RESEARCH ARTICLE Am. J. PharmTech Res. 2015; 5(1) ISSN: 2249-3387

AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Distribution and Limits of Drug Resistance in Gram Negative Bacteria from Hospital Wastewater

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ABSTRACT

The distribution of resistance to ampicillin, penicillin, erythromycin, tetracycline, and amoxicillin among Gram negative bacteria in the hospital wastewater samples was investigated. The emergence of resistance among the Gram negative bacterial population varied considerably in different drug and water sampling sites. Gram negative bacteria showed lower drug resistant viable count ($1.23x10^4$-$7.0x10^2$) in site-II (receiving treated wastewater), as compared to more polluted site-I ($1.28x10^4$-$5.0x10^2$), III ($2.22x10^4$-$5.0x10^2$) and site-IV (1.63 $x10^4$-2.0 $x10^2$) respectively. Viable counts of Gram negative bacterial population were recorded higher against erythromycin from sampling site-III. Lower viable counts of Gram negative bacteria were recorded against tetracycline in site-I and IV. Percentage MIC of antibiotic among the isolates observed higher in tetracycline and erythromycin than the other antibiotics. Isolates of Gram negative bacteria showed their tolerance level (MIC) in the range of 2.5-640μg/ml against the antibiotics. Maximum number of isolates exhibited their MICs at lower concentration range 2.5-5μg/ml against tetracycline. A few number of isolates also showed their MIC at lower concentration of other antibiotics tested from all the sampling sites.

Keywords: Hospital Wastewater, Viable Count, Antibiotics, Gram negative Bacteria, Minimum inhibitory concentration

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Received 10 January 2015, Accepted 23 January 2015

Please cite this article as: Imran M *et al.*, Distribution and Limits of Drug Resistance in Gram Negative Bacteria from Hospital Wastewater. American Journal of PharmTech Research 2015.

INTRODUCTION

Hospital wastewater can be hazardous to public health and ecological balance since it can contain many kinds of pollutants such as radioactive, chemical and pharmaceutical wastes and also pathogenic microorganisms[1]. Hospital environments are a great source of potentially pathogenic microorganisms[2]. Several bacteria are associated to nosocomial infections, mainly representatives of Gram negative rods from the *Enterobacteriaceae* family (GNR), non fermenting Gram negative rods, NFGNR Gram positive cocci *Staphylococcus*, especially coagulase-negative species, CNS and *Enterococus*, ENT[3]. Uncontrolled and excessive use of antibiotics by human and animals results an increase in antibiotic resistance and cause the spread of resistance genes in environmental samples such as hospital waste water[4]. Studies have demonstrated that hospital wastewater is highly selective environments and that they contribute to the high rates of resistant bacteria that are being discharged in the natural environment[5]. Waste effluent from hospitals contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria[6, 7]. As a result, hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria[8].

Hospital effluent with its high content of multidrug resistant enterobacteria and the presence of enteric pathogens could pose a grave problem for the community[9, 10]. The occurrence of strongly selective environments for antimicrobials, such as hospitals, promotes, not only the growth of resistant bacteria, but also leads to an increase in the frequency of resistance bacterial genes and genetic elements such as plasmids. Antibiotic resistance among bacteria is a worldwide problem. The emergence of bacteria resistant to most of the commonly used antibiotics/drugs is of considerable medical significance[11, 12] because of the public health implications[13]. Industrialization and man's activities have partially or totally turned our environment to dumping sites for waste materials. As a result, many water resources have been rendered unwholesome and hazardous to man and other living systems[14, 15]. The aim of our study was to investigate the drug resistant Gram negative bacterial contamination in the hospital wastewater to mitigate the public health risks.

MATERIALS AND METHOD

Sample collection

Water samples were collected from three sites of hospital wastewater along with King George's Medical University Site (I), Sanjay Gandhi Post Graduate Institute of Medical Sciences (treated) Site (II), Sanjay Gandhi Post Graduate Institute of Medical Sciences (untreated) Site (III) and Dr.

Ram Manohar Lohia Hospital Site (IV) at Lucknow city as shown in **Figure 1**. Samples were collected in sterile 250-ml polypropylene bottles, according to internationally recommended methodology[16]. Samples were kept at 4ºC until their arrival to laboratory.

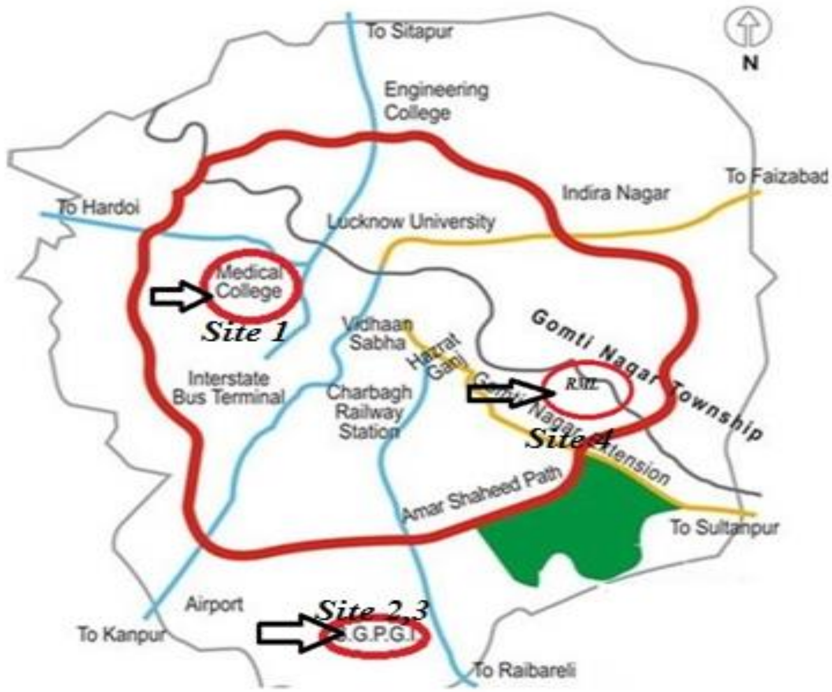

Figure 1: Map showing sampling sites

Isolation of antibiotic tolerant Gram negative bacterial population

Isolation of antibiotic resistant Gram negative bacteria from water samples were done on antibiotic amended Mac conky agar plates at varying concentration (10-160µg/ml). Serial dilutions of the water samples were plated by spreading 0.1 ml on medium for the count of total antibiotic resistant Gram negative bacteria. Plates incubated at 37ºC for 24 hours and Gram negative bacterial counts were expressed as CFU/ml. On Mac conky agar medium.

Determination of minimum inhibitory concentration (MIC) of antibiotics among Gram negative bacterial isolates

The MIC of five different drugs (Penicillin, Erythromycin, Tetracycline, Amoxicillin, and Ampicillin) was determined by the plate dilution method Plate dilution method means the MIC was determined in nutrient agar plates not in broth amended with different concentrations or dilutions of the drugs as adopted by Rennie[17]. The antibiotics were used in varying concentrations ranging from 5 to 640 µg/ml and supplemented individually in nutrient agar, which were then spot inoculated with approximately 3x106 microbial cells with the help of platinum loop of 5mm diameter. The plates were incubated at 37º C for 24 hr. lowest concentration of the antibiotic which inhibits the growth of the microorganisms was considered as MIC.

RESULTS AND DISCUSSION

In this study, antibiotic resistant population of Gram negative bacteria from the sampling sites of hospital waste water was observed against five antibiotics (Erythromycin, Amoxicillin, Ampicillin, Tetracycline and Penicillin) at their varying concentrations (10, 20, 40, 80 and 160 µg/ml). Viable (CFU/ml) count of Gram negative bacteria was observed higher in control plate (non antibiotic supplemented) than antibiotic supplemented plates in all the sites tested.

Table 1: Viable count of antibiotic tolerant Gram negative bacteria from different water sampling sites

Antibiotics	***Conc.*(µg/ml)**	**Site 1**	**Site 2**	**Site 3**	**Site 4**
Control	No antibiotics	$12.5\times10^5\pm1.09$	$13.00\times10^5\pm1.01$	$13.43\times10^5\pm0.95$	$8.13\times10^4\pm0.54$
Erythromycin	10	$7.1\times10^3\pm1.12$	$6.3\times10^3\pm1.0$	$1.35\times10^4\pm0.01$	$1.07\times10^4\pm0.01$
	20	$6.2\times10^3\pm0.95$	$4.9\times10^3\pm0.87$	$1.04\times10^4\pm0.02$	$8.2\times10^3\pm0.59$
	40	$4.6\times10^3\pm0.32$	$4.2\times10^3\pm0.57$	$6.3\times10^3\pm0.52$	$6.8\times10^3\pm0.69$
	80	$1.2\times10^3\pm0.10$	$1.1\times10^3\pm0.12$	$1.9\times10^3\pm0.05$	$4.3\times10^3\pm0.09$
	160	$5.0\times10^2\pm0.49$	$7.0\times10^2\pm0.52$	$5.0\times10^2\pm0.32$	$3.1\times10^3\pm0.12$
Tetracycline	10	$2.9\times10^3\pm0.09$	$5.5\times10^3\pm0.64$	$6.7\times10^3\pm0.67$	$3.2\times10^3\pm0.03$
	20	$2.2\times10^3\pm0.02$	$3.2\times10^3\pm0.12$	$4.2\times10^3\pm0.32$	$1.9\times10^3\pm0.01$
	40	$1.8\times10^3\pm0.18$	$2.6\times10^3\pm0.09$	$3.6\times10^3\pm0.15$	$5.0\times10^2\pm0.59$
	80	$1.1\times10^3\pm0.09$	$1.8\times10^3\pm0.025$	$1.4\times10^3\pm0.07$	ND
	160	$4.0\times10^2\pm0.45$	$1.4\times10^3\pm0.19$	ND	ND
Penicillin	10	$1.28\times10^4\pm0.01$	$6.7\times10^3\pm0.92$	$2.03\times10^4\pm0.02$	$1.63\times10^4\pm0.06$
	20	$1.13\times10^4\pm0.5$	$4.4\times10^3\pm0.32$	$1.63\times10^4\pm0.112$	$1.15\times10^4\pm0.01$
	40	$9.4\times10^3\pm0.75$	$3.8\times10^3\pm0.12$	$1.11\times10^4\pm0.09$	$4.7\times10^3\pm0.012$
	80	$6.5\times10^3\pm0.55$	$3.2\times10^3\pm0.09$	$7.8\times10^3\pm0.65$	$1.8\times10^3\pm0.014$
	160	$3.2\times10^3\pm0.45$	$2.7\times10^3\pm0.08$	$2.7\times10^3\pm0.19$	$1.2\times10^3\pm0.01$
Ampicillin	10	$1.03\times10^4\pm0.01$	$1.23\times10^4\pm0.12$	$2.22\times10^4\pm0.01$	$8.3\times10^3\pm0.65$
	20	$9.6\times10^3\pm0.72$	$1.12\times10^4\pm0.14$	$2.10\times10^4\pm0.05$	$5.3\times10^3\pm0.32$
	40	$8.2\times10^3\pm0.65$	$6.3\times10^3\pm0.54$	$1.16\times10^4\pm0.04$	$2.2\times10^3\pm0.01$
	80	$6.3\times10^3\pm0.12$	$2.5\times10^3\pm0.18$	$6.1\times10^3\pm0.92$	$7.0\times10^2\pm0.61$
	160	$2.4\times10^3\pm0.45$	$9.0\times10^2\pm1.32$	$3.3\times10^3\pm0.42$	$2.0\times10^2\pm0.08$
Amoxicillin	10	$6.8\times10^3\pm1.12$	$4.3\times10^3\pm0.92$	$1.19\times10^4\pm0.01$	$4.9\times10^3\pm0.79$
	20	$6.1\times10^3\pm0.85$	$2.9\times10^3\pm0.05$	$6.8\times10^3\pm0.05$	$2.9\times10^3\pm0.11$
	40	$4.7\times10^3\pm0.34$	$2.5\times10^3\pm0.02$	$4.7\times10^3\pm0.34$	$1.7\times10^3\pm0.10$
	80	$3.6\times10^3\pm0.11$	$1.7\times10^3\pm0.11$	$4.0\times10^3\pm0.39$	$1.3\times10^3\pm0.19$
	160	$2.1\times10^3\pm0.09$	$1.1\times10^3\pm0.08$	$1.2\times10^3\pm0.02$	$4.0\times10^2\pm0.32$

The data are expressed in mean ± SEM). The comparisons were made by ANOVA followed by Dennett's test. $P <$
0.05 significant, $P < 0.01$ very significant, $P < 0.001$ extreme.

The viable count of Gram negative bacteria in different concentrations of antibiotics ranged from ($1.28x10^4$-$5.0x10^2$), ($1.23x10^4$-$7.0x10^2$), ($2.22x10^4$-$5.0x10^2$) and ($1.63\ x10^4$-$2.0\ x10^2$) cfu/ml of water in site I, II, III and IV respectively. Maximum viable count was observed against penicillin and ampicillin in sampling site I, IV and site II, III at 10µg/ml respectively. Minimum viable count was observed against in sites I, III and IV at10 µg/ml against tetracycline. Similar trend was observed at MIC range 20-40 µg/ml against penicillin and ampicillin in all sampling sites tested. Maximum number of viable count was recorded against penicillin at 80 µg/ml in sampling site I, II, III while similar trend was observed at 80-160- µg/ml against erythromycin in sampling site IV. The resistance order in the Gram negative bacterial population at different concentration of the antibiotics were also observed (Table 2). The 200 Gram-negative bacterial isolates were also tested for their MIC (minimum inhibitory concentration) against five antibiotics under test. This test was run at varying concentration (5-640 µg/ml) of the antibiotics.

Table 2: Resistance order in the Gram negative bacterial population at different concentration of the antibiotics.

Conc.(µg/ml)	Resistance order			
	Site-1	Site-2	Site-3	Site-4
10	pen,amp,ery,amx,tet	amp,pen,ery,tet,amx	amp,pen,ery,amx,tet	pen,ery,amp,amx,tet
20	pen,amp,ery,amx,tet	amp,ery,pen,tet,amx	amp,pen,ery,amx,tet	pen,ery,amp,amx,tet
40	pen,amp,amx,ery,tet	amp,ery,pen,amx,tet	amp,pen,ery,amx,tet	pen,ery,amp,amx,tet
80	pen,amp,amx,ery,tet	pen,amp,tet,amx,ery	pen,amp,amx,ery,tet	ery,pen,amx,amp,tet
160	pen,amp,amx,ery,tet	pen,tet,amx,amp,ery	amp,pen,amx,ery,tet	ery,peni,amx,peni,tet

Pen=penicillin, amp=ampicillin, ery=erythromycin, amx=amoxicillin, tet=tetracycline

The tetracycline showed most toxicity against all the isolates from the entire sites tested. In site I, 58% of the total isolates showed their MIC range 2.5-5µg/ml for tetracycline followed by 32%, 16%, 10% and 2% ampicillin, amoxicillin, penicillin and erythromycin respectively. Maximum 46% of the isolates showed their MIC range 320-640 µg/ml against ampicillin while 30% of the isolates demonstrated similar MIC range against both amoxicillin and penicillin. No MIC range was recorded at lower concentration against tetracycline and penicillin while at higher concentration against erythromycin (Figure 2).

In site II maximum 24%, 12% and 10% isolates showed their MIC range 2.5-5 µg/ml against erythromycin, tetracycline, ampicillin and amoxicillin respectively. 74% of the isolates showed their MIC 5-10 µg/ml against tetracycline. 76%, 26%, 22% and 16% of the isolates demonstrated their MIC 320-640 µg/ml against penicillin, ampicillin, amoxicillin and erythromycin respectively. The MIC was not detected at 2.5-20, 5-10 and 160-320 µg/ml against penicillin, ampicillin, amoxicillin and erythromycin respectively (Figure 3). In site III, 76% of the isolates showed their

MIC 2.5-5 µg/ml against penicillin followed by 72%, 70%, and 66% against amoxicillin and erythromycin, tetracycline and ampicillin respectively. Maximum 18%, 16%, and 10%, of the isolates showed their higher MIC (320-640 µg/ml) against penicillin, ampicillin and amoxicillin respectively. No MIC was recorded at varying concentration range of the antibiotic tested (Figure 4). In case of site IV no MIC range was observed at 2.5-5µg/ml against penicillin, erythromycin, ampicillin and amoxicillin respectively. However 62% of the isolates showed their MIC at lower concentration (5-10 µg/ml) against tetracycline. Maximum 38%, 32%, 22% and 18% of the isolates showed their MIC at 320-640 µg/ml against ampicillin, amoxicillin, penicillin and erythromycin respectively. No MIC was observed at 5-20 µg/ml against all the isolates tested except tetracycline. All the antibiotics (penicillin, ampicillin, amoxicillin and erythromycin) showed their MIC at higher concentration (80-640 µg/ml) against all the antibiotic tested (Figure 5).

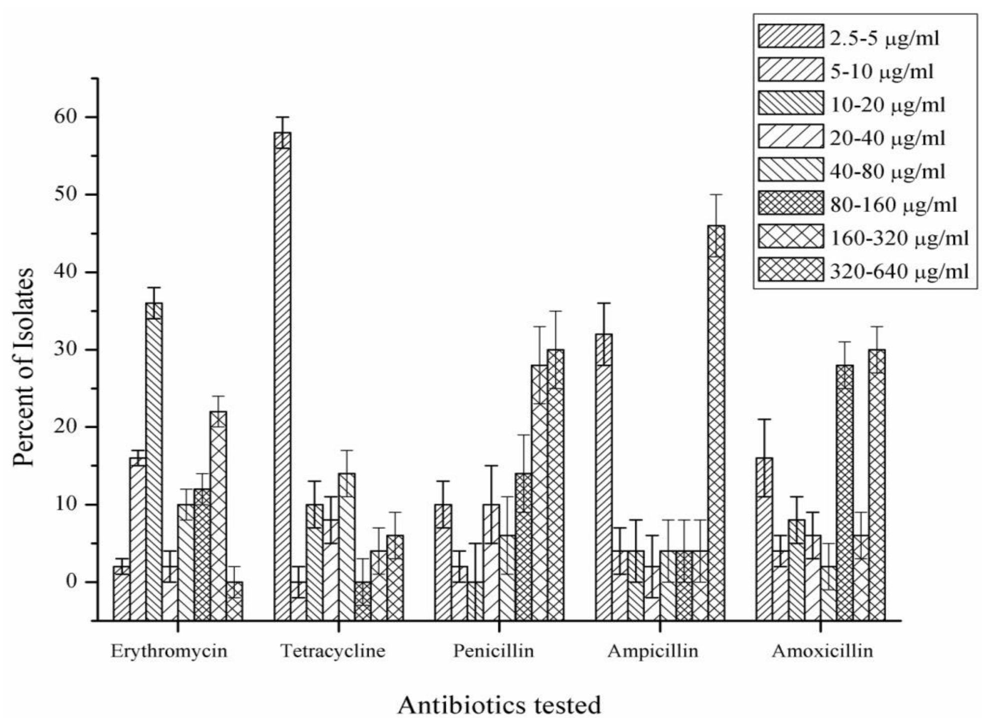

Figure 2: Gram negative isolates showing various ranges of MIC of antibiotics from site 1

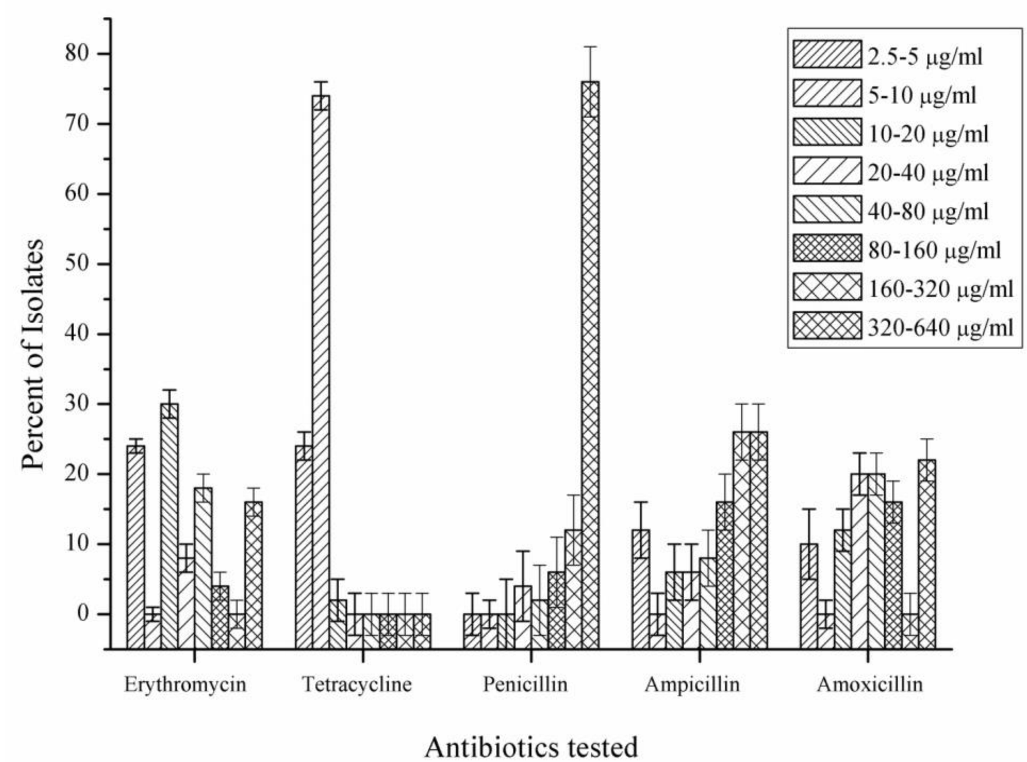

Figure 3: Gram negative isolates showing various ranges of MIC of antibiotics from site 3

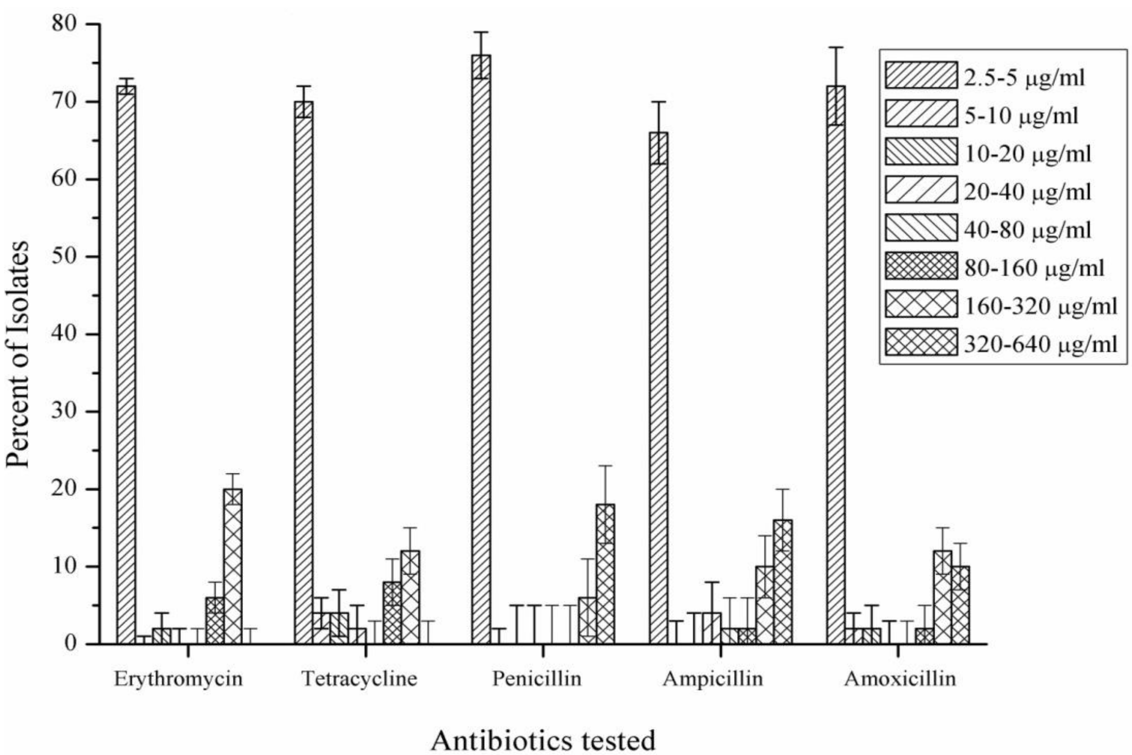

Figure 4 Gram negative isolates showing various ranges of MIC of antibiotics from site 4

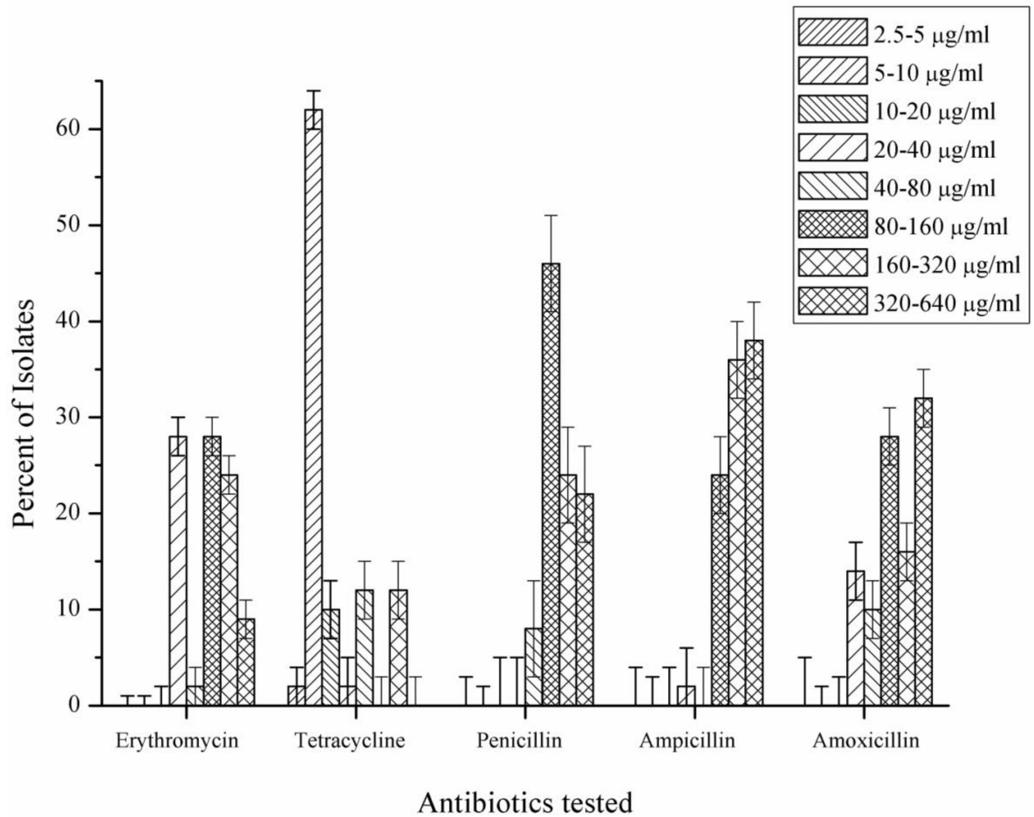

Figure 5: Gram negative isolates showing various ranges of MIC of antibiotics from site 4

Hospital surfaces are contaminated by factors inherent to the presence of patients, such as biological fluids, sometimes associated to assistance techniques and hygiene. Another contamination factor would be the circulation of vectors as carrier agents for fungi and bacteria resistant to antimicrobials. Authors have reported that factor other than the indiscriminate use of antibiotics in human medicine, animal husbandry, and agriculture may disrupt the microbial balance in favour of resistant bacteria[18-20]. Hospital environment have higher Pathogens than the non-hospital environment[21, 20]. In our study a varying trend of antibiotic tolerant Gram negative bacterial population in the hospital wastewater was observed. We recorded a varying viable count of multidrug tolerant Gram negative bacteria in four different sampling sites .Viable count of Gram negative bacteria was insignificantly higher in non antibiotic supplemented control plate than antibiotic supplemented (at lower concentration)plates in all the site. A decrease in viable count was recorded with the increase of antibiotic concentrations tested in all I, II, III and IV sampling sites. The viable count of Gram negative bacteria in different concentrations (10-160 µg/ml) of antibiotics ranged from ($1.28x10^4$-$5.0x10^2$), ($1.23x10^4$-$7.0x10^2$), ($2.22x10^4$-$5.0x10^2$) and (1.63 $x10^4$-2.0 $x10^2$) cfu/ml of water in site I, II, III and IV respectively. The viable count from sampling site-II receiving water from treatment plant showed lower count as compared to site-I, II and IV similar finding are also reported by many people reports[22-24]. They also reported that *E. coli* counts as well as coliform counts were generally high, especially in stagnant, dirty stream water and samples collected from gutters or drainage where microbial counts were too numerous to count from treated wastewater. Hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria[8]. After application, many drugs are excreted non-metabolized by the patients and enter into wastewater. Ekhaise[25] observed a high resistance to ampicillin, tetracycline and chloramphenicol in gram negative bacteria particularly klebsiella, pseudomonas and serratia. They also reported multi-drug resistant isolates more in hospital wastewater than urban wastewater. Reinthaler[23] observed the highest resistance rate in E. coli isolates to reported that the resistance of *E.coli* isolates to tetracycline, amoxicillin, ciprofloxacin and chloramphenicol isolated from sewage treatment plant which treats not only municipal sewage but also sewage from hospitals wastewater and also reported that the resistance of *E. coli* isolates varied according to the site from which the bacteria were isolated. Their findings are also similar to our study that distribution of drug resistance is dependent upon the nature of the wastewater receiving the waste materials containing drugs which are particularly high in hospital wastewater. Our findings are also closely related to other reports[26-28]. In our study we also determined tolerance level of antibiotic among the Gram negative isolates against tetracycline, penicillin, amoxicillin, ampicillin and erythromycin. All the isolates exhibited their MIC in between the 5-640µg/ml against all antibiotics tested. Maximum number of isolates showed their MIC at lower range 5-10 µg/ml against tetracycline while no MIC level was recorded in the range of 10-20 µg/ml against penicillin and maximum no of isolates showed their MIC range of 320-640 µg/ml . Higher number of isolates showed their MIC in the range of 10-20 µg/ml against erythromycin. Most of the isolates exhibited their MIC rang of 320-640 µg/ml against amoxicillin and ampicillin respectively. Similar trends of the drug MIC levels in the members of Enterobacteriaceae have been reported by many workers[29, 30]. The MIC levels revealed that maximum no of the isolates were negatively affected with antibiotics (Penicillin, Ampicillin and Amoxicillin) at lower concentration i.e. the action of such antibiotics on them was merely bacteriostatic and not bactericidal. This finding was also documented by other workers[31, 32]. Ibrahim[33] reported that the Gentamycin was the most active antibiotic against the Gram negative bacilli isolates tested, as only 2.6% of these isolates were resistant to this antibiotic at concentration up to 10µg, while 2.1% showed resistance at concentrations up to 256µg. The least active antibiotic tested was sulphamethoxazole, as 82.9% of the isolates were resistant to 25µg of this antibiotic, while 62.8% were resistant to 1024µ. The second less active antibiotic was ampicillin. Similar trend of drug MICs have been reported in gram negative bacteria by in many other studies[34-36]. Significant rise in drug resistant bacterial contamination exhibited by gram negative bacteria including pollution indicator organisms is a risk to public health, particularly due to the emergence of resistance and microbial diversity in the hospital wastewater. This study may be relevant and useful for the safety of the aquatic environment and human health. In this study, the distribution of resistance to antimicrobial drugs among Gram negative bacteria in hospital wastewater was investigated without differentiating transferable and non transferable resistance. Although, surfaces are not directly connected to transmission in most hospital infections, the impact of hygiene and cleaning procedures in microbial control is evident. It is suggested that microorganisms associated to hospital infections are able to survive during large periods of time, thus being a continuous source of contamination in cases where population control is not efficiently conducted[37, 38].

CONCLUSION

Microbial resistance to antimicrobials has been frequently associated to indiscriminate use of antibiotics. The results show that interventions to reduce excessive antibiotic prescribing to hospital in patients can reduce antimicrobial resistance or hospital-acquired infections, and interventions to increase effective prescribing can improve clinical outcome. This update provides more evidence about unintended clinical consequences of interventions and about the effect of interventions to reduce exposure of patients to antibiotics.

ACKNOWLEDGEMENT

The authors express gratefulness to Syed Waseem Akhtar Vice - Chancellor of Integral University, Lucknow for providing the facilities to execute the research work and thankful to the Heads of the departments of Biosciences and Bioengineering respectfully.

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