



Prevalence of antibiotic resistant bacteria in hospital waste water of Allahabad region

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Abstract

The study was carried out in different hospitals of Allahabad region. Total of 80 hospital waste water samples were subjected to Heterotrophic plate count studies by pour plate method in nutrient agar and incubated at 37°C for 24 hours. Similarly for selective isolation of bacteria the specimens were cultured in MacConkey Agar Media, SS Agar, Cetrimide Agar, MSA Media. Total of 453 strains were isolated which were further identified as *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteric* sub. *arizonae*, *Klebsiella pneumoniae*, *Salmonella enteric* sub. *enteric*, *Enterobacter intermedium*, *Yersinia pseudotuberculosis*, *Enterobacter sakazakii* and *Staphylococcus aureus*. One isolate from each species were subjected to in-vitro antibiotic susceptibility test by modified Kirby Bauer disk diffusion technique for resistance pattern against Ciprofloxacin, Gentamicin, Amikacin, Chloramphenicol, Piperacillin, Amoxicillin, Meropenem, Ceftazidime, Tetracyclin, Nitrofurantoin, Aztreonam and Ampicillin. Heterotrophic plate count of hospital effluents significantly exceeded the standard heterotrophic plate count ($p=0.000$). Out of nine isolates tested all were multi drug resistant showing resistant to more than four antibiotics except *Staphylococcus aureus*. Health care liquid waste were laden with MDR bacteria and seemed to pose a huge public health in transfer of such resistance to the bacterial pathogen causing community acquired infections.

Keywords: hospital waste water, heterotrophic plate count, multi-drug resistant, health care liquid waste.

Introduction

Antimicrobial resistance has become a major clinical and public health problem within the lifetime of most people living today (Stuart, 2002) [16]. The widespread and often inappropriate use of antibiotics in livestock, pests, and humans have been shown to result in the development of antibiotic-resistant bacteria and is generally accepted to be the primary pathway for proliferation of antibiotic-resistant bacteria in the environment (Wegener *et al.*, 1999) [20].

Antibiotics exert a selection in favour of resistant bacteria by killing or inhibiting the growth of susceptible bacteria; resistant bacteria can adapt to environmental conditions and serve as vector for the spread of antibiotic resistance (Wegener *et al.*, 1999) [20]. The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens (Wegener *et al.*, 1999; Kruse, 1999) [20]. Antimicrobial resistance is generally found in hospital environment. The non-metabolized substances excreted by the patients also contain residues of the antibiotics prescribed to patients. The residual quantities of these antibiotics in hospitals waste water expose the bacteria that survive in hospital waste water to the wide range of these antibiotics that could act as selective pressure for development of antibiotic resistance.

Due to heavy antibiotic use, hospital wastewater contains larger number of resistant organisms than domestic wastewater. Majority of healthcare institutions have been discharging their effluents into surface water (rivers). Therefore, even if the hospitals are discharging their healthcare liquid waste into sewerage systems, it is mixed with river water and gets in surface water without proper

treatment. If the hospital effluent are not treated, concentrated forms of infectious agents and antibiotic resistant bacteria are shed into communities resulting in water borne diseases such as cholera, typhoid fever, dysentery and gastroenteritis. Hence along with the benefits of antimicrobial therapy a number of hazards have emerged. The widespread use of antibiotics for human therapy has promoted the emergence and maintenance of multiple antibiotic resistant bacteria. It also resulted in change in ecology of bacterial of bacterial infections and in the types of nosocomial infections.

Thus keeping above in view the study entitled 'Prevalence of antibiotic resistant pathogenic bacteria in Hospital waste water of Allahabad region' was conducted with the following

Objectives

- To isolate and identify the pathogenic bacteria from hospital waste water.
- To evaluate the antibiotic susceptibility pattern of bacterial isolates.

Materials and Method

Place of Work

The present study was conducted in department of microbiology and fermentation technology, Jacob School of Biotechnology and Bioengineering, SHIATS, Allahabad during the period of January to June.

Collection of Samples

80 samples of hospital waste water (from different sites viz.,

burn unit, dressing room, pathology, patient ward, Operation Theater, dental unit and common sewage outlet) and samples of waste water effluent from medical waste treatment plant were collected from Allahabad region:

- Swaroop Rani Hospital
- Nazareth hospital
- Jeevan jyoti hospital
- Hayes memorial hospital
- Parvathi Nursing Home
- Medical waste treatment plant

15ml of hospital waste water sample was collected in sterile sample bottles with the help of sterile syringe from the outermost chamber before discharging into the sewerage system. The collected samples were transported aseptically to the laboratory for investigation. Ten waste water samples each were collected from dressing room, burn unit, pathology, patient ward, Operation Theater, dental unit and common sewage outlet.

Isolation of Bacterial pathogens

Each sample was also inoculated on different selective media namely MacConkey's agar, Mannitol agar, Cetrimide agar, SS agar and incubated at 37°C for 24 hours. The isolated colonies were purified on nutrient agar slants.

Identification of Bacterial pathogens

The colonies were identified on the basis of colony characteristics, morphological characteristics and biochemical tests as given in Bergey's Manual of Systemic Bacteriology (Holt *et al.*, 1984) [5]. Following biochemical tests were performed:

- Catalyse test
- Nitrate reduction
- Oxidase test
- Motility test

Result and Discussion

Table 1a: Cultural and Morphological Characteristics of pathogenic bacteria isolated from hospital waste water

Name of organism	Cultural and Morphological characteristics	Growth on selective Media
<i>Pseudomonas aeruginosa</i>	Gram -ve, Rod shaped, Strictly Aerobic and Motile	Colonies are large, translucent, low Convex with bluish-green pigment Which is diffusible
<i>Escherichia Coli</i>	Gram- ve, Rod shaped, Aerobic and Facultatively Anaerobic, Motile	Pink color, Pin point colonies on MacConkey's agar medium, Colonies are circular, moist, smooth with entire margin and Non- mucoid
<i>Klebsiella pneumonia</i>	Gram- ve, Rod shaped, Aerobic and Facultatively Anaerobic, Non-motile	Pink, mucoid colonies on MacConkey's agar medium
<i>Enterobacter Sakazakii</i>	Gram- ve, Rod shaped, Motile	Light pink color colonies on MacConkey's media, colonies are Round with entire margin
<i>Enterobacter intermedius</i>	Gram- ve, Rod shaped, Motile	Light pink color colonies on MacConkey's media, colonies are Round with entire margin
<i>Yersinia pseudotuberculosis</i>	Gram- ve, Short rod, Aerobic and Facultatively Anaerobic, Non- motile	Small colorless colonies on MacConkey's agar
<i>Salmonella enteric sub. enteric</i>	Gram-ve, Rod shaped, and Facultative anaerobe, Motile	Black centered, colonies on SS a gar
<i>Salmonella enteric sub. arizoniae</i>	Gram-ve, Rod shaped, and Facultative anaerobe, Motile	Black centered, colonies on SS a gar
<i>Staphylococcus aureus</i>	Gram+ve, Aerobic & Facultative anaerobe, cocci In clusters, Non-motile	Yellow colony on Mannitol Salt Agar

+ve=Positive; -ve = Negative; # = Not performed

- Hydrogen sulphide production test
- Urease test
- Methyle Red Test
- Voges-Proskauer test
- Citrate Utilization test
- Indole Production
- Esculin Hydrolysis
- Starch Hydrolysis
- Malonate Utilization test
- Tartarate Utilization test
- Arginine hydrolysis
- Gelatin Liquifaction test
- Carbohydrate fermentation

Antibiotic Susceptibility Test

Antibiotic susceptibility testing was performed by the disk diffusion method according to Kirby-Bauer method (Bauer *et al.*, 1966) [2]. The nutrient broth inoculums suspension was swab inoculated on Nutrient agar plates and the antibiotic disks were placed on the plates were incubated at 37°C for 24-48 hours. Zone of inhibition around the antibiotic disks were recorded and the results were interpreted accordingly. Antibiotics for which the resistance was tested includes Ampicillin (10µg), Piperacillin (100µg), Nitrofurantoin (300µg), Ceftazidime (30µg), Aztreonam (30µg), Ciprofloxacin (5µg), Amikacin (30µg), Methicilin (5µg), Chloramphenicol (30µg), Vancomycin (30µg), Tetracyclin (30µg), Meropenem (10µg), Gentamicin (10µg) and Amoxicillin (30µg).

Statistical Analysis of Results

The data recorded during the course of investigation were subjected to statistical analysis using analysis of variance (ANOVA) one way classification and χ^2 - test (Panse and Sukhatme, 1967) [12].

Table 1 b: Biochemical characteristics of pathogenic bacteria isolated from hospital waste water

Biochemical tests	Name of organisms								
	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Enterobacter sakazakii</i>	<i>Enterobacter intermedius</i>	<i>Yersinia tuberculosis</i>	<i>Salmonella enteric sub. enteric</i>	<i>Salmonella enteric sub. arizonae</i>
Catalase Test	#	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve
NO ₃ Reduction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Motility	+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve
Urease Production	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Indole Production	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
MR Test	-ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
VP Test	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Citrate Utilization Test	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve
Triple sugar iron test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Pigmented Growth on NA	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
ONPG Test	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
Arginine Hydrolysis	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Lysine Hydrolysis	#	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Malonate utilization Test	#	#	-ve	+ve	-ve	+ve	-ve	+ve	+ve
Tartarate Utilization Test	#	#	+ve	-ve	-ve	+ve	-ve	+ve	-ve
Esculin Hydrolysis Test	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve
Gelatin Liquifaction	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Starch Hydrdrolsis Test	-ve	-ve	#	#	#	#	#	#	#
Growth a 4°C and 41°C	+ve at both the temp.	#	#	#	#	#	#	#	#
Non- flurescent Diffusible Pigment	+ve	#	#	#	#	#	#	#	#
Oxidase test	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

+ve= Positive, -ve = Negative; # = Not performed

Table 1c: Carbohydrate fermentation test of pathogenic bacteria isolated from hospital waste water

Carbohydrate	Organisms								
	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Enterobacter sakazakii</i>	<i>Enterobacter intermedius</i>	<i>Yersinia pseudotuberculosis</i>	<i>Salmonella enterica sub. enteric</i>	<i>Salmonella enterica sub. arizoniae</i>
Sucrose	A-G-	A+G+	A+G+	A+G+	A+G+	A-G-	A-G-	A-G-	A-G-
Lactose	A-G-	A+G-	A+G+	A+G+	A+G+	A-G-	A-G-	A-G-	A-G-
Mannitol	A+G-	A+G-	A+G+	A+G+	A+G+	A+G+	A-G-	A+G+	A+G-
Arabinose	A-G-	A-G-	A+G+	A+G+	A+G+	A+G+	A-G-	A+G+	A+G+
Fructose	A-G-	A+G-	A+G+	A+G+	A+G+	A+G+	A+G-	A+G+	A+G+
Mannose	A-G-	A-G-	A+G+	A+G+	A+G+	A+G+	A-G-	A+G+	A+G+
Sorbitol	A-G-	A-G-	A+G+	A+G+	A-G-	A+G+	A-G-	A+G+	A+G+
Inositol	A-G-	A-G-	A-G-	A+G+	A+G+	A+G+	A-G-	A+G+	A+G+
Trehalose	A-G-	A+G+	A+G+	A+G+	A+G+	A+G+	A-G-	A+G+	A+G+
Xylose	A+G-	A-G-	A+G-	A+G	A+G+	A+G+	A+G-	A+G+	A+G+
Cellobiose	#	A-G-	A-G-	A+G+	A+G+	A+G+	A+G-	A+G-	A+G-
Dulcitol	#	A-G-	A+G+	A+G+	A-G-	A-G-	A-G-	A+G-	A-G-
Glycerol	#	A-G-	A+G+	A+G+	A+G-	A+G+	A+G-	A+G+	A+G+
Maltose	#	A+G-	A+G+	A+G+	A+G+	A+G+	A+G-	A+G+	A+G+
Raffinose	#	A-G-	A-G-	A+G+	A+G+	A+G+	A-G-	A-G-	A-G-
Rhamnose	#	A-G-	A+G-	A+G+	A+G+	A+G+	A+G-	A+G+	A+G-
Salicilin	#	A+G-	A+G+	A+G+	A+G+	A+G+	A+G-	A+G-	A-G-
Glucose	A+G-	A+G-	A+G+	A+G+	A+G+	A+G+	A+G-	A+G+	A+G-

A+G+=Acid positive, gas positive; A+G- = Gas negative; A-G- = Acid negative, Gas negative, # = Not tested

Percentage incidence of gram- negative pathogenic bacteria is much higher (96.59%) in comparison to that of gram-positive pathogens which is only 4.41%, present findings were in agreement with several other studies where the higher incidence rate of gram- ve pathogens were also reported, in comparison to the presence of gram- positive

bacteria (Elmanana *et al.*, 2006; Yang *et al.*, 2009). The overall distribution of isolated bacteria in the whole study showed the dominance of gram –ve rods (70%), followed by the gram- positive rods (27%), gram- positive cocci (2%) and gram –ve cocci (1%). Higher isolation rate of Gram-negative bacteria were reported by several other authors.

Table 2: Percentage incidence of pathogenic bacteria isolated from hospital associated waste water $\chi^2_{(Cal)} = 284.4 > \chi^2_{(tab)}$ at 5% = 15.51 S

Total samples	Total isolates	Organisms								
		GRAM –ve (95.59%)								GRAM +ve (4.41%)
		<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Enterobacter sakazakii</i>	<i>Enterobacter intermedius</i>	<i>Yersinia pseudotuberculosis</i>	<i>Salmonella enteric sub. erizonae</i>	<i>Salmonella enteric sub. enterica</i>	<i>Staphylococcus aureus</i>
80	453	155 (34.21%)	72 (15.89%)	37 (8.16%)	22 (4.85%)	34 (7.50%)	27 (5.96%)	49 (10.81%)	37 (8.16%)	20 (4.41%)

$\chi^2_{(Cal)} = 284.4 > \chi^2_{(tab)}$ at 5% = 15.51 S = Significant; -ve = negative; +ve = positive

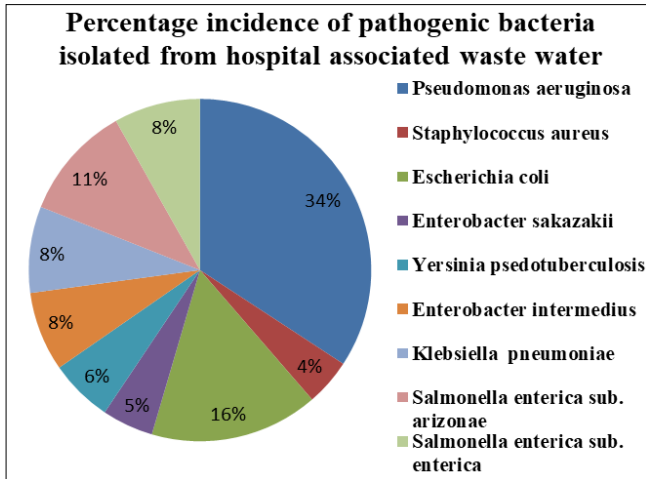


Fig 1: Percentage incidence of pathogenic bacteria isolated from hospital associated waste water

Antibiotic Susceptibility test of bacterial pathogens isolated from hospital waste water

The pathogens isolated from hospital waste water samples from different sites were tested for their sensitivity towards various antibiotics. Even when the heterotrophic plate count of bacteria in the hospital waste water is low, the biological risk of bacteria actually present in the effluent must be high. The risk is mainly associated with the presence of bacteria resistant to antibiotics.

Out of nine isolates eight were MDR bacteria showing resistance towards four or more than four antibiotics except *Staphylococcus aureus*. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed resistance towards most number of antibiotics (eight). Simultaneous resistance to Aztreonam, Ampicillin, Piperacillin, Ceftazidime, Amoxicillin, Tetracyclin formed the common MDR pattern. The findings of present study are similar to the findings of

Chitnis *et al.* (2000)^[3], where almost the same MDR pattern was reported for the hospital effluent isolates. Toroglu *et al.* (2005)^[17] also reported the high MDR rate in the bacterial isolates of Asku River (Kahramanmaras), Turkey. In other studies conducted by several authors MDR bacteria were isolated from hospital waste water samples and urban waste water samples. Multiple drug resistance can spread through transfer of resistance plasmids (R-Plasmids) (Watanabe, 1963), and such transfer has been shown to occur in extra intestinal environment like the sewage system (Anderson, 1975)^[1]. Moreover antimicrobial agents used in medicine are mainly released into the aquatic environment via waste water (Heberer, 2002), therefore the prevalence of MDR bacteria in hospital waste water might be due to transfer of R- Plasmids among bacteria in the waste water, which resulted in enriching the MDR strains by antimicrobial selection pressure. Relatively high level of antibiotic resistance is a reflection of misuse or abuse of these drugs (Lateef, 2003)^[8]. Antibiotic prescriptions in hospitals are given without clear evidence of infection or adequate medical indication. Broad- spectrum antibiotics are sometimes given in place of narrow- spectrum antibiotics as substitute for culture and sensitivity testing, with the consequent risk of dangerous side effects, super infections and selection to drug resistant mutants (Prescott *et al.*, 1999). In developing countries, such as ours drugs are available to public and people may practice self-administration of antibiotics and further increase the prevalence of drug resistant strains (Lateef, 2003)^[8].

Staphylococcus aureus was found to be most susceptible organism while *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* was most resistant.

Among all the antibiotics aztreonam, ceftazidime and amoxicillin were found to be the least effective while meropenem, ciprofloxacin were most effective followed by gentamicin.

Table 2: antibiotic susceptibility pattern of bacterial pathogens isolated from hospital waste water

Organisms	Antibiotics (inµg)													
	Nf (300)	Ao (30)	A (10)	Cf (5)	G (10)	Ak (30)	C (30)	Pc (100)	Ca (30)	Ac (10)	TE (30)	MR (10)	M (5)	Va (30)
<i>Enterobacter sakazakii</i>	R	R	IR	S	S	S	S	S	R	S	R	S	-	-
<i>Salmonella enterica sub. enteric</i>	S	R	R	S	S	S	S	R	IR	R	R	S	-	-
<i>Klebsiella pneumoniae</i>	R	R	R	S	R	S	S	R	R	R	R	S	-	-
<i>Escherichia coli</i>	IR	R	S	S	S	S	S	R	R	R	IR	S	-	-
<i>Enterobacter intermedium</i>	S	R	R	S	S	S	S	R	R	R	IR	S	-	-
<i>Yersinia pseudotuberculosis</i>	S	R	R	S	S	S	S	R	R	R	IR	S	-	-
<i>Salmonella enterica sub. arizoniae</i>	S	R	R	S	S	R	IR	R	R	R	R	S	-	-
<i>Pseudomonas aeruginosa</i> R	R	R	R	S	S	S	R	R	R	R	R	S	-	-
<i>Staphylococcus aureus</i>	-	-	S	S	S	S	IR	S	R	S	S	S	R	S

R = Resistant; S = Susceptibility; IR = intermediately resistant; - = not tested

Conclusion

Waste water samples were collected from different sites of different hospitals and analyzed for their HPC (Heterotrophic plate count) and all the samples were also screened for the presence of pathogenic bacteria viz. *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* spp., *Enterobacter* spp., *Klebsiella pneumoniae* and *Staphylococcus aureus*. The organisms were identified on the basis of cultural and biochemical characteristics.

The pathogens isolated were tested for their susceptibility towards commonly prescribed antibiotics viz. Ciprofloxacin, Gentamicin, Amikacin, Choloramphenicol, Piperacillin,

Amoxicillin, Meropenem, Ceftazidime, Tetracyclin, Nitrofurantoin, Aztreonam, Ampicillin, Methicillin and Vancomycin.

From the present study the following observations were made:

1. Highest mean HPC (1004.4 cfu/ml) was observed for the samples taken from medical waste treatment plant and lowest mean HPC (30.8 cfu/ml) was observed for the samples of burn unit.
2. Incidence of *Pseudomonas aeruginosa* in hospital associated waste water is highest (34.21%), followed by *Escherichia coli* (15.89%), *Salmonella enteric* sub.

- arizonae* (10.81%), *Klebsiella pneumoniae* (8.16%), *Salmonella enterica* sub *enteric* (8.16%), *Enterobacter intermedius* (7.50%), *Yersinia pseudotuberculosis* (5.96%), *Enterobacter sakazakii* (4.85%) and *Staphylococcus aureus* (4.41%).
- Percentage incidence of gram-positive pathogens were very less (4.41%) in comparison to gram-negative pathogens (95.59%).
 - Meropenem (10µg) and ciprofloxacin (5µg) were most effective antibiotics while Aztreonam (30µg) and ceftazidime (30µg) were least effective antibiotics.
 - Staphylococcus aureus* was most susceptible pathogen among all the pathogens isolated while *klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the most resistant pathogens.

Most of the isolates showed resistance towards a number of antibiotics, suggesting that there has been misuse of these drugs. The high resistance rate also indicates the negative impact of antibiotic therapy. High isolation rate and HPC count suggests that untreated liquid hospital waste could result in dissemination route of multi resistant bacteria and consequently their genes of resistance in environment. Therefore in view of this, strict quality control measures should be implemented to ensure proper treatment of hospital generated waste. This will ensure the discharge of properly treated water to the water bodies which will prevent the spread of water borne diseases. To evaluate the extent of transmission and impact of such transmission on the effectiveness of the antibacterial use in human medicine further study is imperative. Periodic monitoring of antibiotic resistance pattern to detect any change in it would be necessary for effective treatment against these pathogens.

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