



International Journal of Medical Research & Health Sciences

www.ijmrhs.com

Volume 2 Issue 4 Oct-Dec

Coden: IJMRHS

Copyright ©2013

ISSN: 2319-5886

Received: 27th Aug 2013

Revised: 16th Sep 2013

Accepted: 28th Sep 2013

Research article

SCREENING OF ANTIBIOTIC RESISTANT GRAM NEGATIVE BACTERIA AND PLASMID PROFILING OF MULTI-DRUG RESISTANT ISOLATES PRESENT IN SEWAGE ASSOCIATED WITH HEALTH CARE CENTERS

Khan Md. Anik Ashfaq, Sutradhar Pijush, Islam Mohammad Majharul, Ojha Ravi Kant, Biswas Gokul Chandra*

Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh

*Corresponding author e-mail: gcbiswas1985@gmail.com

ABSTRACT

Background: Healthcare effluent acts as the store house of harmful infectious agents such as the pathogens and microorganisms possessing multiple drug resistant genes. Potential health risk includes spreading of diseases by these pathogens and wide dissemination of antimicrobial resistance genes. Gram-negative bacteria are particularly important for causing most of the hospital and community acquired infections. **Aim:** This study was carried out to highlight the incidence of antibiotic resistant bacteria in hospital generated effluent discharged into municipal sewage system of Sylhet city, Bangladesh. **Methodology:** Standard biochemical tests were used to isolate and identify 29 gram negative bacteria from 6 effluent samples. Antibiotic susceptibility test was assessed by Kirby-Bauer disc diffusion method. Plasmid isolation and gel electrophoresis were performed using standard protocols. **Results:** Antibiogram study showed that the percentage of isolates resistant to amoxicillin, ceftriaxone, ciprofloxacin, gentamicin, imipenem, azithromycin, and sulphonamethoxazole-trimethoprim were 93.10%, 55.17%, 27.6%, 24.14%, 20.7%, 13.8% and 10.34% respectively. Ten of the isolates showed resistance to three or more commonly used antibiotics. Plasmid profiles of the multi-drug resistant isolates showed to harbor two or more plasmids and almost all of them showed a common band for plasmid DNA size of 24.5kb. **Conclusion:** Resistance to the bacterial pathogens causing community acquired infections may, thus, exert a serious public health threat through confining the antibiotic pool. Hospitals should follow, monitor and regulate proper sanitary measures of hospital generated effluents to forestall the dissemination of multi drug resistant bacteria transfer from hospital waste to the environment.

Keywords: multi-drug resistance, hospital effluents, antibiogram, plasmid profiling.

INTRODUCTION

Waste effluent from hospitals and clinics contain high numbers of resistant bacterial strains and residual antibiotics at a concentration to which

susceptible bacterial growth is inhibited.¹ One study by Schwartz et. al.,² has evaluated the microbiological content of hospital and

household waste quantitatively and qualitatively and found that general hospital waste contains bacteria with pathogenic potentials for humans compared to household waste.² Grabow and Prozesky³ showed that two mechanisms, introduction and selection for resistant bacteria results in increased number of resistant bacteria in the sewers where hospital effluents are immediately discharged³. Excretion mechanism is responsible for the addition of a significant amount of un-metabolized antibiotics.⁴ Long-term exposure of microorganisms to low concentrations of antibiotics in wastewater and surface water has the potential for the development of antibiotic resistance, which was reported by Smith et. al.,⁵ If the hospital effluents are not treated, concentrated forms of infectious agents and antibiotic resistant microbes are shed into communities resulting in water borne diseases such as cholera, typhoid fever, dysentery and gastroenteritis.⁶ Grabow and Prozesky stated that, although, sewage treatment reduces the number of bacteria in wastewater but the effluent generally contains a large number of both resistant and susceptible bacteria³.

The basic principle of underlying wastewater management is the strict limit on the discharge of hazardous liquids into sewers without prior treatment so that living pathogenic organisms are not introduced into the environment.⁷ In Bangladesh, at present no government rule is prevailing for regulating the hospital waste management. No institutional mechanism is working on the government side specially for handling this kind of waste, which was reported by 'Prism Bangladesh'.⁸ Also, unfortunately, there is no structured form of medical waste treatment in Bangladesh, and most wastes are dumped in open areas for natural degradation or re-sold by scavengers⁹, although, hospitals usually maintain local measures (e.g., incineration, chemical sterilization etc.) of waste treatment before disposal. The sewerage network of Sylhet city consists of many small drains

connected with some natural hilly channels, which fall into the Surma River.¹⁰ Therefore, even if the hospitals are discharging their health care liquid waste into the sewerage system, it mixes with the sewage and gets in surface water without proper treatment.

Haque (1994) reported that some 5.2 million people (including 4 million children) die each year from waste-related diseases in Bangladesh.¹¹ Therefore, untreated hospital liquid waste discharges into surface water directly or indirectly have been causing additional problems. Gram-negative bacteria are crucial in this aspect because they are the most common causes of hospital and community acquired infections and they are inherently resistant to many hydrophobic antibiotics.¹²⁻¹⁵ In such scenario, this study was carried out to screen the antibiotic susceptibility pattern of gram-negative bacterial isolates from healthcare liquid waste generated in Sylhet metropolitan area of Bangladesh and investigate on their role in developing antibiotic resistance.

MATERIALS AND METHODS

Sample collection, isolation and identification of bacteria: A total of six sewage samples associated with hospital and clinical effluents were collected from six different spots in the Sylhet Metropolitan area, where hospitals effluents are immediately discharged. Sample from each site was collected in 150 ml sterile containers and transported to the laboratory within an hour of collection in cold conditions. 100 ml diluted samples were spread over MacConkey agar media that inhibits the growth of gram positive bacteria and incubated for 24 hours at 37°C. Differentiation as lactose fermenter and non-lactose fermenter could be made on MacConkey agar for the isolates based on pigmentation.¹⁶ Five colonies from each plate were selected with different colony morphologies by using five-colony method and further purified twice.¹⁷ A total of twenty nine isolates thus

obtained were identified on the basis of morphological characteristics, gram staining and biochemical tests according to Bergey's Manual of Determinative Bacteriology.¹⁸ Other media used in this study include Eosine Methylene Blue Agar (EMB), Salmonella Shigella (SS) Agar and Thiosulfate Citrate Bile Salts (TCBS) agar.

Antibiotic sensitivity test: Antimicrobial susceptibility testing of all 29 bacterial isolates was performed following the modified Kirby-Bauer disk diffusion method on Muller-Hinton against selected antibiotics namely Amoxicillin (AMX) 10µg, Ceftriaxone (CTR) 30µg, Azithromycin (AZM) 15µg, Gentamycin (CN) 10µg, Imipenem (IPM) 10µg, Ciprofloxacin (CIP) 5µg and Sulphamethoxazole-Trimethoprim (SXT) 10 µg. *Klebsiella* ATCC and *Pseudomonas* ATCC were the only standard controls used to screen antibiotic resistance of *Klebsiella* sp. and *Pseudomonas* sp. isolates respectively. Inhibition zone size was interpreted using standard recommendation of the National Committee for Clinical Laboratory Standards¹⁹ now known as the Clinical Laboratory Standard Institute (supplementary data: Table 1).

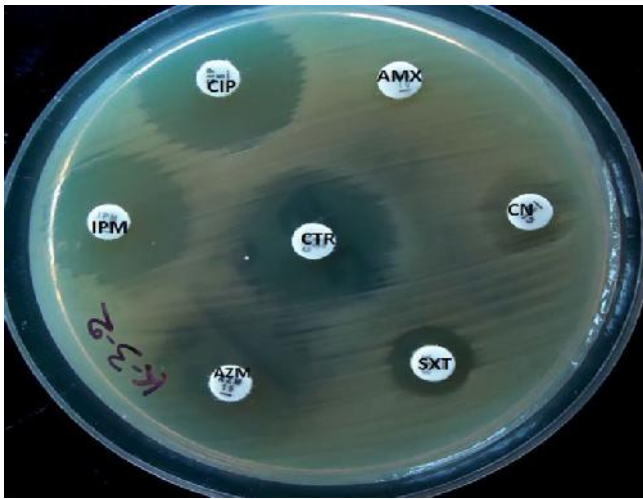
Plasmid analysis: The present study emphasized on investigation on the correspondence between antibiogram and plasmid profile of the multiple drug resistant (MDR) isolates. Therefore, plasmid DNA of MDR isolates was extracted from cultured cells following alkaline lysis method of plasmid preparation.²⁰ The samples were processed using gel electrophoresis to identify the number of plasmid copies present in different isolates. Agarose gel electrophoresis was carried out in a horizontal gel apparatus (My Run Cosmo bio co. Ltd, IMR-201). After electrophoresing of all the plasmids in 0.7% agarose (Merck, Mumbai), the gel stained with ethidium bromide were visualized by Ultraviolet Transilluminator (UVP, High Performance transilluminator; USA) and photographed by Samsung camera. The number and molecular

sizes of the plasmid DNA were determined on the basis of mobility through agarose gel and was compared with the mobility of Supermix DNA Ladder, (Genei Pvt. Ltd., Bangalore, India) which was used to estimate the plasmid size.

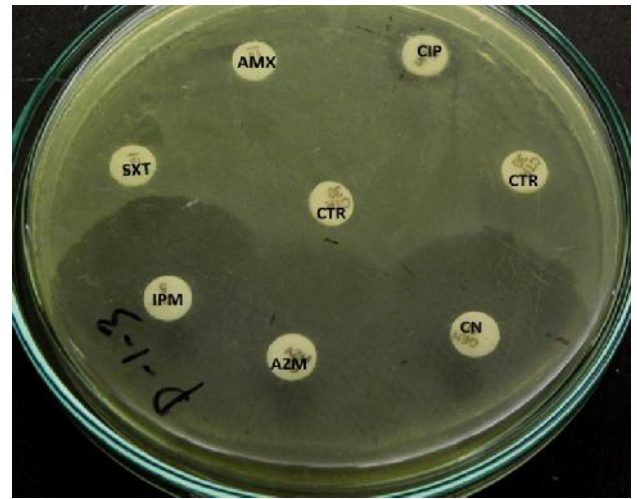
RESULTS

In the present study, we collected effluent samples from hospitals and clinics which were immediately released into the corresponding municipal sewerage. As gram negative bacteria are the most common causes of hospital and community acquired infections¹⁵, an effort was given to isolate and identify gram negative probable pathogens. Of the 29 isolates, eight (27.58%) were found to be of *Pseudomonas* sp., followed by seven (24.14%) of *Escherichia coli*, six (20.69%) of *Klebsiella* sp., three (10.74%) of *Shigella* sp., two (6.90%) each of *Yersinia* sp. and *Vibrio* sp., and one (3.49%) of *Serratia* sp. respectively.

Antibiogram study of the 29 isolates (supplementary data: Table 2, 3, 4) showed that the percentage of isolates resistant to amoxicillin, ceftriaxone, ciprofloxacin, gentamicin, imipenem, azithromycin and sulphamethoxazole-trimethoprim were 93.10%, 55.17%, 27.6%, 24.14%, 20.7%, 13.8% and 10.34% respectively. Highest rates of sensitivity pattern were found to imipenem (68.96%), followed by SXT (65.52%), ciprofloxacin (62.06%) and azithromycin (62.06%) (**Fig. 2**). Among the predominant isolates (*Pseudomonas* sp., *E. coli*, and *Klebsiella* sp.), The highest percentage of resistance to ciprofloxacin and azithromycin was shown by *E. coli* isolates (42.86%) whereas 66.66% of *Klebsiella* sp. showed resistance to ceftriaxone and gentamycin which was highest (supplementary data: Table 2). All of the isolates of *Pseudomonas* and *Klebsiella* sp. expressed resistance pattern to amoxicillin whereas in case of *E. coli* it was 85.71%.



(A)



(B)

Fig. 1: Antibiogram of two of the isolates- (A) *Klebsiella sp.* and (B) *Pseudomonas sp.*

Although, imipenem showed highest sensitivity pattern to the isolates, both of the two *Yersinia sp.* expressed resistance to this antibiotic. Bacterial isolates resistant to three or more of the

antimicrobial agents tested, hence multi-drug resistant (MDR) were found to be 10 in number (34.48%) out of 29.

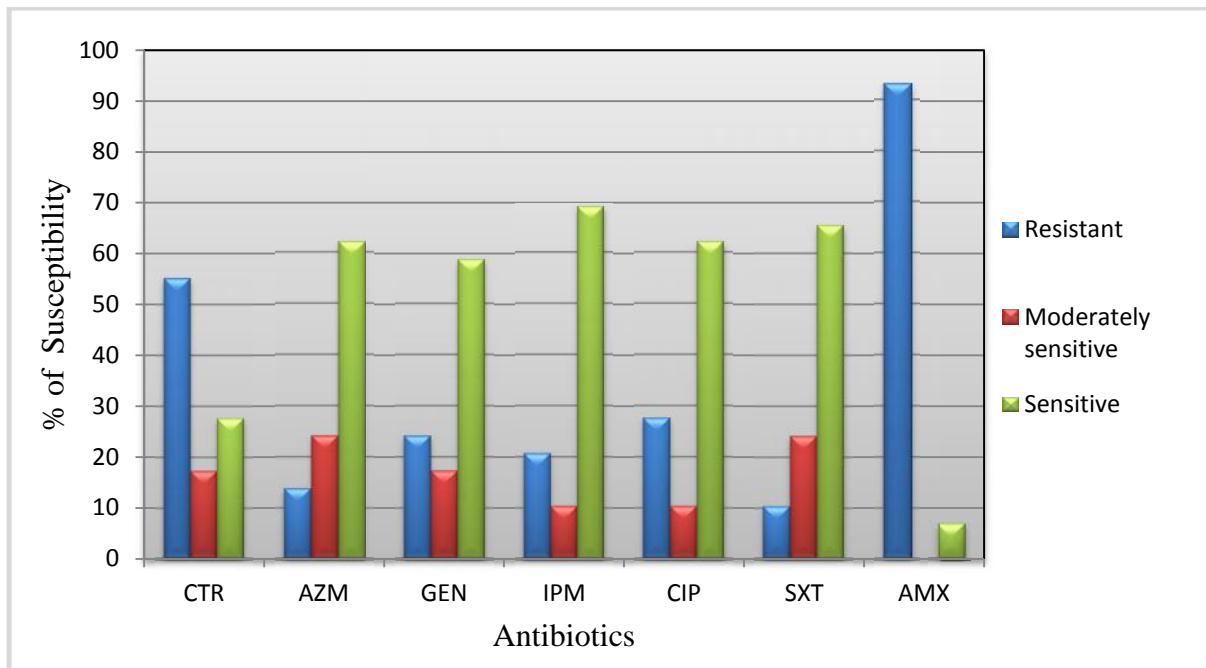


Fig. 2: Comparative antibiogram study of twenty nine isolated bacteria.

Plasmid profiling of ten isolates showed that eight of the isolates show plasmid DNA of varying sizes (1.5 kb to 24.5 kb) (Fig. 3 and Table 1). One of the isolates possessed single band of plasmid while others had more than one with different sizes as shown in the table. On the other hand, only two isolates have no plasmid even though they were resistant to antibiotics.

Almost all of the isolates that showed presence of plasmid contained common plasmid DNA size of 24.5kb. Earlier, antibiogram of MDR bacterial isolates showed that all isolates were resistant to amoxicillin. Four of the isolates were resistant to ciprofloxacin and three of them (P-1-3, K-1-2 and K-6-4) harbored a plasmid each of size around 2kb.

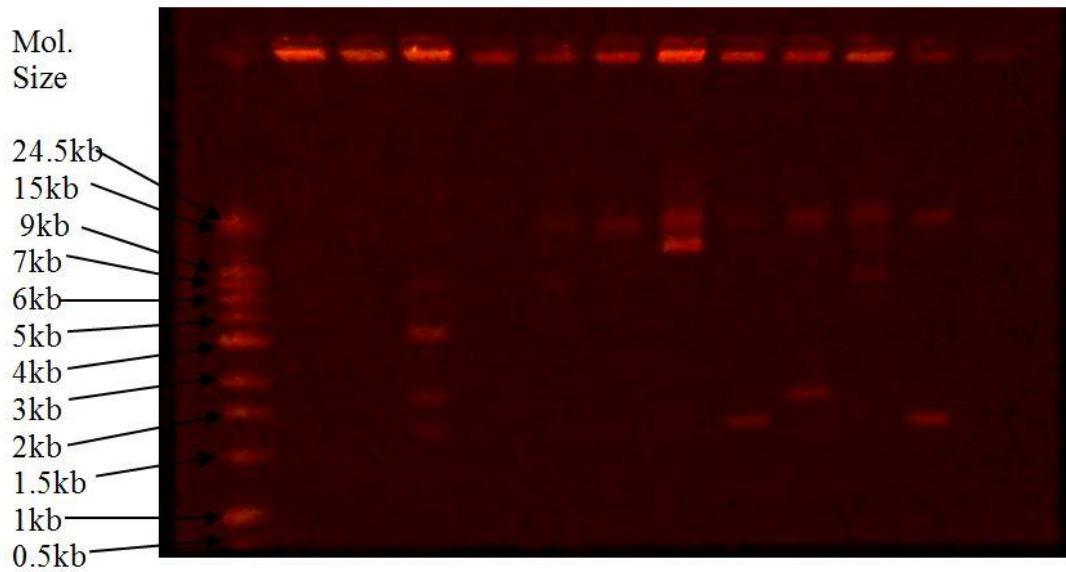


Fig. 3: Agarose gel electrophoresis of plasmid DNA from ten MDR isolates.

*L= Ladder DNA, †P-A=*Pseudomonas* ATCC, ‡K-A= *Klebsiella* ATCC

Table: 1. Plasmid profiling and correlation with antibiogram.

Isolates	Antibiotics resistance pattern	No. of bands	Band size (kilo base pair)
*P-4-5	AMX, CTR, SXT	0	-
P-1-3	AMX, CTR, CIP, SXT	4	7kb, 4.2kb, 2.4kb, 1.7kb
†E-1-4	AMX, CTR, AZM	0	-
E-3-1	AMX, CTR, CIP	2	24.5kb , 7kb
‡S-6-2	AMX, CTR, IPM	1	24.5kb
§Y-4-4	AMX, CTR, IPM, CN	2	24.5kb , 14.5kb
K-1-2	AMX, CTR, CIP, CN	2	24.5kb , 2kb
K-3-2	AMX, SXT, CN	2	24.5kb , 2.8kb
K-2-2	AMX, CTR, CN	2	24.5kb , 9kb
K-6-4	AMX, CTR, CIP	2	24.5kb , 2kb
ATCC- <i>Pseudomonas</i>	AMX	0	-
ATCC- <i>Klebsiella</i>	AMX	0	-

*P=*Pseudomonas* sp., †E= *E. coli*, ‡S=*Shigella* sp., §Y=*Yersinia* sp., || K=*Klebsiella* sp.

DISCUSSION

Both lactose fermenting and non-lactose fermenting bacteria were isolated and identified in which most predominant was *Pseudomonas* sp. with 27.58%, followed by *Escherichia coli*-24.14%, and *Klebsiella* sp.- 20.69%. One of the hospital effluent studies carried out in India involved isolation of gram negative bacilli, in which *E. coli* (37.19%) was predominant organisms, which is contrasted to this study

whereas percentages of *Pseudomonas* Sp. (22.31%) and *Klebsiella* sp. (19.83%) were fairly consistent. The study carried out in India showed higher percentage of resistant isolates from hospitals to ciprofloxacin (42.97%) and gentamicin (27.27%) whereas the less percentage of isolates was found to show resistance to imipenem (16.66%)²¹ In contrast to this, less percentage of isolates showed resistance to

ciprofloxacin (27.6%) whereas almost same pattern of resistance was found in the present study in case of gentamycin (24.14%). Amoxicillin was the antibiotic to which highest number of resistant bacteria (93.1%) was found, followed by ceftriaxone (55.17%). Discharge of antibiotic residues from hospitals and households usage into effluent and municipal sewage provides an indication of a selection pressure on bacteria.²² Islam et al (2008) carried out a similar type of study in Bangladesh and found that the resistance development was directly related to the use of antibiotics.²³ It can be assumed that isolated organisms in this study have been persistently exposed to the antibiotic residues and as a result, they might have developed resistance mechanisms to them. Walton (1988) pointed out that hospital environment appears to be the origin of MDR bacterial strains and the selective pressure responsible for expanding such bacterial populations in hospitals must have been through the use of drugs in humans and veterinary and agricultural uses are not responsible.²⁴ Therefore, the results suggest that the MDR bacteria containing multi drug resistant genes present in the hospital effluent of this particular locality may act as a possible source of transfer of these resistant genes to the bacterial population.

Plasmid profiling of two of the MDR isolates did not show up any visible band. Possibly, some resistant genes were not located on plasmid and perhaps exist on the bacterial chromosome as it is known that genetic origin of drug resistance may be plasmid or chromosomal.²⁵ Beta-lactams are particularly important because they are the most prevalently used antibiotics all over the world, and because clinical crisis resulted due to resistance to this antibiotic.²⁶ -lactamases, including extended-spectrum -lactamases (ESBLs), AmpC -lactamases (AmpC) and metallo- -lactamases (MBLs) genes that are responsible for prevalent emergence of antibiotic resistance in gram-negative bacteria are most

often carried on plasmid, thus, facilitate rapid spread between microorganisms.²⁷ Being the types of beta-lactam, this may be attributed to the widely dissemination of amoxicillin, ceftriaxone and imipenem resistant genes, because a significant percentage of bacterial isolates tend to show resistance to antibiotics in the present study. It is suggested to carry out further research including plasmid curing and transformation in order to examine plasmid-mediated mode of resistance to the antibiotics used in this study.

As this study was performed over limited number of samples and isolates, studies in broad manner are needed to fully understand the extent to which hospitals and clinics in big cities of Bangladesh contribute to resistance properties of bacterial strains. In addition, the samples were collected from the spots where the effluents immediately discharged into municipal sewage, with a view to isolating those bacteria which have a better chance to come from effluents or persist to effluent materials i.e., antibiotic residues. In this circumstance, some other bacterial strains that have attained resistant properties from other sources other than hospital effluents may be mixed. Despite the possibility of this rare pitfall, the present observations suggest that hospital effluents may contribute to health hazard by adding MDR bacteria to city sewage of this particular locality, which ultimately falls into river.

CONCLUSION

The present study was carried out on a preliminary basis, in order to investigate on the development of antibiotic resistance and their possible mode of dissemination. Finally, it can be concluded that the isolated strains were showing resistance mechanisms against various antibiotics especially to beta lactams and these drug resistant strains may cause to induce health hazard. Hence, solution to the problem of MDR bacteria in healthcare liquid waste is crucial. Good safety sterilization methods should be

implemented before releasing of waste materials to the environment or sewage. Proper regulation and monitoring of an integrated health care liquid waste management practice is essential in order to diminish the risk of disseminating multiple drug resistant microorganisms for the safeguard of public health.

ACKNOWLEDGEMENTS

This research work was carried out both in the former 'USDA laboratory' and in the 'Microbiology, Fermentation and Environmental Biotechnology laboratory' of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet. We want to thank the personnel of both laboratories for their support and technical help.

REFERENCES

1. Asha P, Jyothis M, Shini Z. Antibiotic resistant Enterococci from drinking water sources. *Asian Journal of Pharmaceutical and clinical research*. 2012; 5(3):158-60.
2. Schwartz T, Kohlen W, Jansen B, Obst U. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water and drinking water bio films. *FEMS Microbiol Ecol*. 2003; 43(3):325-35.
3. Grabow WO, Prozesky OW. Drug resistance of coliform bacteria in hospital and city sewage. *Antimicrob Agents Chemother*. 1973; 3(2):175-80.
4. Saini S, Das BK, Kapil A, Nagarajan SS, Sarma RK. The study of bacterial flora of different types in hospital waste, evaluation of waste treatment at AIIMS Hospital, New Delhi. *Southeast Asian J Trop Med Public Health*. 2004; 35(4):986-9.
5. Smith KE, Besser JM, Hedberg CW, Leano FT, Bender JB, Wicklund JH et al. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. *The New England Journal of Medicine*. 1999; 340:1525-32.
6. Sharma DR, Pradhan B, Mishra SK. Multiple drug resistance in bacteria isolates from liquid wastes generated in central hospitals of Nepal. *Kathmandu University Medical Journal*. 2010; 8(29):40-4.
7. WHO [homepage on the internet]. Geneva: World Health Organization [cited on July 2008]. *Healthcare Waste Management Manual*. [http://www.who.int/topics/medical_waste/en/]
8. A booklet "*HaspitalBorjo Babosthapon Nirdeshika*" ("*Directives for Hospital Waste Management*") Prism Bangladesh, 1stEdn; 2005. p29.
9. Akter N, Acott RE, Chowdhury SA. Medical waste disposal at BRAC health centres, an environmental study. BRAC Research, Research and Evaluation Division, 75 Mohakhali C/A, Dhaka 1212, 1998.
10. Alam R, Nath SK, Siddique MSM. A study on municipal wastewater characteristics monitoring of Sylhet city. An undergraduate thesis, Department of Civil & Environmental Engineering, Shahjalal University of Science and Technology, Sylhet, 2002.
11. Haque. Waste Landfill and Hospital Waste Incineration. Roteb-Solid Waste Consultancy, Kleinpolderplein 5, P.O.Box 10902- 3004. Rotterdam, The Netherlands, 1994.
12. Hancock RE. Alterations in outer membrane permeability. *Annu Rev Microbiol*. 1984; 38:237-64.
13. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 2003; 67(4):593-656.
14. Vaara M. Agents that increase the permeability of the outer membrane. *Microbiol Rev*. 1992; 56(3):395-411.
15. Bastopcu A, Yazgi H, Uyanik M H, Ayyildiz A. Evaluation of quinolone resistance in Gram negative bacilli isolated from community and hospital acquired infections. *The Eurasian Journal of Medicine*. 2008; 40:58-61.

16. Levy SB, Marshall B, Schluederberq S, Rowse D, Davis J. High frequency of antimicrobial resistance in human fecal flora. *AntimicrobAgents Chemother.* 1988; 32(12):1801–6.
17. Osterblad M, Leistvuo T, Huovinen P. Screening for antimicrobial resistance in fecal samples by the replica plating method. *J ClinMicrobiol.* 1995; 33(12):3146–9.
18. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Group 11: Oxygenic phototrophic bacteria. In: Hensyl WR (eds.), *Bergey's Manual of Determinative Bacteriology*, 9th Edn., Williams & Wilkins, Baltimore, 1994; pp. 377–425.
19. National committee for clinical laboratory stands. Methods for dilution aerobically NCCLS Approved standard M7-A5 and informational supplement M-100-S19 Wayne, PA USA, 2000.
20. Sambrook J, Russel D W 2001. *Molecular Cloning*, 3rd Edn. 3 vol. Cold Spring Harbor Laboratory Press, New York
21. Usha K, Kumar E, SaiGopalDvr. Occurrence of various beta-lactamase producing gram negative bacilli in the hospital effluent. *Asian J Pharm Clin Res.* 2013; 6(3):42-46.
22. Kummerer K, Henninger A. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *ClinMicrobiol Infect.* 2003; 9(12):1203-14.
23. Islam MJ, Uddin MS, Hakim MA, Das KK and Hasan MN. Role of Untreated Liquid Hospital Waste to the Development of Antibiotic Resistant Bacteria. *Journal of Innovation and Development Strategy.* 2008; 2(2):17-21.
24. Walton JR. *The Veterinary Record.*1988; 122:249-51.
25. Oktem IM, Gulay Z, Bicmen M and Gur D. HITIT project study group qnrA prevalence in extended spectrum beta-lactamase-positive enterobacteriaceae isolates from Turkey. *Jap J Infect Dis.*2008; 61:13-17.
26. Jean SS, Teng LJ, Hsueh PR, Ho SW, Luh KT. Antimicrobial susceptibilities among clinical isolates of extended-spectrum cephalosporin-resistant Gram-negative bacteria in a Taiwanese University Hospital. *J AntimicrobChemother.*2002; 49:69-76.
27. Gupta, V. An update on newer beta-lactamases. *Indian J Med Res.* 2007; 126(5):417-27.