciprofloxin; IMP: imipenem; PTZ: piperacillin tazobactam; CL: colistin; MRP: meropenem.

may be killed in the equalizer tank, thus *Staphylococcus aureus* might be eliminated from this stage, which has to be proved. Organisms like *Enterococcus faecalis* and *Bacillus subtilis* were also not observed in the lagoon; this shows that they are sensitive when subjected to continuous treatment process.

The antibiotic susceptibility pattern was different for each organism, we represented the most resistant organisms of particular species. Among all isolates of Gram-negative bacteria isolated from treated hospital effluent, 97% of the isolates were resistant to Ampicillin (Balaji *et al.* 2011). Our study also showed that the organism which we isolated from hospital effluent showed more resistance to ampicillin and also tetracycline. *Proteus mirabilis* were resistant to first-line drugs which was similar to another study in 2011, where identified antibiotic resistance pattern of bacteria species was isolated from hospital waste water in Ede South Western, Nigeria (Balakrishnan *et al.* 2003). Antibiotic susceptibility testing for *Bacillus subtilis* is reference based (Balakrishnan *et al.* 2003).

*E. coli* which was isolated from recycled hospital effluent was capable of producing ESBL (Sadowy & Luczkiewicz 2014), the result was similar to our study. *E. coli* isolated from hospital waste water was resistant to cefotaxime (including extended-spectrum betalactamase (ESBL) producers), ciprofloxacin, and ceftiofloxacin. ESBL-producing *Enterobacteriaceae* are resistant to strong antibiotics including extended spectrum cephalosporins. ESBL are the result of mutations in the ubiquitous class A TEM or SHV beta lactamases. The ESBL strains are commonly sensitive to inhibition by clavulanic acid (Kim & Aga 2007). Since ESBL producers vary in their substrate profiles CLSI recommends the use of more than one antibiotic to increase the sensitivity of detection (Clewell 1990).

Wastewater treatment plants (WWTPs) constitute an important source for Enterococcal strains carrying antimicrobial resistance determinants, thus increasing a pool of such genes and capable of transfer to other organisms (Russell 2001). The Enterococcal strains isolated in our study also showed resistance to ciprofloxacin, gentamycin, high level gentamycin and erythromycin. As CLSI guidelines have not established a susceptibility pattern of different antibiotic discs for *Bacillus subtilis*, the values are interpreted on the basis of Balakrishnan *et al.* (2003).

The bulk of the bacteria in the hospital effluent remain firmly adhered to solid particles. Aeration and clarification removes the bulk of the bacteria by physical processes like flocculation but still the treated liquid effluent contains sizeable loads of multi-drug resistant (MDR) bacteria and so chlorination should be adequate and up to the mark of water quality.

The isolated microbes in our study showed the resistance nature towards the first-line drugs and to some second-line drugs. Even after treatment they are not removed; this shows that there is acquired resistance in the organisms.

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## CONCLUSIONS

In the present scenario of water scarcity in most of the developing countries, recycling of water for reuse is a welcome step if the recycled water is quality controlled. The recycled water with resistant microbes, even though helping in conservation of water, will be of no use in the protection of human health. Our study results showed the presence of multi-drug resistance pathogens in hospital effluent and there may be a chance of contaminating the hospital environment. Proper regulation and monitoring of an integrated health care liquid waste management practice is essential all over the hospitals in order to diminish the risk of disseminating multiple drug-resistant microorganisms for the safeguard of public health and re-use of the water to maintain an ecological balance. Proper training in the handling of waste will enable healthcare organizations to diffuse this critical problem safely and cost effectively.

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