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BACTERIAL LOAD, MULTIPLE ANTIBIOTIC-RESISTANCE PATTERNS, AND CYTOTOXIC EFFECTS OF COLIFORM AND COLIFORM-RELATED BACTERIA ASSOCIATED WITH THE SURFACE WATER OF DHAKA CITY

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Abstract

The surface water quality of Dhaka Metropolitan City was studied from June to December 2022, with special attention to coliform bacterial pollution. The aerobic heterotrophic bacteria, total coliform, and fecal coliform were high in Buriganga and Shitalakshya River water compared to other selected water bodies. The physico-chemical parameters of the selected rivers and lakes were unsatisfactory. A total of 110 isolates were obtained, of which 26 were selected for further study. Twelve Gram-positive isolates belonged to the genera *Enterococcus, Micrococcus, Planococcus* and *Bacillus.* Fourteen Gram-negative isolates belonged to the genera *Escherichia, Klebsiella, Enterobacter, Alcaligenes, Pseudomonas, Plesiomonas, Aeromonas* and *Salmonella.* Most of the Gram-negative bacteria were multiple antibiotic resistant (MAR). *Plesiomonas shigelloides* showed the highest MAR (50%). *Klebsiella oxytoca* (MAR 40%) and *E. coli* (MAR 30%) showed a positive cytotoxic effect on the non-cancer cell line. Coliform bacteria associated with the surface water of Dhaka City pose a serious health threat to city dwellers.

Introduction

Surface water contamination by fecal waste is a major public health concern due to the presence of pathogenic microorganisms, particularly bacteria derived from feces. Coliforms serve as indicators of fecal pollution, and their abundance correlates with the presence of other pathogenic bacteria (Patel *et al.* 2016). Bacteria found in the guts of humans and other warm-blooded animals can enter the aquatic environment through feces, causing serious infections through drinking or contact with contaminated water (Florio *et al.* 2018). Dhaka, the capital of Bangladesh and one of the most densely populated metropolitan cities in the world, faces the serious problem of water pollution (Saha *et al*. 2017, Sarker *et al.* 2019). Fecal pollution of surface water is prevalent in Dhaka City due to poor sanitation management, inadequate fecal sludge management, and rapid unplanned urbanization (Amin *et al.* 2019).

Bangladesh, a developing country in Southeast Asia, is at high risk of antibiotic resistance due to inadequate regulations, self-medication, and indiscriminate use of antibiotics in agriculture and livestock production (Khan *et al.* 2020). The emergence of resistance in aquatic environments might harm human health through potential transmission via different pathways, including drinking water, bathing water, and the food chain (Döhla *et al.* 2019).

Some strains of coliform bacteria have been associated with carcinogenesis, such as *Klebsiella* spp.*, Enterococcus* spp.*, Helicobacter pylori* and pathogenic *Escherichia coli,* due to the production of harmful cytotoxins (Collins *et al.* 2010). Thus, the present investigation aimed to examine surface water samples of Dhaka City for the presence of antibiotic-resistant coliform bacteria and the cytotoxic activity of the selected isolates on Vero cells.

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Materials and Methods

During the summer and winter months, surface water samples from the ten natural water bodies inside the Dhaka Metropolitan City were gathered in plastic bottles that had been disinfected with ethanol. The water features that were chosen were Shahidullah Hall Pond, Jagannath Hall Pond and Zahurul Haque Hall Pond of Dhaka University, Ramna Temple Pond, Azimpur Pond, the Dhanmondi Lake, the Hatirjheel Lake, the Gulshan Lake and the Buriganga River (Sadar ghat) and the Shitalakshya River.

Measurements of the physico-chemical properties of the collected water samples were performed according to American Public Health Association Standard Methods (APHA 2012). The pH of the collected water samples was measured with an electric pH meter (HANA HI 8424). EC was determined by an EC meter (HANA HI 8733). DO and BOD_5 were measured by the DO-3IP meter (DKK-TOA).

Different culture media were used for the isolation and enumeration of different bacteria, including nutrient agar (NA) for aerobic heterotrophic bacteria, MacConkey agar and SS agar for enteric bacteria, and m-FC agar media for fecal coliform bacteria. The pH of each culture medium was adjusted to 7.2 during media preparation. Serial dilution (Clesceri *et al.* 1998), spread plate (Sharp and Lyles 1969), and membrane filtration technique (Atlas and Snyder 1995) techniques were used to enumerate and isolate bacteria. The inoculated plates were incubated at 37°C for 24 hrs. Bacterial colony-forming unit (cfu) was recorded using a digital colony counter (DC-8 OSK 100086, Japan). After counting, individual bacterial colonies were immediately isolated. Based on morphological characteristics, primary selection was done, and the selected bacterial isolates were purified by streak plate method. Gram staining was done for the identification of Gram-positive and Gram-negative bacteria. Significant physiological and biochemical tests were performed for the provisional identification of the selected isolates. Provisional identification of Gram-positive bacteria was performed according to Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986), and Gram-negative bacteria were identified following the Manual of WHO (Krieg and Holt 1984). The selected isolates were subjected to culture and sensitivity tests (C/S) based on CLSI (2018) guidelines to determine resistance patterns against 10 common antibiotics. The tested common antibiotics were Ampicillin (AMP 10), Penicillin G (10), Ciprofloxacin (CIP 5), Streptomycin (S 10), Erythromycin (E 15), Cefixime (CFM 5), Doxycycline (DO 30), Tetracycline (TE 30), Neomycin (NE 30), and Gentamicin (CN 10).

For the cytotoxicity test, overnight cultures of the selected bacterial isolates were suspended in phosphate buffer saline (PBS), and pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The pellets were re-suspended in 1.5 ml of PBS, and to prevent overheating, the re-suspended pellets were sonicated on ice for 5 min. After sonication, samples were centrifuged at 12,000 rpm for 10 min at 4°C. Cell-free supernatants (CFS) were collected and filter-sterilized via 0.2 µm filters (McCoy *et al.* 2021). The Vero cells are kidney epithelial cells of African green monkey, which were collected from the Cell and Tissue Culture Research Laboratory, Centre for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh, and maintained in DMEM (Dulbecco’s Modified Eagles’ medium). Trypsin was used for the detachment of monolayer cells and the preparation of cell suspensions. Viable cells were counted by a hemocytometer and the cell suspension was diluted with DMEM containing 10% FBS in order to obtain a final density of $15x10^4$ cells/ml. 48-well plates were seeded with 200 microliters of cell suspension per well and incubated for cell attachment at 37°C and 5% CO_2. The Vero cells were treated with the test sample CFS after 24 hrs. Bacterial growth medium served as a control. After the addition of the bacterial cell-free extracts, plates were incubated for an additional 48 hrs at 37°C, 5% CO_2, and 100% relative humidity. After 48 hrs of incubation, cytotoxicity was examined under an inverted light microscope (Optika, Italy). Duplicate wells were used for each sample.

Results and Discussion

The physico-chemical properties of the collected water samples are given in Table 1. The pH is a key variable that indicates pollution levels in any aquatic environment. In the rainy season, the pH values ranged from 6.67 to 7.75, and in the winter season, they ranged from 6.94 to 7.86 (Table 1), which is considered ideal for the survival of aquatic species (Kumar *et al.* 2020). The highest pH (7.86) was recorded in Shahidullah Hall pond and Azimpur pond water during the winter season. During the winter, the pH levels were slightly higher due to the dilution of effluents caused by the rainwater flow in the rainy season. Saha *et al.* (2012) showed a pH of 7.31 in Azimpur colony pond water. Alam *et al*. (2020) reported a similar pH (6.50–7.60) in Shitalakshya River water.

Table 1. Physicochemical parameters of surface water during rainy and winter season.

Sampling sites	pH		EC (µs/cm)		DO (mg/l)		BOD (mg/l)	
	Rs	Ws	Rs	Ws	Rs	Ws	Rs	Ws
Shahidullah Hall Pond	7.21	**7.86**	268	314	6.55	4.78	4.48	3.83
Jagannath Hall Pond	6.88	7.57	177	356	4.22	4.18	2.85	3.97
Ramna Temple Pond	6.89	7.46	240	135	2.97	5.81	1.98	2.85
Azimpur Pond	7.75	**7.86**	626	543	6.38	3.07	4.40	4.13
Zahurul Hall Pond	7.65	7.50	223	215	5.25	3.96	5.20	4.77
Gulshan Lake	7.64	7.44	**694**	580	3.35	2.09	3.08	2.60
Hatirjheel Lake	7.50	7.19	567	577	4.75	3.80	2.26	5.50
Dhanmondi Lake	6.94	7.32	324	332	6.24	4.67	5.16	4.59
Buriganga River (Sadar ghat)	6.87	6.94	248	597	3.12	**1.30**	**5.52**	2.66
Shitalakshya River	6.67	7.44	219	192	**0.75**	1.05	0.89	2.20

Rs = Rainy season; Ws = Winter season; EC = Electrical conductivity; DO = Dissolved oxygen; BOD = Biochemical oxygen demand.

According to standard (De 1999), the electrical conductivity (EC) value of surface water is <350 µs/cm. The highest EC value (694 µs/cm) found in Gulshan Lake water in the rainy season indicates that the Lake Gulshan may receive industrial wastewater containing high levels of ion concentration, which decreases the quality of the water. In a previous study, Ahmad *et al.* (2018) reported an EC value of 428.24 µs/cm in Gulshan Lake. If any water body has a <5 mg/l DO value and a >5 mg/l BOD_5 value, then it will be highly polluted (De 1999). The lowest DO values in this investigation were obtained in the Shitalakshya and Buriganga rivers (0.75–1.05 mg/l and 1.30–3.12, respectively) for both seasons. Results showed that the water quality in these rivers is lower than that of ponds and lakes (Table 1). The highest BOD_5 was 5.52 mg/l for the River Buriganga (Sadar ghat) water during the rainy season, and this value was slightly higher than the acceptable range, indicating the highest level of water pollution. Hasan *et al.* (2019) reported a BOD_5 value of 9.1 mg/l in Buriganga River (Sadar ghat) water.

The total aerobic heterotrophic bacterial load in the water samples tested varied between 3.2×10^5 and 1.72×10^6 cfu/mL during the rainy season and between 2.4×10^4 and 6.8×10^4 cfu/ml during the winter. The Buriganga River water had the greatest concentrations of heterotrophic bacteria, total coliform, and fecal coliform, with respective values of 6.8×10^4 to 1.72×10^6 cfu/ml, 4.5×10^4 to 2.4×10^6 cfu/ml and 2.3×10^4 to 3.2×10^5 cfu/ml (Table 2). The bacterial load was found to be high in the Shitalakshya River as well, ranging from 5.7×10^4 to 1.36×10^6 cfu/mL, 3.8×10^4 to 1.0×10^6 cfu/ml and 1.5×10^4 to 2.8×10^5 cfu/ml, respectively (Table 2).

Table 2. Bacterial load (cfu/mL) of the collected water samples.

Water sampling sites	Rainy season			Winter season		
	Aerobic heterotrophic bacteria	Total coliform	Fecal coliform	Aerobic heterotrophic bacteria	Total coliform	Fecal coliform
Shahidullah Hall Pond	4.3×10^5	3.9×10^5	None	3.0×10^4	2.3×10^4	None
Jagannath Hall Pond	4.0×10^5	1.8×10^5	None	3.1×10^4	1.8×10^4	None
Ramna Temple Pond	3.8×10^5	1.9×10^5	None	3.8×10^4	2.3×10^4	None
Azimpur Pond	3.9×10^5	3.4×10^5	None	4.4×10^4	3.1×10^4	None
Zahurul Hall Pond	3.6×10^5	2.7×10^5	None	3.1×10^4	2.2×10^4	None
Gulshan Lake	6.5×10^5	3.0×10^5	2.1×10^5	4.0×10^4	2.8×10^4	1.3×10^4
Hatirjheel Lake	4.8×10^5	2.2×10^5	1.3×10^5	3.3×10^4	2.5×10^4	1.1×10^4
Dhanmondi Lake	3.2×10^5	1.5×10^5	None	2.4×10^4	1.1×10^4	None
Buriganga River (Sadar ghat)	1.72×10^6	2.4×10^6	3.2×10^5	6.8×10^4	4.5×10^4	2.3×10^4
Shitalakshya River	1.36×10^6	1.0×10^6	2.8×10^5	5.7×10^4	3.8×10^4	1.5×10^4

The Gulshan Lake and Hatirjheel Lake water were also investigated, and a significant number of different groups of bacteria were noticed compared to Dhanmondi Lake. Respectively, Azimpur pond, Shahidullah Hall pond, Jagannath Hall pond, Ramna Temple pond, and Zahurul Haque Hall pond water were investigated. Interestingly, no fecal coliform was found in the selected pond water or in Dhanmondi Lake during the rainy and winter seasons (Table 2). Because of the excessive fecal matter caused by human waste, animal excrement, and significant rainfall during the rainy season, there was an increased load of bacterial populations, particularly coliform bacteria. Saha *et al.* (2009) found in a previous investigation that the maximum count of heterotrophic bacteria in the Buriganga River varied from 1.0×10^5 to 42.0×10^5 cfu/ml. The Turag River had very similar total coliform loads (4.55×10^4 cfu/ml) during the monsoon and 2.76×10^4 cfu/ml during the winter, according to Begum *et al*. (2018). The current findings demonstrated that the water in the Buriganga and Shitalakshya Rivers was heavily contaminated with bacterial populations, including coliforms, but significantly safe for the ponds at the Dhaka University campus.

During the present investigation, 110 bacterial colonies were isolated from the selected water bodies, of which 26 were finally selected based on their size, shape, elevation, margin, texture, appearance, color, and optical density. Among the 26 selected isolates, 12 were Gram-positive bacteria belonging to the genera *Bacillus, Planococcus, Micrococcus*, and *Enterococcus*. On the other hand, 14 isolates were Gram-negative rods and belonged to the genera *Escherichia, Enterobacter, Klebsiella, Alcaligenes, Pseudomonas, Plesiomonas, Aeromonas*, and *Salmonella* (Table 3). Yasmin *et al.* (2023) reported the presence of similar bacteria, including *Bacillus subtilis, Klebsiella* sp., *Escherichia coli, Alcaligenes* sp., and *Salmonella* sp., in the surface water of the Gomati River.

Fig. 1 showed that, among the 26 bacterial isolates, *Plesiomonas shigelloides* (D-7) exhibited the highest percentage (50%) of MAR, and *Bacillus alvei, B. schlegelii, Micrococcus* sp., *Enterococcus faecalis, Escherichia coli, Enterobacter* sp., and *Salmonella typhi* showed the lowest percentage (30%) of MAR. Most of the isolates were resistant to penicillin and erythromycin, which is very alarming. In a previous study, Odonkor *et al.* (2021) reported that *E. coli* showed 32.99% resistance against penicillin, and other Enterobacteriaceae isolates obtained from surface water showed high levels of resistance to ciprofloxacin (51.6%) and tetracycline (40.2%). These resistant bacteria might have arisen due to the misuse or overuse of antibiotics without proper prescription. The result clearly indicated that the presence of these multiple antibiotic-resistant coliforms, fecal coliforms, and other related pathogenic bacteria in surface water is very alarming for human life. Ingestion and contact with this contaminated water can somehow enter the food chain and also cause serious health problems.

Table 3. Provisional identification of the selected Gram-positive and Gram-negative bacteria.

Source	Gram-positive bacteria and isolate designation	Gram-negative bacteria and isolate designation
Shahidullah Hall Pond	*Enterococcus faecalis* F-1	
Azimpur Pond	*Bacillus alvei* A-5, *Planococcus citreus* A-8	*Aeromonas* sp. A-4
Zahurul Hall Pond	*Enterococcus faecalis* Z-2, *Bacillus alvei* Z-5	
Gulshan Lake	*Micrococcus* sp. G-1, *Bacillus alvei* G-8	*Escherichia coli* G-3, *Pseudomonas aeruginosa* G-4, *Salmonella typhi* G-6
Hatirjheel Lake	*Bacillus alvei* H-3	*Plesiomonas shigelloides* H-5, *Aeromonas* sp. H-9, *Plesiomonas* sp. H-11
Dhanmondi Lake	*Enterococcus faecalis* D-1, *Bacillus schlegelii* D-4	*Plesiomonas* sp. D-5, *Plesiomonas shigelloides* D-7
Buriganga River (Sadar ghat)	*Enterococcus faecalis* B-8	*Alcaligenes* sp. B-1, *Enterobacter aerogenes* B-7, *Pseudomonas aeruginosa* B-12
Shitalakshya River	*Enterococcus faecalis* S-11	*Plesiomonas* sp. S-3, *Klebsiella oxytoca* S-14

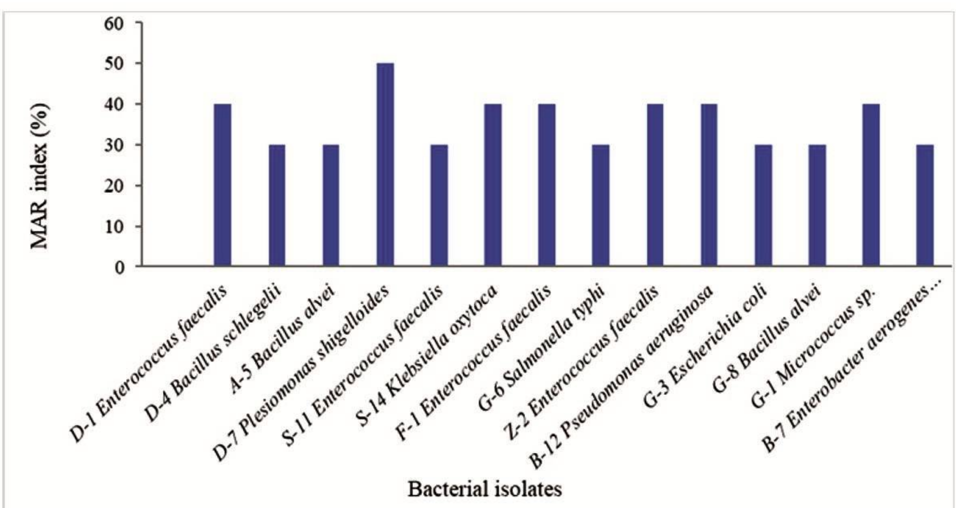

Fig. 1. Multiple Antibiotic Resistance (MAR) index of the selected bacterial isolates.

A cytotoxicity test was conducted for 26 bacteria on the Vero cell line. The untreated Vero cells (control) were healthy spindle-shaped and showed no apoptotic or necrotic bodies, while the treated cells with the cell-free extracts (CFS) of two isolates, *Escherichia coli* (G-3) and *Klebsiella oxytoca* (S-14), from Shitalakshya River and Gulshan Lake water, showed cytotoxicity. The cell-free extract completely damages Vero cells after 48 hrs, causing the death of the cell culture when compared to the control (Fig. 2). Kumar *et al.* (2008) reported that the toxins from two (8.3%) serotypes out of 24 *E. coli* strains produced a significant cytotoxic response in Vero cells. Some strains of *E. coli* have the potential to toxin and damage the cells of the colon. In humans, the toxin cyclomodulin, encoding fecal coliform (*E. coli*) has been shown to be cytotoxic in patients with colorectal cancer (McCoy *et al.* 2021). Studies have shown that the pathogenic coliform,

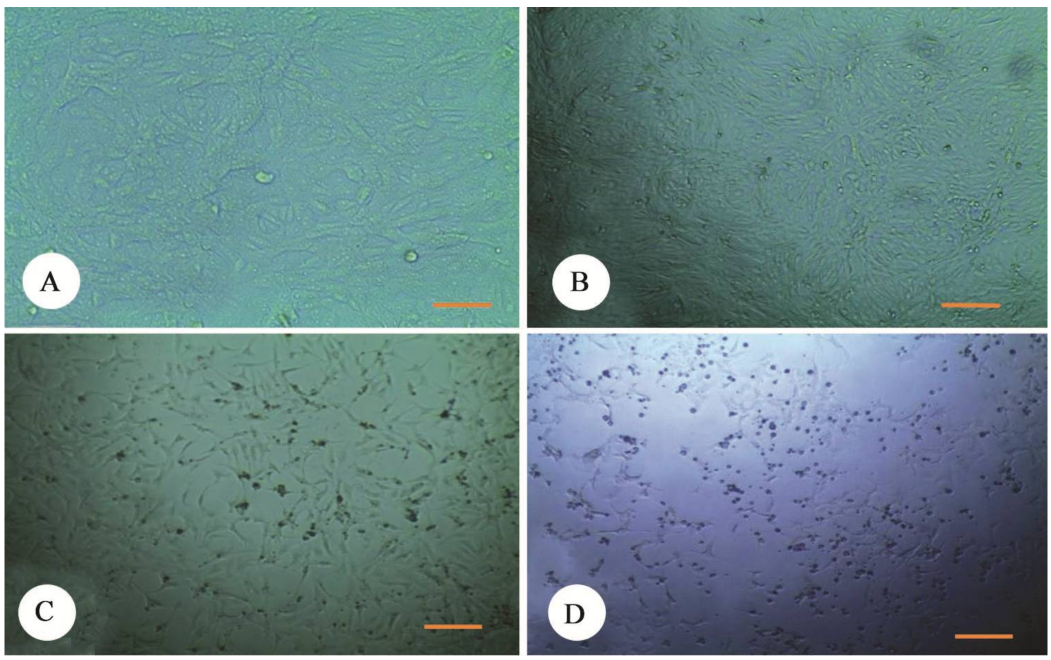

Fig. 2 Cytotoxic activity of cell-free extract of selected bacteria on the Vero cell line. A. Untreated control, (B) Treated with CFS of *Plesiomonas shigelloides* (D-7) showing no cytotoxicity, (C) *Escherichia coli* (G-3) and (D) *Klebsiella oxytoca* (S-14) showing dead cells. 1 Bar = 20 µm

K. oxytoca, can produce cytotoxins and is associated with human colitis and mucocutaneous infections (Dallal *et al.* 2017). The present investigation also demonstrated the same result that *Klebsiella oxytoca* has the ability to produce harmful toxins associated with carcinogenesis. The cytotoxicity test revealed that toxins released by coliform bacteria after the ingestion of contaminated water can damage normal cells.

The present investigation of surface waters in Dhaka city revealed severe water pollution, particularly in the Buriganga and Shitalakshya Rivers, by enteric bacteria. Gulshan and Hatirjheel Lakes were also polluted, while Dhanmondi Lake and several other ponds were found to be safe in the context of enteric pathogens. Culture and sensitivity tests indicated widespread multiple antibiotic resistance (MAR) among bacteria, with a maximum MAR of 50% and a minimum of 30%. Two isolated bacteria (*Klebsiella oxytoca* and *E. coli*) were cytotoxic positive coliforms. In summary, bacteria associated with the surface water of Dhaka city could pose a threat to public health.

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