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Full Length Research Paper

Prevalence of multiple antibiotic resistant bacteria and chromosomal determinants in surface water of Bangladesh

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A total of 210 different bacteria were isolated from water samples collected in different surveillance sites in Bangladesh between May and July 2004. Of these, 147 isolates were analyzed and 103 were identified by different biochemical tests as well as stereotyped with specific antiserum. Sixty five percent of these isolates were identified as *Escherichia coli* which were resistant to multiple drugs including tetracycline, nalidixic acid, kanamycin, streptomycin and sulphamethoxazole-trimethoprim. The remaining thirty five percent of the isolates were *Vibrio cholerae*, *Pseudomonas*, *Aeromonas*, *Staphylococcus* and *Klebsiella*. Most of the *V. cholerae* were nalidixic acid, streptomycin and sulphamethoxazole-trimethoprim resistant. Almost 90% of nalidixic acid resistant *V. cholera* were stereotyped as non O1. The remaining 10% were O1 and O139. All bacteria were used to isolate plasmid DNA to compare plasmid patterns. The plasmid contents of representative drug resistant strains were analyzed by electroporation to determine the location of antibiotic resistant gene whether it was chromosomal or extrachromosomal. The results of these experiments suggested that Bangladeshi environmental water including rivers, lakes and ponds contained a large number of multiple antibiotic resistant bacteria and almost all antibiotic resistant determinants were located in the chromosome instead of plasmid.

Key words: Antibiotic resistant bacteria, chromosomal determinants, surface water.

INTRODUCTION

Multiple antibiotic resistant bacteria are considered as a global threat to public health. This is because antibiotics, namely sulfamethoxazole-trimethoprim and tetracycline are the drugs of choice and often the last option for the treatment of hospital infections caused by multiresistant bacteria. The transferable nature of the gene clusters encoding high-level multiple antibiotic resistances in bacteria in the environment has caused concern in the scientific community. Antibiotic resistant organisms including bacteria have developed a resistance to certain drugs. Some bacteria build living walls in response to exposure to antibiotics, creating a physical barrier that shields them and contributes further to the growing problem of drug resistant infections (Matic et al., 1996). The tremendous therapeutic advantage afforded by antibiotics is being threatened by the emergence of increasingly resistant strains of microbes. Selective pressure favoring resistant strains arises from misuse and overuse of antimicrobials (File, 1999; Livermore, 2005). The pathogenic organisms are responsible for acquisition of resistance.

The worldwide use of antimicrobials in different fields has created enormous pressure for the selection of resistance among opportunistic bacterial pathogen (Balotescu et al., 2003; Sharma et al., 2005). It has been the focus of many studies to elucidate some unique aspects of molecular biology, including the adaptive mechanisms responsible for emergence and spread of multiresistance

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Table 1. Distribution of different bacteria in the investigated water samples.

No. of strain's isolated	No. of strains analyzed	Identified bacteria		Percentage of total	No. of strain's carrying plasmid
210	147	79	*E. coli*	63.7%	58
		20	*Pseudomonas*	16.12%	0
		2	*Staphylococcus*	1.61%	0
		1	*Aeromonas*	0.80%	0
		1	*Klebsiella*	0.08%	0

Some *Vibrio cholerae* strains were isolated on TTGA plate that were resistant to only nalidixic acid and some of them were nalidixic acid, streptomycin and sulphamethoxazole-trimethoprim resistant.

(Tambic, 2004; Kim and Park, 1998). Prior antibiotic exposure is the major risk factor for amplification and perpetuation of resistance (Lynch and Martinez, 2002).

Inadequate and prolonged antimicrobial prophylaxis increases resistance to antimicrobial drugs (Harbarth et al., 2000). In the changing pattern of drug resistance, a strain of bacteria exposed to a growth inhibitory lethal drug responds normally at first, then the growth rate is reduced or the population size is diminished (Acar et al., 1985; Rideg et al., 2005). The drug played some directive role for a long period of time in causing a biochemical adaptation in the cells. The resistance is acquired by mutation or by the acquisition of resistance-encoding genetic material which is transferred from other bacteria (Dzidic et al., 2003; Kim and Park, 1998).

This study was conceived to provide information on the occurrence of multiple antibiotic resistant bacteria collected from different environmental samples of Bangladesh and the genetic location of the antibiotic resistant determinants.

MATERIALS AND METHODS

Bacterial strains/source/preservation

Six different types of isolates were isolated from the aquatic environment of different surveillance sites of Dhaka city in Bangladesh. These isolates were biochemically identified as *Ecsherichia coli*, *Vibrio cholerae*, *Pseudomonas*, *Aeromonas*, *Staphylococcus* and *Klebsiella*. Almost 80% bacteria were at least three antibiotic resistant. Most of the *E. coli* were plasmid containing. *V. cholerae*, *Staphylococcus*, *Klebsiella* and *Aeromonas* were also used for plasmid isolation. Samples were spread on the selective antibiotic plates of gelatin agar (GA), luria agar (LA) and MacConkey agar media. Sometimes samples were enriched by 6 h incubation with bile peptone (BP) media if needed. Mueller-Hinton Agar (MHA) was used for antibiotic susceptibility test. Samples were preserved in glycerol broth at -80°C. For electroporation study, *E. coli* DH5α was used as competent cell.

Biochemical characterization and identification of environmental isolates

The biochemical reactions of the strains were determined by standard methods described in manual for laboratory investigation of acute enteric infections (Chakraborty et al., 2005). Several selective biochemical tests including gram staining, oxidase test, catalase test, kligler iron agar (KIA) test, motility indol urea (MIU) test, indole test, urease test, citrate test, carbohydrate fermentation test, amino acid decarboxylation reaction, salt tolerance test, analytical profile index (API) were carried to find out analyzed bacteria. Pure culture of the test organism was obtained by streaking onto gelatin agar (GA), tellurite taurocholate GA (TTGA) and MacConkey agar plates. The characteristic colonies were sub-cultured further and were subjected to different tests. The identified strains were *V. cholerae*, *E. coli*, *Pseudomonas*, *Aeromonas*, *Staphylococcus* and *Klebsiella*. The different identified strains are summarized in a table (Table 1).

Isolation of plasmid DNA

Plasmid DNA was isolated by the method of Birnboym and Doly, modified by Maniatis (Maniatis et al., 1989). Isolation was carried out from mini preparations. Cells pellet from 1.5 ml O/N culture was collected by centrifugation and dissolved in 50 mM Glucose, 25mM Tris-HCl and 10 mM EDTA that were used for cell wall disruption. 0.2 N NaOH, 1% SDS were used as lysis buffer to lyse cell wall and potassium acetate and glacial acetic acid were used to stop the reaction. Supernatant was collected by centrifugation and extracted with phenol: chloroform: isoamyl alcohol (25:24:1) followed by a precipitation with double volume absolute ethanol. The pellet was dried in vacuum drier and dissolved in 40 µL of TE. 1% agarose gel electrophoresis was carried out to observe plasmid size and band patterns.

Electroporation of plasmid DNA

Electroporation was carried out by Maniatis et al. (1989). For electroporation, cells were grown to mid-log phase, harvested and then washed extensively with water to eliminate all salts. Usually, glycerol was added to the water to a final concentration of 10% so that the cells can be stored frozen and saved for future experiments. To electroporate plasmid into cells, washed *E. coli* DH5α were mixed with the plasmid to be transformed and then pipetted into a plastic cuvette containing electrodes. A short electric pulse, about 2,400 volts/cm, was applied to the cells causing smalls holes in the membrane through which the DNA enters. The cells are then incubated with broth before plating.

RESULTS

Isolation of different antibiotic resistant microorganisms from environmental water sample

The combination of four different antibiotics (tetracycline, nalidixic acid, kanamycin and gentamicin) was used to

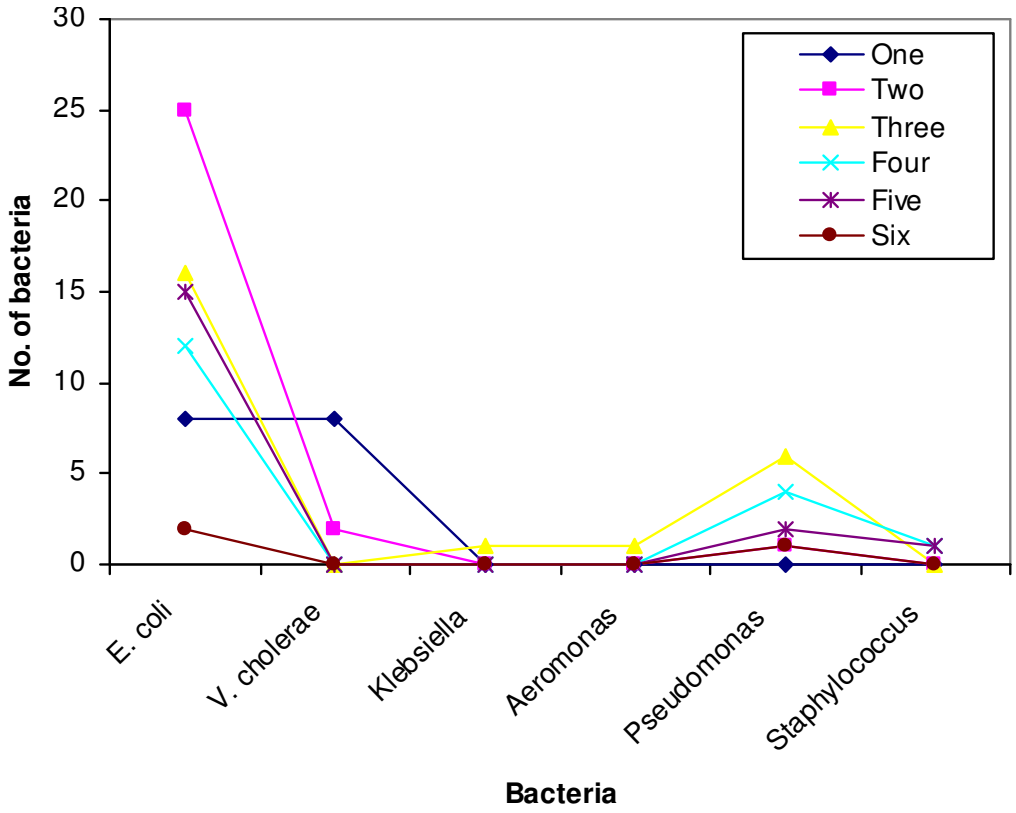

Figure 1. Multiple antibiotic resistant bacteria used for this study.

isolate resistant bacteria from the water samples. Most of the strains were resistant to tetracycline, nalidixic acid, kanamycin and gentamicin (Figure 1). A few of those are only tetracycline and nalidixic acid resistant. Among all these antibiotics kanamycin sensitive strains were available. Nalidixic acid resistant strains were 5% whereas tetracycline and kanamycin resistant strains were 1% and gentamicin resistant was 0.5%. In combination with two antibiotics, tetracycline and nalidixic acid resistant were 1% and the rest were less than 1%. In combination with four antibiotics, 0.6% resistant microbes were isolated from Turag river, a major sampling site in Bangladesh and the rest were isolated from other sampling sites. The number and percent of resistant colonies of one sampling are presented in Table 2. After collecting the colonies from the plates of the combinations of four different antibiotics, the representative colonies were further checked by gradually increasing another different antibiotics i.e. ampicillin, streptomycin, chloramphenicol and sulphamethoxazole-trimethprim. In combination with five, six, seven and eight antibiotics, ~0.03% resistant organisms were isolated from Turag River and the rest from other sampling sites. It was observed that a five antibiotic resistant strain were also resistant to eight antibiotics. In Table 3, the number and percent of multiple antibiotic resistant isolates of two sampling and their mean are shown.

Prevalence of nalidixic acid resistant non O1 *V. cholerae*

Most of the *V. cholerae* isolated from water sample were nalidixic acid resistant and serotypically non O1. According to the data, it was found to grow *V. cholerae* non O1 only in the nalidixic acid plate whereas tetracycline, kanamycin and gentamicin plates were blank. Approximately, 90% of *V. cholerae* were serotyped and biochemically identified as non O1. The remaining 10% were O1 and O139.

Plasmid profile analysis

Plasmid DNA was isolated from multiple antibiotic resistant bacteria and was detected by agarose gel electrophoresis. Most of the *E. coli* were plasmid containing whereas *Pseudomonas*, *Aeromonas*, *Staphylococcus* and *Klebsiella* were non-plasmid containing. Some *V. cholerae* Non O1 also possessed a less number of plasmid. All strains of environmental sample did not contain plasmid in spite of carrying multi drug resistance capacity. Almost all three, four and five antibiotics resistant bacteria were plasmid containing. Most of the *E. coli* contain plasmid of different molecular weight (M.W.). Out of 67 strains, 58 strains were plasmid containing. On

Table 2. Isolation of different antibiotic resistant bacteria from environmental water sample in Dhaka.

Sampling site	CFU/ml (NA)	No. and percent of colonies per ml water														
		One antibiotic				Two antibiotics						Three antibiotics				Four antibiotics
		Tet	Nal	K	G	Tet, Nal	Tet, K	Tet, G	Nal, K	Nal, G	K, G	Tet, Nal, K	Tet, Nal, G	Tet,K, G	Nal, K, G	Tet, K, Nal, G
Turag River	$1.0x10^5$	0	$5.0x10^3$ (5%)	$1x10^3$ (1%)	0	300 (.3%)	200 (.2%)	300 (.3%)	400 (.4%)	200 (.2%)	$1.0x10^3$ (1%)	130 (.13%)	110 (.11%)	100 (.10%)	100 (.10%)	60 (.06%)
Buriganga River	$2.0x10^5$	$3.0x10^3$ (1.5%)	$1.2x10^4$ (6%)	$2x10^3$ (1.5%)	0	500 (.25%)	0	300 (.15%)	300 (.15%)	300 (.15%)	$1.6x10^3$ (0.8%)	70 (.03%)	90 (.04%)	90 (.04%)	60 (.03%)	10 (.005%)
Mohakhali Lake	$6.0x10^5$	$1.1x10^4$ (1.83%)	$1.2x10^5$ (20%)	$5x10^3$ (.8%)	$1x10^3$ (.16%)	$1.4x10^4$ (2.3%)	$1.3x10^3$ (.21%)	$1.9x10^3$ (.31%)	$2.1x10^3$ (.35%)	$3.5x10^3$ (.58%)	700 (.12%)	550 (.09%)	$1.8x10^3$ (.3%)	200 (.03%)	300 (.05%)	80 (.013%)
Rampura & Khilkhet Lake	$2.0x10^4$	0	0	$1x10^3$ (5%)	$1x10^3$ (5%)	0	0	0	100 (.5%)	0	200 (1%)	10 (.05%)	80 (.4%)	10 (.05%)	40 (.2%)	10 (.05%)

Tet, Nal, K and G represents tetracycline, nalidixic acid, kanamycin and gentamicin, respectively.
NA = No antibiotic.

the basis of plasmid pattern, sixteen differrent patterns were observed as shown in Figure 2. The size of the plasmid ranges from 1 to 15 kb.

Analysis of electroporation

The representative plasmid DNA were electroporated into the *E. coli* DH5α that was prepared for competence by 10% glycerol due to the intention to find out the antibiotic coding region whether it was chromosomal or extrachromosomal. That is the reason electrporated plasmid DNA containing *E. coli* DH5α were plated onto the selective antibiotics containing plate after over night incubation at 37°C. For these purpose, those antibiotics were used for plating that were resistant to the native bacteria from which plasmid DNA was isolated. No detectable transformed antibiotic resistant plasmid containing *E. coli* DH5α was found after analyzing thirty five bacteria. These results suggested that all environmental bacteria were genotypically resistant by their chromosomal locus.

DISCUSSION

The emergence of drug resistant diseases is one of the most serious health problems in modern society, particularly in Bangladesh. The interest of this study was to determine the content of drug resistant strain and the rate of the change of natural strain to drug resistant strain in the aquatic environment. This happens, for instance, when antibiotics are misused or overused (Nuermberger and Bishai, 2004).

Frequent use of antibiotics in medicine and in food of animal origin production has resulted in an increase in the prevalence of bacterial strains resistant to these antimicrobial agents (Kummerer, 2004; Levy, 2002; Salyers, 2002; Witte, 1998). The low effectivity of antibiotics results in infections that are more difficult to treat (Hall, 2004). In the medical community, the need for prudent use of antibiotics is accepted worldwide. Furthermore, the European Union has banned the use of several antibiotics as growth promoters (avoparcin, bacitracin, spiramycin, tylosin, and virginiamycin) in the animal industry, and there are proposals to withdraw more antibiotics (Aarestrup et al., 2001). In contrast, in the United States, antimicrobial agents are used widely as food additives to improve growth and feed conversion in many types of animal operations, including poultry, swine, and cattle operations. As a result, antibiotic resistance in the bacterial communities in the intestinal tracts of domestic animals has become common (Aarestrup et al., 2002; Aarestrup et al., 2000; Haack and Andrews, 2000).

The main result of this analysis is that with an efficient drug against the natural strain, if there is

Table 3. Checking for multiple antibiotic resistance.

No. of Sampling	Sampling site	CFU/ml (No antibiotic)	No. and percent of colonies per ml water			
			Five antibiotics (Tet, K, G, Nal, Amp)	Six antibiotics (Tet, K, G, Nal, Amp, S)	Seven antibiotics (Tet, K, G, Nal, C, AMP, S)	Eight antibiotics (Tet, K, Nal, G, AMP, S, C, SXT)
1	Turag River	$1.0x10^5$	38 (.03%)	35(0.035)	30 (.03%)	27 (.027%)
2	Turag River	$3.16x10^5$	33 (.01%)	0	0	0
Mean		$2.18x10^5$	57.81	54.9	61.23	13.25

Tet, Nal, K, G, Amp, S, C and SXT represents tetracycline, nalidixic acid, kanamycin, gentamicin, ampicillin, streptomycin, chloramphenicol and sulphamethoxazole-trimethoprim, respectively.

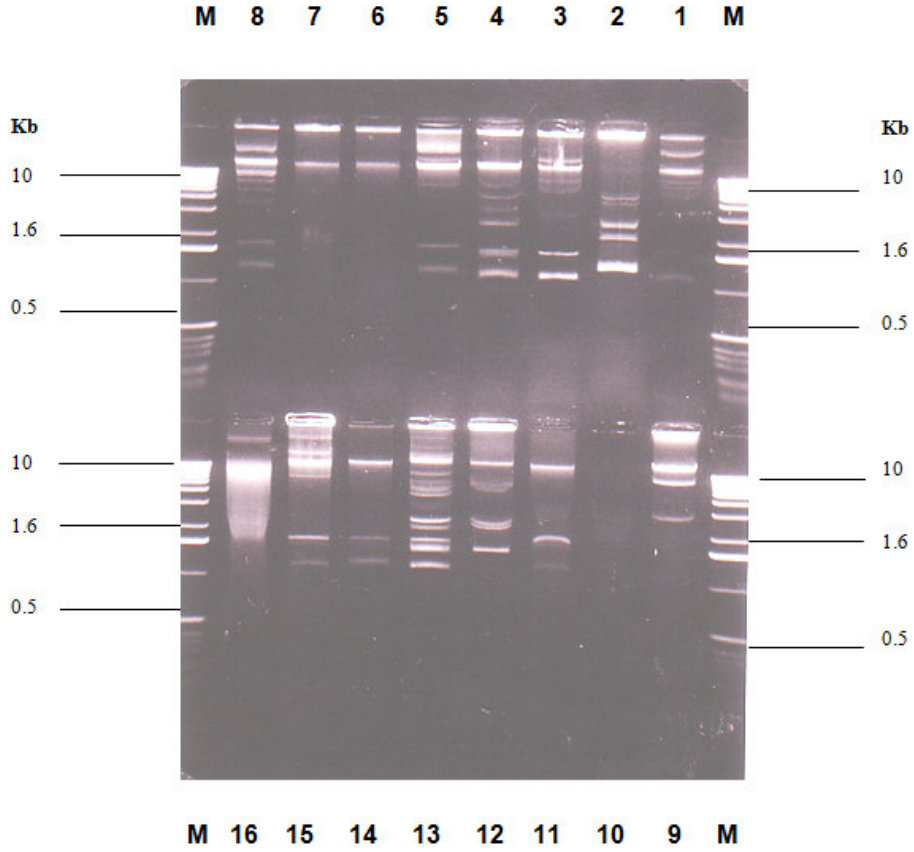

Figure 2. Electrophoretic pattern for the plasmid profile of the multiple antibiotic resistant bacteria. Agarose gel electrophoresis of plasmid DNA isolated from representative *E. coli*. Sixteen plasmid patterns are shown in the figure after analyzing fifty two strains. Lane: (1-16): Samples of plasmid DNA, M: 1 Kb DNA ladder.

even a small chance that the natural strain alters into the drug resistant one, then there will eventually be an outbreak of the drug resistant strain throughout the population. In that case the natural strain disappears and is replaced by the drug resistant strain. The disturbing part of this is that an efficient treatment of the natural strain gives an edge to the drug resistant strain. This pathogen is responsible for potentially severe infections in the community, and has a great capacity for acquisition of resistance to antibacterial agents (Carbon and Isturiz, 2002).

In this study, initially a series of the combination of four different antibiotics (tetracycline, nalidixic acid, kanamycin and gentamicin) were used to isolate multi-drug resistant bacteria from the environmental water samples in Dhaka. The major percent of isolates were resistant to tetracycline and nalidixic acid. A few of them were only nalidixic acid and gentamicin resistant. As the data of one

sampling site, nalidixic acid resistant strains were 5%, tetracycline resistant 1%, kanamycin resistant 1%, gentamicin resistant 0.5%. In combination with two antibiotics, tetracycline and nalidixic acid resistant were 1% and the rest were less than 1%. Six percent resistant isolates were found in case of four antibiotics. The representative resistant colonies were further checked gradually by increasing the number of antibiotics includeing ampicillin, streptomycin, chloramphenicol and sulphamethoxazole-trimethprim by replica plating. In combination with five, six, seven and eight antibiotics, ~0.03% resistant organisms were isolated from Turag River and the rest from other sampling sites. Five antibiotics resistant strain were also resistant to eight antibiotics.

Six types of multidrug resistant bacteria i.e. *E. coli*, *V. cholerae*, *Pseudomonas*, *Aeromonas*, *Klebsiella* and *Staphylococcus* were identified by different biochemical test from the isolated microbes. *V. cholerae* O1 and O139 were stereotyped using the commercially available antisera that contains somatic 'O' antigen. River water of Dhaka city contained the highest proportion of *E. coli* and *V. cholerae* non O1. Isolation rate of pathogenic *V. cholerae* O1 and O139 were relatively lower than *V. cholerae* non O1. Sixty five percent of these isolates were identified as *E. coli* which were resistant to multiple drugs including tetracycline, nalidixic acid, kanamycin, streptomycin and sulphamethoxazole-trimethoprim. The remaining thirty five percent of the isolates were *V. cholerae*, *Pseudomonas*, *Aeromonas*, *Staphylococcus* and *Klebsiella*. In a recent study, totally 3,500 gram-negative bacterial strains were isolated from 13 hospitals in Guangzhou in the past two years, and the top 3 most common pathogens of them were *E. coli* (1,244 strains, 35.5%), *Klebsiella pneumoniae* (900 strains, 25.7%), and *Pseudomonas aeruginosa* (547 strains, 15.6%) (Xiao et al., 2005).

Most of the *V. cholerae* were nalidixic acid, sulphamethoxazole-trimethoprim and streptomycin resistant and a few number were sulphamethoxazole-trimethoprim and streptomycin resistant. *E. coli* were tetracycline, kanamycin, gentamicin, ampicillin, streptomycin, sulphamethoxazole-trimethoprim and nalidixic acid resistant. The most frequent resistance was resistance to nalidixic acid and sulfamethoxazole-trimethoprim, although resistance to high levels of aminoglycosides (streptomycin, kanamycin and gentamicin) and tetracycline was also detected. A total of 210 different bacteria were isolated from water samples collected in different surveillance sites in Dhaka between May 30, 2004 and July 30, 2004. Of these bacterial isolates, 147 isolates were analyzed and 103 were identified by different biochemical tests. From this result, it can be concluded that, water is the natural reservoir of multi-drug resistant bacteria. In 2002, a literature mentioned 4 ecological compartments considered as "reservoirs for dissemination and transfer of microbial antibioresistance i.e. humans, animals, plants and natural soil and water (Kang et al., 2005).

Plasmid DNA was isolated from these multiple antibiotic resistant bacteria. Most of the *E.coli* were plasmid containing whereas other identified strains such as *Pseudomonas*, *Aeromonas*, *Staphylococcus*, *Klebsiella*, *V. cholerae* O1 and O139 were non-plasmid containing but resistant to seven or eight different types of antibiotics. Some *V. cholerae* non O1 were plasmid containing. All strains of environmental sample did not contain plasmid in spite of carrying multi drug resistance capacity. Seventy five percent of the *E. coli* contained plasmid of different molecular weight (M.W.). Out of 67 strains of *E. coli*, 58 strains were plasmid containing. Analysis of plasmid DNA pattern of *E. coli* revealed that most of the *E. coli* strains contained multiple numbers of plasmid ranging from 1 to 15 kb. Plasmid patterns were formed according to the number and molecular weight of the plasmid and strains were grouped in a particular pattern. Sixteen different plasmid patterns were found after analyzing fifty-two strains of *E. coli*. Electroporation experiment of this plasmid DNA was carried out to assess the genetic location of antibiotic resistant determinants whether it was chromosomal or extrachromosomal and thus it can be suggested that almost cent percent of bacteria were resistant by their chromosomal locus.

In conclusion, Bangladeshi contaminated aquatic environment contained a high content of multiple antibiotic resistant bacteria as a natural reservoir.

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