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ORIGINAL ARTICLE

Antibiotic Resistant Bacteria in Urban Hospital Wastewater : A Preliminary Report

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Water, one of the most available natural bacterial habitat, can act as a major reservoir of antibiotic resistant bacteria (ARB). The role of waste water in the development and dissemination of antibiotic resistance is a major concern nowadays. Among the different types of urban waste water, hospital effluent is a primary source of antibiotic residues and antibiotic resistant pathogenic bacteria released from patient excreta. Thus hospital waste water can serve as a hotspot for generation of new resistant population both through vertical and horizontal gene transfer. Lack of proper management of hospital effluent can lead to dissemination of new ARB and associated antibiotic resistance genes to the environmental water sources. Here we have assessed the prevalence of antibiotic resistance among bacterial isolates, mainly fecal coliforms, from a hospital wastewater of Kolkata. Majority of the isolates were resistant to more than one antibiotic. Of them five isolates were found to be resistant to ciprofloxacin, ofloxacin, tetracycline, cefepime, cefotaxime and amoxicillin. Four of them were found to be plasmid bearing. Our study established the presence of plasmid bearing MDR strains in hospital effluent which may facilitate horizontal gene transfer. As traditional waste water treatment plants are not designed for the removal of ARB. So ARBs are entering the aquatic environment which is a major point of concern and needs constant monitoring.

Key words: Antibiotic resistance, hospital effluent, MDR

Antibiotic, the wonder drug, is fast losing its efficacy due to rapid emergence of antibiotic resistance. The major causes of emergence of antibiotic resistance are overuse of antibiotics, inappropriate prescribing and misuse, extensive use in agriculture and scarcity of new antibiotics (Ventola, 2015). Subinhibitory and subtherapeutic level of antibiotics often lead to development of new antibiotic resistance by genetic alteration and strain diversification (Viswanathan, 2014). A fraction of the antibiotics administered to patients remain unmetabolized and are subsequently released in their liquid excreta. Thus considerable concentration of antibiotic can be detected in hospital sewage and waste water treatment plant which can exert sufficient selection pressure for new resistant strain development (Kümmerer, 2009; Rodriguez-Mozaz, *et al*., 2015).

Hospital wastewater harbours normal non-pathogenic environmental bacteria in addition to pathogenic resistant and non-resistant population. Thus it can act as a reservoir and bioreactor for generation and dissemination of new resistant strains by facilitating horizontal gene transfer among these bacterial populations (Hocquet, *et al*., 2016). There is constant recycling between the two phases of urban water cycle, namely, the unclean waste water and the clean surface and drinking water (Vaz-Moreira, *et al*., 2014). Moreover it has been reported that conventional wastewater treatment often cannot eliminate the resistant bacterial population (Łuczkiewicz, *et al*., 2010; Novo, *et al*., 2013) and hence there is high probability of interchange of resistant pathogenic bacteria between the two phases. To tackle antibiotic resistance constant monitoring of development of clinically important novel antibiotic resistance in the environment has been advocated (Bush, 2011). Thus there is an urgent need for surveillance of hospital effluent for antibiotic resistant bacteria as it serves as the major source of emerging drug resistance pathogen in the environment (Hocquet, *et al*., 2016; Weingarten, *et al*., 2018).

In India the scenario of antibiotic resistance is quite alarming due to rampant overuse and misuse of antibiotics (Laxminarayan & Chaudhury, 2016). Both clinical and environmental resistance is on the rise (Kakkar, *et al*., 2017). An investigation of antibiotic resistome of hospital effluent from city of Mumbai indicates presence of novel integron-borne antibiotic resistance genes (Marathe, *et al*., 2019). However, in our region, study of antibiotic resistance from hospital effluent is very limiting. A survey among clinicians of different teaching hospitals of Kolkata acknowledged the misuse of antibiotics in these hospitals and recommended regular surveillance as one of the effective remedial measures (Chatterjee, *et al*., 2015). In this present study we have assessed the prevalence of antibiotic resistant bacteria, with reference to fecal coliforms, in hospital wastewater collected from the city of Kolkata.

MATERIAL AND METHODS

Sample Collection: Sewage water was collected in autoclaved sterile container from the drainage system of a hospital in Park Circus area of Kolkata, India.

Isolation of Antibiotic Resistant Enteric Bacteria: The sewage sample was serially diluted up to 10-4 dilutions. 0.1 ml of stock and all dilution were plated on nutrient agar medium to determine the total viable count. 0.1 ml of stock and all dilution were plated on Eosin Methylene Blue (EMB) agar plates containing either ciprofloxacin (10ug/ml) or tetracycline (15ug/ml) antibiotics. The nucleated colonies appearing on EMB-Cipro plates were again restreaked on EMB-Tet plates and those of EMB -Tet were restreaked on EMB-Cipro plates. Five of the double resistant colonies were sub-cultured on antibiotic containing EMB plates till pure culture was obtained. All the five isolates were gram stained.

Confirmatory Test for Fecal Coliforms: All the isolated colonies were inoculated in lactose broth to check for gas production. IMViC tests were performed with all the cultures to test for fecal coliforms.

Antibiotic Disc Diffusion Assay: The five isolates were individually plated on LA plates and discs of the following antibiotics cefipime (50µg/disc), cefotaxime (10µg/disc), amoxicillin (30µg/disc), ciprofloxacin (10ug/disc) ofloxacin (2µg/ disc), doxycycline (10µg/disc) were placed aseptically and the plates were incubated at 37oC for 18 hrs. Diameter of zone of inhibition was

measured. All antibiotic discs used in the study were purchased from Himedia, Mumbai, India.

Plasmid Isolation: Plasmid isolation was performed from all the five isolates. LB broth was inoculated with individual isolate and incubated at 37ºC for 18 hrs. 1 ml of the culture was centrifuged at 6000 rpm for 8 minutes and supernatant was discarded. The pellet was resuspended in 100 ul of solution I (25mM Tris.HCl, 10 mM EDTA, 50 mM glucose pH 8). Then 200 ul of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and mixed properly. To it 150 ul of ice cold solution III (3M KOAc pH 4.8) was added, mixed properly and incubated on ice for 10 minutes. The tubes were centrifuged at 12000 rpm for 15 minutes. The supernatant was collected in a fresh tube and to it 0.7 volume isopropanol was added, mixed and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12000 rpm for 20 minutes and supernatant discarded. The pellet was washed with 70% ethanol and then air dried. Finally the pellet was dissolved in 20 ul TE buffer. From it 10 ul of sample was loaded in 0.8% agarose gel. The gel was run at 100 V for 40 minutes and observed on a uv-transilluminator.

RESULTS AND DISCUSSION

Enumeration of Antibiotic Resistant Bacterial Population

The total viable count of the sewage sample was found to be 8.1 x 10^5 cfu/ml. Resistant bacterial colonies were observed for both the antibiotics, ciprofloxacin and tetracycline. EMB agar was used to specifically isolate gram negative enteric bacteria from the sewage sample. The ciprofloxacin resistant gram negative enteric bacterial count, 2.2 x 10^3 cfu/ml, was found to be higher than that of tetracycline resistant population of 5.8 x 10^2 cfu/ml.

Both nucleated and non-nucleated colonies were observed on both plates. Nucleated colonies which are indicators of fecal coliforms were tested for double resistance. Almost thirty out of them were found to be resistant to both ciprofloxacin and tetracycline. Pure cultures of five double resistant colonies were isolated and further work was performed with them. All these cultures were found to be gram negative small rod and two of them produce green metallic sheen on EMB plates.

Gas formation was observed for all cultures when inoculated in lactose broth. All the five cultures were confirmed to be fecal coliforms from the results of IMViC tests as given in Table 1.

Antibiotic Susceptibility

The resistance pattern of the isolates was assessed for five other most commonly used antibiotics. Since initial selection of bacterial population was based on ciprofloxacin resistance, ciprofloxacin and another commonly used second generation quinolone, ofloxacin discs were used. Isolates were found to be resistant to both the quinolones. Isolates were also resistant to the poketide antibiotic tetracycline as already reported above. A second generation tetracycline, doxycycline, which is nowadays used more due to its lower toxicity than tetracycline was also tested. Zone of inhibition was observed for all isolates in case of doxycycline (Table 2).

Two groups of beta-lactam antibiotics, penicillin and cephalosporin were also tested. A second generation penicillin, amoxicillin, a third generation cephalosporin, cefotaxime as well as cefipime, a fourth generation cephalosporin were assayed. No zone of inhibition was observed for ciprofloxacin, ofloxacin, amoxicillin, cefipime and cefotaxime. All the isolates were resistant to six of the total seven antibiotics tested.

Search for Mobile extrachromosomal Genetic material

The key factors leading to emergence of new antibiotic resistance are horizontal acquisition of resistance genes (carried by plasmids or insertion sequences), by recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci (Davies, 1997; Martínez & Baquero, 2000; Davies, 1994). Selection pressure in hospital waste water will easily result in plasmid acquisition by new species. Hence we have looked for the presence of plasmids, the mobile extrachromosomal genetic material in our isolates. Of the five isolates four were found to harbour plasmid DNA molecule inside them. The band pattern as observed from agarose gel indicates different sized plasmids in the four isolates.

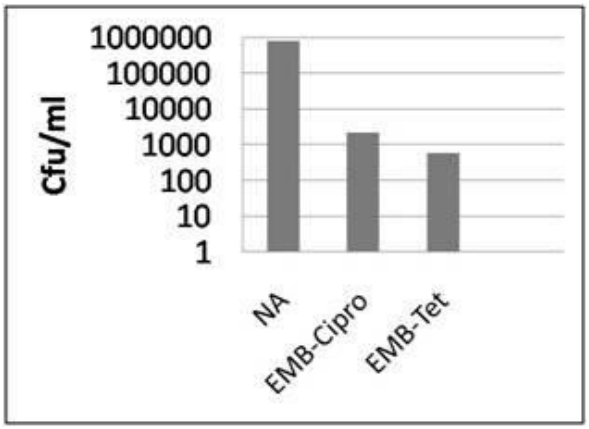

Figure 1: Relative Proportion of Bacterial Population

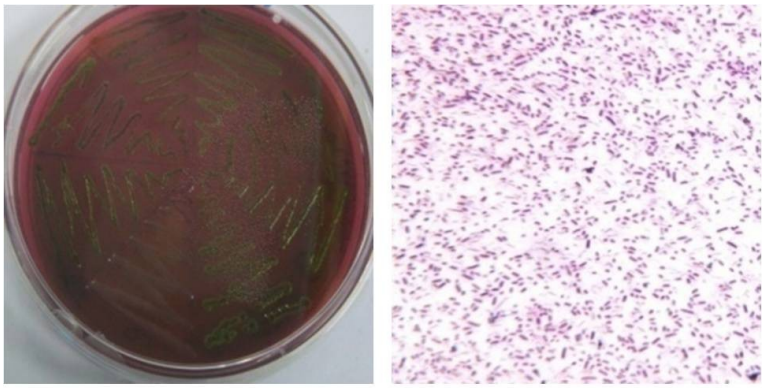

Figure 2: (A) EMB plates showing metallic sheen (B) Gram Stained cells of culture giving metallic sheen as viewed under microscope

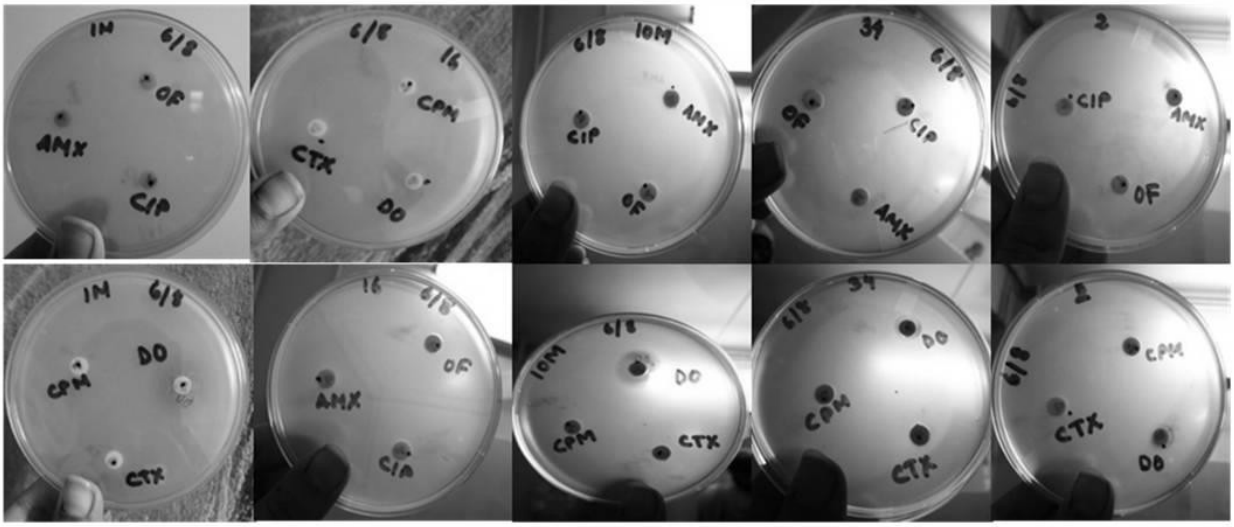

Figure 3: Results of Antibiotic Disc Diffusion Assay. On plates antibiotics are represented as OF-ofloxacin, AMX-amoxicillin, DO-doxycycline, CIP-ciprofloxacin, CPM-cefipime and CTX-cefotaxime. Zone of inhibition observed only in case of doxycycline.

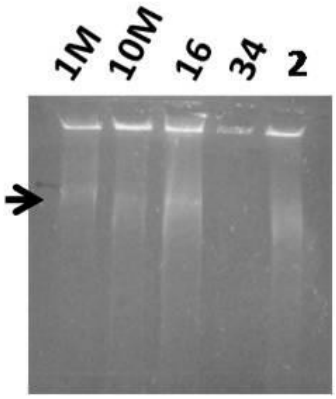

Figure 4: Agarose gel showing isolated plasmid DNA. Individual sample IDs 1M, 10M, 16, 34 and 2 are represented on top of each lane. The arrowhead indicates the position of the plasmid band.

Table 1: Results of IMViC Tests

Sample ID	Indole Test	Methyl Red Test	VP Test	Citrate Test
1M	+ve	+ve	-ve	-ve
10M	+ve	+ve	-ve	-ve
2	+ve	+ve	-ve	-ve
16	+ve	+ve	-ve	-ve
34	+ve	+ve	-ve	-ve

Table 2: Zone of Inhibition for Doxycycline

Sample ID	Average Zone Diameter(mm)
1M	14.6
10M	15.3
2	10
16	15.6
34	9.6

CONCLUSION

The burden of infectious diseases is extremely high in India. The antibiotics are readily available even without prescription and hence misuse and overuse of antibiotics are rampant in India. These two are the key driving forces behind the unregulated increase of antibiotic resistance in India (Laxminarayan & Chaudhury, 2016). Kakkar *et al*. have highlighted the keys points of National Action Plan on Antimicrobial resistance (AMR) to tackle this menace in India. One of the key features of that is to 'strengthen knowledge and evidence through surveillance'. Regular surveillance should include clinical and community settings (Kakkar, *et al*., 2017).

Hospitals wastewater is the reservoir of antibiotic residue and antibiotic resistant pathogens released from

patients on antibiotic therapy. This study illustrates the presence of multidrug resistant fecal coliforms in hospital wastewater. The isolates were found to be resistant to second generation quinolones, ciprofloxacin and ofloxacin as well as second generation penicillin, amoxicillin. A really alarming result is that the isolates showed resistance to even third and fourth generation cephalosporins. Moreover the isolates carry plasmid DNA which often acts as the carrier of antibiotic determinant genes in horizontal gene transfer. Hence these resistance detrminants if present on the plasmid could be easily transferred to sensitive pathogens or even to non-pathogens present in the waste water. Since conventional wastewater treatment is incapable of removing the entire resistant population (Łuczkiewicz, *et al*., 2010) these MDR population can pollute the river to which wastewater is discharged post treatment. Thus dissemination of AMR to normal environmental bacterial population is inevitable. Thus constant surveillance and improved techniques of waste water treatment to remove resistant population prior to discharge should be adopted.

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CONFLICTS OF INTEREST

The author declare that they have no potential conflicts of interest.

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