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Short Communication

Characterization of Coliform Bacteria Isolated From Surface Water

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ABSTRACT: The present study was undertaken to assess the phenotypic and genotypic characteristics of total coliforms bacteria. The work involved in isolation, identification and characterization of the coliform bacteria e.g. *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae and Citrobacter freundii* from surface waters in and around Dhaka, Bangladesh. Among the 278 environmental isolates, 48 were *Escherichia coli*, 19 *Klebsiella pneumoniae*, 18 *Enterobacter cloacae* and 37 *Citrobacter freundii* which were primarily identified by cultural, biochemical and analytical profile index test (API) tests. *Escherichia coli* were further confirmed by using the fluorescence agent, 4-methylumbelliferyl-β-D-glucuronide (MUG). At 37^{o}C, 84% *E. coli* gave positive result for MUG whereas only 46% *E. coli* were MUG positive at 44.5^{o}C. Only 16% *K. pneumoniae* was MUG positive at 37^{o}C and *E. cloacae* and *C. freundii* could not grow on MUG medium. In this study, Ceftazidime, Aztreonam, Cefixime and Amoxycillin/Clavulanic acid (2:1) antibiotics were used to assess extended spectrum β- lactamase producing bacteria. Total 86% of the coliform showed positive result for ESBL. In this study, sixteen different antibiotics were used for observation of drug resistance pattern by disk diffusion method. Most of the strains were resistant to penicillin, oxacillin, erythromycin, bacitracin respectively and the lowest resistance was against imipenam. Correlation with the tolerance/susceptibility of antibiotics and the plasmid profile of coliforms was studied. Based on the result, the most resistant isolate was identified as *Klebsiella pneumoniae* and its plasmid size was 20.92 KB. Congo red binding test was also performed to differentiate between virulent and avirulent *E. coli*. Within coliform, 27% and 11% of *E. coli* and *Klebsiella* sp. gave positive result for congo red binding assay respectively. The finding of this study revealed that drug resistance and other virulence properties potential of the members of the coliforms was observed in many isolates that reflects an alarming signal for water quality. It also presents a scenario of the acquiring of virulence properties and drug resistance to a group of bacteria that are considered to be avirulent commensals to human.

KEYWORDS: Coliforms, Surface water, Water quality

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INTRODUCTION

Water plays a vital role in the development of healthy human communities. Degradation of water quality is the unfavorable alteration of the physical, chemical, biological properties of water that prevents its domestic, commercial, industrial, agricultural, recreational and other beneficial uses(1). In the developed world, water related diseases are rare due to the presence of efficient and safe water supply. However, in the developing world like Bangladesh, water, if contaminated, has greater potential for transmitting a wide variety of diseases and illnesses. Traditionally, indicator bacteria have been used to determine the possible presence of fecal contamination and to estimate the amount of contamination in water, foods, and other samples. The detection of indicator bacteria is preferred over direct pathogen detection because the former are considered to be normal, non pathogenic intestinal inhabitants that are present in feces and waste water in much higher numbers than are pathogenic microorganisms and because they are technically easier to detect and quantify than pathogens. Present standards for the sanitary quality of water, foods and other materials, with respect to fecal contamination, are based on concentrations of indicator bacteria. Originally, total coliform bacteria were considered to be from four genera of the family Enterobacteriaceae that could all ferment lactose. These genera were Escherichia, Klebsiella, Enterobacter and Citrobacter. Of the total coliforms present in the human gut, E. coli represents the majority of the population. Total coliforms represent only about 1% of total population of bacteria in human feces in concentrations of about 109 bacteria/gm (2).

In general terms, the greatest microbial risks are associated with ingestion of water that is contaminated with human or animal feces. Wastewater discharges in fresh waters and coastal seawaters are the major source of fecal microorganisms, including pathogens (3).

Acute microbial diarrheal diseases are a major public health problem in developing countries. People affected by diarrheal diseases are those with the lowest financial resources and poorest hygienic facilities. Children under five, primarily in Asian and African countries, are the most affected by microbial diseases transmitted through water. The direct detection of pathogens in the water would provide the best evidence for their existence (4). Therefore, in this study we attempt to detect and characterize the total coliforms as indicator from surface water of Dhaka city to assess the water quality.

MATERIALS AND METHODS:

Sampling sites: Samples were collected from different rivers like Turag, Balu, Birulia, Shitolakkha, Meghna, Buriganga, Kalna and Narod. Special care was taken for sampling and source selection. A mercury thermometer graduated between 0°C to 100°C was used to record temperature. A pH meter (HANNA instruments HI 8520) was used to determine the pH of the collected water samples.

Isolation and presumptive identification of coliforms from water samples: 100 µl of surface water was spread on different selective media and incubated at 37oC for overnight. After incubation, two to five morphologically different typical of 4 coliforms colonies were picked up and streaked onto MacConkey, Xylose Lysine Deoxycholate agar (XLD), Eosin Methylene Blue (EMB), Deoxycholate Citrate Agar (DCA), Sorbitol MacConkey (S-MAC) agar plates for pure culture as well as for presumptive confirmation.

Identification of isolates using API system: This is a standardized identification system for member of Enterobacteriaceae and other non fastidious Gram negative rods, which uses 23 miniaturized biochemical tests and a database. The API 20E strip (BioMerieux, France) consists of 20 micro-tubes containing dehydrated substrates.

Biochemical Tests: Biochemical tests were performed according to the methods described in the Bergey`s Manual of Systematic Bacteriology (5). This test included Oxidase test, Simmons Citrate tests, Kligler Iron Agar (KIA) test, Motility Indole Urease test, as well as some sugar fermentation and amino acid utilization test.

Identification of E.coli isolates using the fluorogen MUG (C16H16O9): Fluorogenic procedure with the substrate MUG has become common for the identification of E. coli isolated from urine sample. E. coli are able to produce glucoronidase enzyme that cleaves the MUG substrate, providing a fluorescent end product methylumbelliferons that is detectable under a long-wave UV light. For satisfactory results, incubation temperatures were kept between 22oC and 44.5oC (6). In this test, agar plates containing MUG were incubated at 37oC and 42oC. But in this study, we also carried out the MUG test for Klebsiella sp.

Determination of antimicrobial susceptibility of the coliforms: Antimicrobial susceptibility was determined on Muller-Hinton agar plates following the Bauer-Kirby method (7). The following antimicrobial agents such as ampicillin (10 µg), nalidixic acid (300 unit), oxacillin (10 µg), ceftotaxime (30 µg), ceftazidime (30 µg), imipenem (10 µg), tetracyclin (30 µg), chloramphenicol (30µg), erythromycin (15 µg), amoxicillin/clavulanic acid 2:1 ratio (10µg), penicillin (10µg), bacitracin (10µg), methicilin (5µg), ciprofloxacin (5µg), ceftriaxone (30µg) and gentamicin (10µg) were used for drug sensitivity test of total coliforms. All antibiotics disks were commercially available and purchased from Oxoid, In Vitro Diagnosticum, USA.

Congo red binding assay: Congo red (CR) dye containing defined medium are able to produce pigmented colonies which can be used to differentiate avirulent and virulent strain of Enterobacteriaceae. In this experiment three different congo red concentration was employed. Out of three concentration, 0.015% permitted the best discrimination between virulent and avirulent E. coli and Klebsiella spp. isolates (7).

Determination of β-lactamases activity by double disk diffusion method: Phenotypic detection of ESBL production was performed using a modified double-disk synergy test (mDDST)(8). In this test, conjugate antibiotic Amoxycillin/Calvulanic acid and three other Cephalosporin group antibiotics (Ceftazidime, Aztreonam and Imipenem) were used.

Plasmid analysis: Plasmid DNA was prepared by SDS/alkali lysis method (9).

RESULT AND DISCUSSION

Isolation and Identification of coliforms

The presence of coliforms was confirmed by various cultural, biochemical and Analytical Profile Index (API) 20E. According to cultural and biochemical properties of coliforms, among the 278 isolates, 48 (17.27%) isolates were E. coli, 19 (6.83%) isolates were K. pneumoniae, 18 (6.47%) isolates were E. cloacae and 37(13.31%) isolates were C. freundii respectively.

Methyl-Umbelliferyl (MUG) test – a confirmatory test for E.coli

The MUG assay is based on the enzyme activity of β-glucoronidase (GUS) which cleaves the substrate 4-methylumbelliferyl β-D-glucoronide, to release 4-methyumbelliferron (MU). When exposed to long-wave UV light (365nm) light 4-methylumbelliferone exhibit a bluish fluorescence that is easily visualized in the medium or around the colonies. Several report suggested that over 95% E. coli produces GUS, including anaerobic, non-gas producing strains. Enterohemorrhagic E. coli (EHEC) of serotype O157:H7 has been shown to be GUS negative strains (10). In the present study, 84% of the total E. coli showed MUG positive at 37oC whereas 46% of E. coli was able to grow on MUG medium at 44.5oC. Only 16% K. pneumoniae was MUG positive at 37oC while E. cloacae and C. freundii could not grow on MUG medium respectively.

Antibiotic sensitivity test and plasmid profiling

In this study, 100% E. coli were resistant against erythromycin, penicillin G, bacitracin, and oxacillin respectively (Figure 1A). K. pneumoniae isolates (100%) were resistant against seven antibiotics which were ampicillin, amoxicillin, penicillin G, bacitracin, oxacillin and nalidixic acid and all (100%) showed sensitivity to imipenem (Figure 1B). All E. cloacae were resistant against six antibiotics i.e ampicillin, oxacillin, erythromycin, amoxicillin, bacitracin, penicillin G and 93.33% showed highest sensitivity to imipenem (Figure 1C). On the other hand, 100% C. freundii were resistance against eight antibiotics such as ampicillin, erythromycin, amoxicillin, bacitracin, penicillin G, cefotaxime, methicillin and oxacillin (Figure 1D).

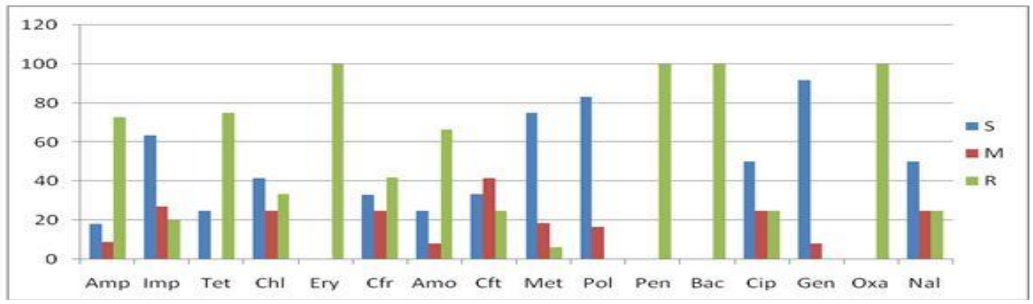

Fig: 1A

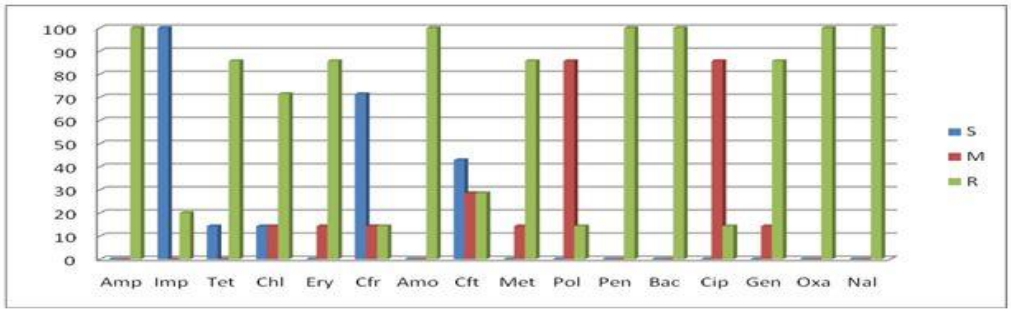

Fig: 1B

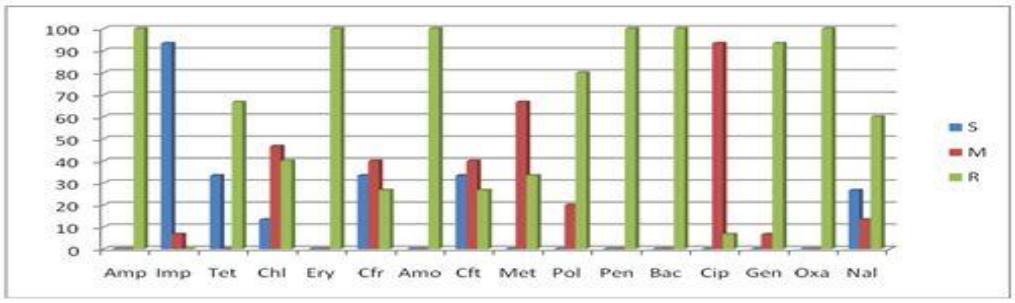

Fig: 1C

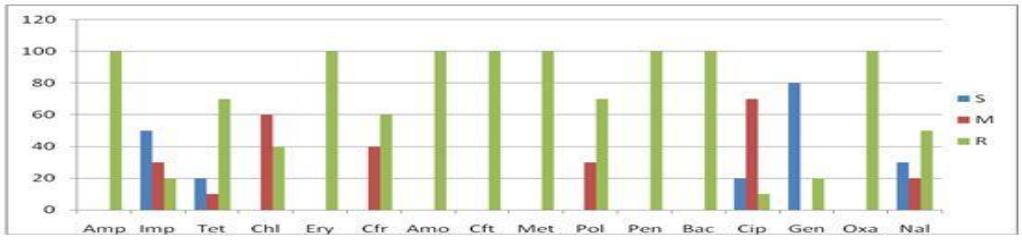

Fig: 1C

Figure 1. Antibiotic sensitivity pattern of A. *E. coli* B. *Klebsiella spp.* C. *Enterobacter cloacae* D. *Citrobacter freundii* (Here, S for sensitive, M for moderate and R for Resistant)

By observing the results, it was concluded that all of the coliforms were resistant to Ceftazidime. The second most resistant pattern was against Aztreonam and Augmentum. Since the 4th generation antibiotics were used, so these data revealed an alarming indication of the drug resistant *E. coli* strains that seems to be increasing day by day.

Relationship between plasmid profiles and drug resistance

Plasmid profiling was done for multidrug resistant coliforms. Among the 28 multidrug resistant isolates, three of *E. coli* had the same plasmid size (18.96 KB) and they showed resistance to Ceftazidime, Imipenem, Augmentum (Clavulanic acid 2:1/Amoxycillin). Four isolates of *K. pneumoniae* contained the same plasmid size (20.92 KB), this was the largest plasmid and they gave resistant pattern to Aztreonam, Imipenem and Ceftazidime. Three isolates of *E. cloacae* had the same plasmid size (16.21KB) and they were resistant to Aztreonam, Ceftazidime and Augmentum (Clavulanic acid2:1/Amoxycillin). In case of *C. freundii*, two types of plasmid size were observed (19.35 and 16.21 KB). The first one were resistant to Ceftazidime, Aztreonam and Augmentum (Clavulanic acid2:1/Amoxycillin) and the second one gave resistant pattern against Ceftazidime, Aztreonam, Imipenem and Augmentum. It was observed that 20 *E. coli* isolates contained plasmids of the same size (18.96 KB) with similar antibiotic resistance pattern.

Treatment of diseases with antibiotic and chemotherapeutic agents was responsible for the occurrence of drug resistance trait among enteric pathogens and the majority of drug resistant bacteria carry transferable drug resistance (R) factor [(11)]. Through discharge of human and animal

fecal material, drug resistant bacteria are distributed in the surface water where exchange of R plasmids can occur under certain physical, chemical and biological conditions [(12)]. Thus drug resistant bacteria can spread in the environment where man and animal acquire infection with bacteria carrying drug resistant plasmids. In Bangladesh, there is clear evidence of abuse of antibiotics which was responsible for occurrence of drug resistance of the pathogenic bacteria [(13)].

Congo red binding test of coliforms

E. coli isolates bind to Congo red (CR) dye and produce pigmented colonies on defined media can be used to differentiate avirulent and virulent *E. coli*. The concentration 0.015% permitted the best discrimination between avirulent and virulent *E. coli* isolates.

The table-2 showed that 27% of the *E. coli* samples gave positive result in Congo red binding test which indicated that these strains were possibly virulent. Usually *Klebsiella* spp. does not give positive result for Congo red test. But out of 18 isolates 2 isolates had given positive result. This pie chart indicates that 11% of the *K. pneumonia* isolates were positive for Congo red binding test and the other 89% were negative.

Table-1: Congo red binding assay

Organisms	Positive (%)	Negative (%)
E. coli	27	73
*Klebsiell*a spp	11	89

Extended Spectrum β- Lactamase (ESBL) producing coliforms: β- lactamases are enzymes produced by some bacteria and are responsible for their resistance to β-lactum antibiotics like penicillins, cephamycins and carbapenems (ertapenem), (Cephalosporins are relatively resistant to β-lactamase). These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactum. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties [(14)]. In this study we found that 86% of the total coliforms were positive for ESBL activity as shown by double disk diffusion method and the rest 14% were negative for β-lactamase activity.

Concluding Remarks

In the present study, we found that the coliform bacteria mostly *E. coli* and *Klebsiella* was becoming multidrug resistance and also virulent strains were increasing which is very alarming and a public health concern for using surface water.

Acknowledgement

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