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Molecular characterization and human health risk assessment of multi-drug and heavy metals tolerant bacteria from urban river water

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ABSTRACT

The present study characterized the multi-drug and heavy metal-resistant bacteria and their human health risks in the six major urban rivers. The bacterial strains were identified by molecular techniques based on 16 s rDNA gene sequence analysis, where most of the isolates belong to *Bacillus* spp. and *Staphylococcus* spp. The minimum inhibitory concentration (MIC) of the bacterial strains to different heavy metals like chromium, lead, cadmium, cobalt, mercury, and nickel ranged up to 3000 mg/L. The antibiotic susceptibility tests revealed that 69.23% of the strains resistant to ceftriaxone, while 61.54% were resilient to cefotaxime, 53.85% to ampicillin, 46.15% to amoxicillin, 30.77% to streptomycin, 15.38% to azithromycin, 15.38% to chloramphenicol, 7.69% to tetracycline, 7.69% to gentamycin, 7.69% to vancomycin. Interestingly, ciprofloxacin was found highly sensitive to all the bacterial strains in the present study. The multiple heavy metals resistance (MHMR) index of all the bacterial strains in the present study was very high compared to the standard value of 0.50. The multiple antibiotics resistance (MAR) index was highest for *Bacillus cereus* MKSMPbT1 (0.45), while it was lowest in *B. xiamenesis* MKSMCrB1 and *B. pumilus* MKSMNiT1 (0.09). The results of the hemolytic assay revealed that almost all the bacterial strains identified in the present study are highly pathogenic in nature. In essence, the bacterial strains identified in the present study could pose significant environmental and public health concerns that draw strict government attention.

1. Introduction

The presence of both heavy metals and antibiotics in the environment poses a significant threat to human health, with escalating concerns arising over recent decades. Metals and antibiotic-resistant bacteria are now ubiquitous, in nearly every environment, particularly in water resources. This proliferation of antibiotic-resistant bacteria and genes in the environment heightens the risk to both humans and animals. Bacteria established the aptitude to tolerate metals, at low concentration, thereby accelerating the proliferation of antibiotic-resistant bacteria, particularly in newly contaminated environments. This phenomenon may accelerate the spread of resistance to human pathogens [1]. While certain metals are essential micronutrients necessary for biological macromolecules and cellular functions, they can become

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toxic above a certain concentration [2]. Microorganisms demonstrate a robust response to low concentrations of heavy metals, but elevated levels can have adverse effects on microbial communities within the ecosystems. In response to high metal concentrations, microorganisms develop resistance through specific mechanisms such as efflux and uptake systems [3,4]. Interestingly, metal-resistant organisms can also be found in unpolluted environments, where they adapt to high metal concentrations [5].

Antibiotic-resistant organisms are prevalent in water environments where human and animal activities facilitate the transmission of resistance genes to indigenous waterborne microbes [6]. Within this ecosystem, even non-pathogenic bacteria can serve as reservoirs for resistance genes. The introduction of antimicrobial agents, disinfectants, detergents, and industrial pollutants further facilitates the proliferation of such resistant organisms [7]. However, it is noteworthy that in some cases, the pollution of water environments by heavy metals may also contribute to the spread of antimicrobial resistance. A recent study by Gupta et al. [8] revealed that heavy metal selective pressure could drive the emergence of multi-resistant bacteria in heavy metal-polluted settings by concurrently transmitting heavy metals and antibiotic resistance through integrons. The study found significant correlations between class 1 integrons and heavy metals, heavy metal resistance, and antibiotic resistance, indicating that antibiotic resistance co-selection occurs more frequently in heavy metal-polluted environments. Additionally, the study demonstrated that antibiotic resistance and heavy metal resistance share similar mechanisms or are linked by the same mechanisms [8]. Numerous studies have isolated microorganisms with simultaneous resistance to multiple heavy metals and antibiotics from natural environments such as water and soil. The co-occurrence of antibiotic and heavy metal resistance among bacterial isolates is often attributed to the selective pressure exerted by the presence of metals in those specific environments [9–13].

River water is found to be contaminated with metals and antibiotic-resistant organisms [14]. In Bangladesh, major rivers like the Buriganga, Turag, Shitalakhya, and Karnaphuli rivers, which flow through cities such as Dhaka and Chattogram, are highly polluted with heavy metals and antibiotic residues [15,16,68]. Consequently, there is a risk that bacteria in these rivers will establish antibiotic resilience throughout the genetic revolution from indigenous metal-tolerant bacteria [17,18]. Furthermore, bacterial communities in river water can acquire antibiotic resistance from pathogenic or potentially pathogenic bacteria harboring antibiotic-resistant genes, which are continuously released into the water environment through wastewater and subsequently mixed with environmental bacteria [19–21]. The presence of antibiotic-resistant bacteria in natural environments poses a significant public health threat that warrants urgent attention [22,23,67].

The major urban rivers in Dhaka and Chattogram, Bangladesh, including the Buriganga, Turag, Shitalakhya, Balu, Dhaleswari, and Karnaphuili, which serve as the primary water sources for these cities, have experienced severe environmental pollution in recent years [24,25]. This pollution has made it increasingly challenging to meet the growing demand for safe drinking and potable water. However, there is no significant scientific study highlighting the status of multiple antibiotics and metals-resistant organisms in these waterways, which could further be exacerbated by growing environmental and public health risks to the communities dependent on these rivers and relevant stakeholders. Given these concerns, it is imperative to investigate the water quality of these urban rivers to explore the extent of the problems. Thus, the objectives of the present study are to (i) isolate and characterize the bacteria showing multiple resistance to metals and antibiotics employing molecular techniques by 16 s rDNA gene sequence analysis, (ii) to assess their antibiotic susceptibility and heavy metals tolerance patterns, and (iii) to evaluate the potential human health risks associated with these bacterial strains by multiple heavy metals resistance (MHMR) and multiple antibiotics resistance (MAR) indexes and hemolytic assay.

2. Experimental

2.1. Study area and sampling

Six major rivers around Dhaka and Chattogram City were selected for this study. The Balu, Turag, Dhaleswari, Sitalakhya, and Buriganga rivers were chosen from Dhaka city. From Chattogram City, Karnaphuli river was selected. Eighteen water samples of around 200 mL were collected from six major rivers around Dhaka and Chattogram City during the period of March to June 2021 in the morning. We used sterilized plastic bottles to collect the water samples. The sampling depth was 10–15 cm below the water surface. The water samples were collected from the specific points of each river, particularly from the site where discharged effluents were mixed with river water as well as upstream and downstream of that mixing point. Fig. 1 shows the river water sampling locations.

2.2. Determination of physicochemical parameters of water samples

The physicochemical parameters of the river water samples were determined following the guidelines of APHA, 1998 [26]. The pH value of the sample was measured using a portable pH meter (HI 8424, HANNA, Romania). Electrical conductivity (EC), total dissolved solid (TDS) and temperature readings were collected from an HI 99300 EC/TDS meter (HANNA, Romania). The dissolved oxygen (DO) value was obtained from a portable dissolved oxygen meter, HI 9141 (HANNA, Romania).

2.3. Determination of total aerobic bacterial count

The total microbial count in river water was determined using nutrient agar (NA) media (Hi Media, India). For this, 1 mL of water sample was added to 9 mL of sterile normal saline, and a serial dilution (10^{-1} to 10^{-6}) was prepared. Then, 0.1 mL of the suspension of each dilution was distributed to NA plates. Plates were put in an incubator at 37 °C for 24 h. Then, the total number of bacteria per mL of water was determined triplicate by the viable plate count method.

2.4. Isolation and enumeration of metal-resistant bacteria

The nutrient agar medium with 100 mg/L concentration of Cr^{+6}, Ni^{+2}, Pb^{+2}, Cd^{+2}, Co^{+2}, and Hg^{+2} was used to isolate heavy metal-resistant bacteria from these water samples. The stock solution of 1000 mg/L concentration of $K_2Cr_2O_7$, $NiSO_4$, Pb $(NO_3)_2$, $CdCl_2{\cdot}H_2O$, Co $(NO_3)_2$ and $HgCl_2$ was prepared for this. Then, 50 µl of water sample was spread on the agar media and incubated at 37 °C for 48–72 h. After the incubation period, the number of isolates was counted, and the desired colonies were selected. The isolates were purified in NA media by streaking plate method and preserved at − 70 °C in 30% glycerol until further study.

2.5. Tolerance of isolates towards different concentrations of heavy metals

Initially, isolates having distinct colony morphology from each river were tested for their metals tolerance in Luria-Bertani (LB) agar media added with various concentrations of Cr^{+6}, Ni^{+2}, Pb^{+2}, Cd^{+2}, Co^{+2}, and Hg^{+2}. The bacterial colonies were streaked on LB agar media containing 200–3000 mg/L concentration of the above metals until their growth completely ceased [33,34]. The plates were kept in an incubator at 37 °C for 72 h. The media without heavy metals was served as control. The minimum concentration of heavy metal at which bacterial growth was inhibited was considered as minimum inhibitory concentration (MIC).

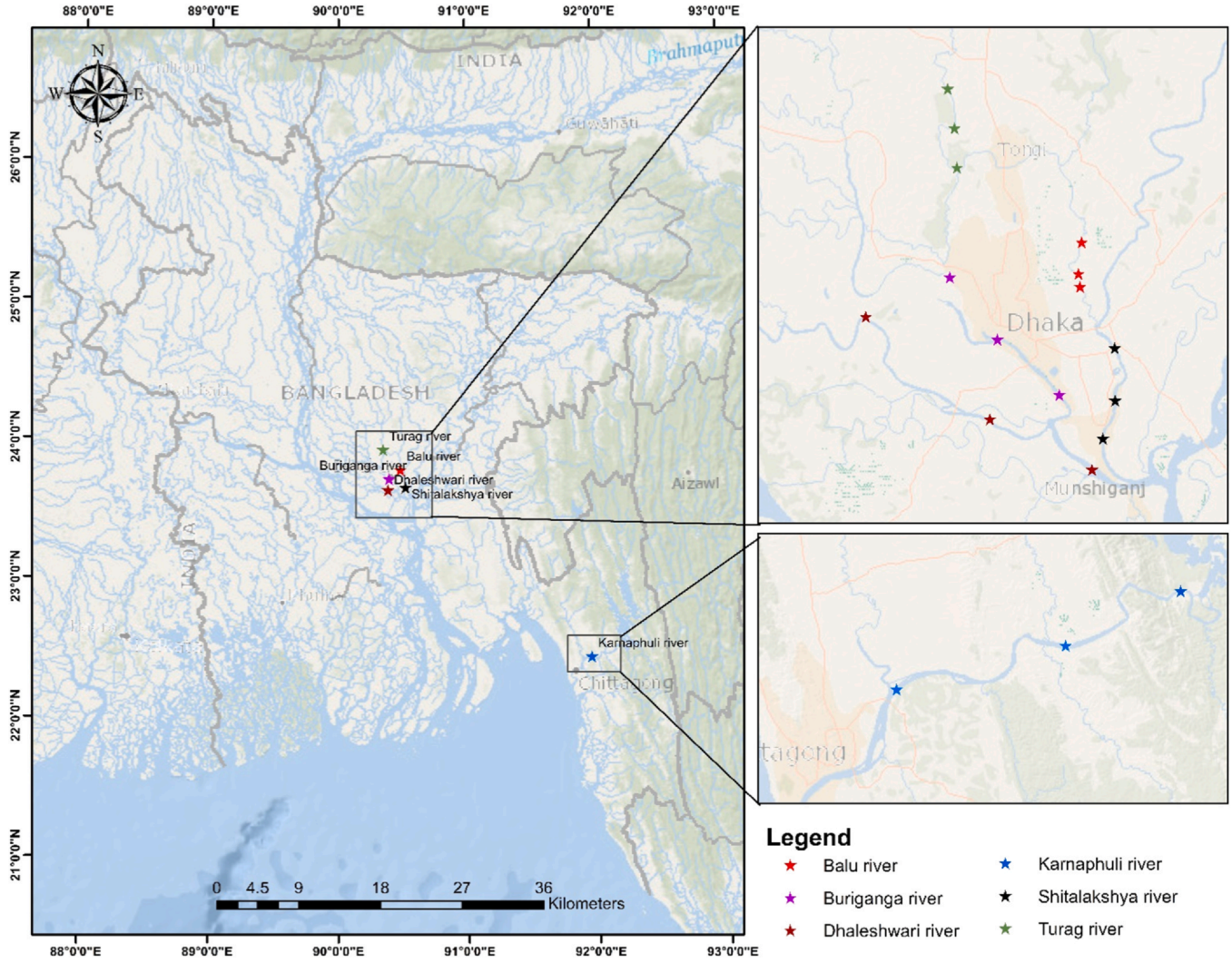

Fig. 1. Study area with sampling locations.

2.6. Susceptibility of metal-resistant isolates to antibiotics

The antibiotic sensitivity test of the isolates was carried out using the disc diffusion method followed by Bauer et al., 1966 [30]. Different antibiotics were used as ciprofloxacin (CIP) (5 μg), amoxicillin (AMX) (10 μg), ampicillin (AMC) (10 μg), streptomycin (STRP) (10 μg), gentamycin (GEN) (10 μg), azithromycin (AZM) (15μG), vancomycin (VAN) (30μG), tetracycline (TET) (30 μg), chloramphenicol (CHLMP) (30 μg), ceftriaxone (CTR) (30 μg) and cefotaxime (CTX) (30 μg). Firstly, an aliquot of 0.1 mL overnight fresh culture equivalent to 0.5 McFarland standard was spread on Mueller-Hinton agar (Hi Media, India) media and an antibiotic disk was placed on it under aseptic conditions. Then, it was kept in an incubator at 37 °C for 18–24 h. The bacterial growth on the media was observed after 24 h, and the bacteria were recorded as resistant, intermediate susceptible or susceptible following Clinical Laboratory Standards Institute (CLSI) guidelines 2011 by measuring the diameter of their zone of inhibition. Finally, the multiple antibiotic resistance (MAR) index of respective rivers was estimated according to Tao et al. [31–33].

2.7. Molecular characterization of selected isolates and their phylogenetic analysis

The molecular characterization of the bacterial strains was performed by PCR techniques employing 16 S rDNA gene sequencing methodologies, according to Ovreas et al. (1997) [27,28]. The detailed techniques have been provided in the supplementary data S1. The 16 S rDNA sequences were then recorded to the gene bank of NCBI for accessing the unique gene bank accession numbers. The phylogenetic exploration of the sequenced bacteria was done using MEGA 6 software [29] to analyze the relationships among the sequences of the 16 s rDNA genes of the present study and related organisms from the gene bank database.

2.8. Hemolytic assay

A hemolytic experiment was performed to differentiate between pathogenic and nonpathogenic bacterial species of our isolates. The test was done by streaking of the isolates in blood agar (Oxoid, UK) media with 5% sheep blood. The change of color of blood from red to yellow grey/ dark green is indicative of α-hemolysis, whereas a clear zone is considered β-hemolysis. However, no change in the color of the blood indicates γ-hemolysis [33].

3. Results

3.1. Determination of water quality parameters

The pH value of the water samples ranged from 6.95 to 7.19, whereas the EC ranged from 150 to 495 μs/cm, TDS ranged from 45 to 304 mg/L, and DO value ranged from 0.65 to 5.9 mg/L. Temperature variations within the samples were also evident from 24 °C to 30 °C (Table 1).

Table 1
Water quality parameters of six major urban river water in Bangladesh.

Sample ID	pH	EC (µs/cm)	TDS (mg/L)	DO (mg/L)	Temperature (°C)
Balu-1	7.09	121	61	1.34	27.5
Balu-2	7.12	231	116	1.54	28
Balu-3	7.1	150	75	1.4	28.2
Mean	7.1	167.33	84	1.43	27.9
Buriganga-1	7.2	419	209	1.18	30
Buriganga-2	7.16	450	260	1.45	28.75
Buriganga-3	7.19	480	230	1.31	29
Mean	7.18	449.67	233	1.31	29.25
Shitalakkha-1	6.91	407	203	5.3	24.2
Shitalakkha-2	6.95	469	234	5.9	25
Shitlakkha-3	7.05	425	215	4.7	24.67
Mean	6.97	433.67	217.33	5.3	24.62
Turag-1	7.04	495	299	2.23	29
Turag-2	7	410	304	2.97	28.67
Turag-3	7.02	405	302	2.46	28.5
Mean	7.02	436.67	301.67	2.55	28.72
Dhaleshwari-1	6.95	436	218	4.11	28.3
Dhaleshwari-2	6.95	433	216	4.46	28.75
Dhaleshwari-3	6.94	435	215	4.35	28.68
Mean	6.95	434.67	216.33	4.31	29.02
Karnaphuli-1	6.94	98	49	0.7	24.5
Karnaphuli-2	6.93	93	46	0.65	25.25
Karnaphuli-3	6.93	95	45	0.73	24
Mean	6.93	95.33	46.67	.69	24.58

3.2. Enumeration of total microbial load from six urban rivers

The total microbial count in the water samples is presented in Table 2. The total microbial count of Balu river ranged from 1×10^5 to 1.2×10^5 CFU/mL, and the mean bacterial count was 1.1×10^5 CFU/mL. The mean aerobic count of bacterial isolates in the Balu, Buriganga, Shitalakkha, Turag, Dhaleshwari and Karnaphuli rivers were1.1×10^5, 1.1×10^7, 1.3×10^6, 1.2×10^4, 1.05×10^5 and 1.75×10^9 CFU/mL. However, the highest average microbial count was found in the Karnaphuli river, and the lowest average microbial count was found in the Turag river.

3.3. Enumeration of the total count of heavy metal-resistant bacteria

The bacteria resistant to Cr (VI), Pb (II) and Ni (II) were found in all rivers water. The highest number of Cr (VI) resistant bacteria (log 5.73) was found in the Karnafuli river, whereas the Balu river was found to have a maximum number of Pb (II) resistant bacteria (log 5.43) than the other rivers water. In terms of Ni (II) resistance, the number of bacteria (log 5.86) enumerated in the Buriganga river was the highest. We did not find any Hg-resistant bacteria in our water samples. Co (II) resistant bacteria were only found in the case of Buriganga (log 3.81), Turag (log 3.56) and Dhaleswari (log 3.11) rivers. Cd (II) resistant bacteria were found in the Karnafuli river (log 3.85) and Buriganga river (log 3.53) (Fig. 2).

Table 2
Enumeration of the total microbial load of six major urban rivers of Bangladesh.

River Name	Total microbial count (CFU/mL)	Average microbial count (CFU/mL)
Balu-1	1.2×10^5	1.1×10^5
Balu-2	1.0×10^5	
Balu-3	1.1×10^5	
Buriganga-1	1.0×10^7	1.1×10^7
Buriganga-2	1.1×10^7	
Buriganga-3	1.2×10^7	
Shitalakkha-1	1.0×10^6	1.3×10^6
Shitalakkha-2	1.5×10^6	
Shitalakkha-3	1.4×10^6	
Turag-1	8.0×10^3	1.2×10^4
Turag-2	1.6×10^4	
Turag-3	1.2×10^4	
Dhaleshwari-1	1.1×10^5	1.05×10^5
Dhaleshwari-2	1.0×10^5	
Dhaleshwari-3	1.05×10^5	
Karnaphuli-1	2.0×10^9	1.7×10^9
Karnaphuli-2	1.5×10^9	
Karnaphuli-3	1.6×10^9	

CFU = Colony forming unit.

3.4. Selection and identification of the bacterial strains

We selected 30 isolates from six river water for our study. Among them, thirteen isolates were chosen for further study based on the morphology. All the isolates were gram-positive bacteria, and they were finally identified by the 16 S rDNA sequencing method. The identified bacterial strains are *Bacillus aerius* MSMCrD2, *B. thuringiensis* MSMCrS2, *B. stratosphericus* MSMCrT2, *Staphylococcus sciuri* MSMPbD1, *B. anthracis* MSMPbBL2, *B. mobilis* MSMNiBL1, *B. xiamenensis* MSMCrB1, *S.*

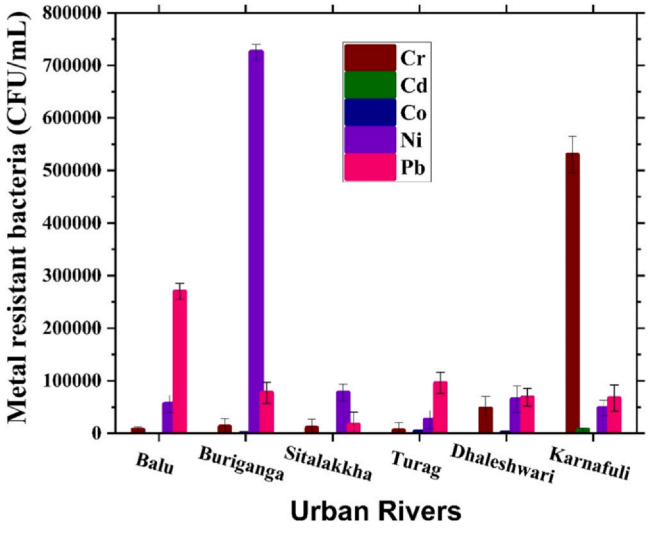

Fig. 2. Enumeration of heavy metals tolerant bacteria in the urban river water.

Table 3
Gene bank accession numbers of thirteen bacterial isolates found in the present study.

SI. No	Bacterial strains	Isolates number	Gene bank accession number
1	*Bacillus pacificus MSMNiB1*	S_1	MN620453
2	*Bacillus xiamenensis MSMCrB1*	S_2	MN620454
3	*Bacillus thuringiensis MSMCrS2*	S_3	MN620455
4	*Staphylococcus hemolyticus MSMNiS2*	S_4	MN620456
5	*Bacillus aerius MSMCrD2*	S_5	MN620457
6	*Bacillus cereus MSMPbT1*	S_6	MN620458
7	*Bacillus pumilus MSMNiT1*	S_7	MN620459
8	*Bacillus stratosphericus MSMCrT2*	S_8	MN620460
9	*Bacillus mobilis MSMNiBL1*	S_9	MN620461
10	*Bacillus anthracis MSMPbBL2*	S_{10}	MN620462
11	*Bacillus wiedmannii MSMPb1K1*	S_{11}	MN620463
12	*Bacillus cereus MSMK2Cd*	S_{12}	MN620464
13	*Staphylococcus sciuri MSMPbD1*	S_{13}	MN620465

haemolyticus MSMNiS2, *B. pacificus* MSMNiB1, *B. pumilus* MSMNiT1, *B. cereus* MSMPbT1, *B. wiedmannii* MSM K1Pb1 and *B. cereus* MSMKPb1K2. All the bacterial sequences were submitted to the gene bank database, and a unique accession number was provided for each of the strains (Table 3). The phylogenetic trees were constructed based on the most similar sequences obtained from BLAST results and the sequences of 16 S rDNA of the present study using neighbor joining methods of MEGA 6 software. The sequences of other bacterial isolates extracted from the NCBI database showed homology > 99% of the bacterial isolates of the present study. The phylogenetic trees of thirteen bacterial strains are presented in Fig. 3. Most of the strains belong to the *Bacillus* group, whereas some are in the *Staphylococcus* group. The phylogenetic tree analysis revealed that S_{12}, S6, S_3, S_1, and S_{10} are most distantly related to all the other bacterial strains. The genetic distance between each of the isolates is 0.1 nucleotides per site in their alignment.

3.5. Tolerance of multiple heavy metals and their MHMR index

The MIC for metals ranged 100–3000 mg/L. In the case of Cr (VI) tolerance, the S_4 isolate exhibited the highest MIC value (2500 mg/L) and the lowest MIC value (200 mg/mL) was observed for S_{13}. The MIC value of other isolates ranged between 300–500 mg/L. The isolates were also tested for their tolerance against Ni (II). The maximum MIC (600 mg/L) value was found for various isolates, including S_1, S_3, S_4, S_8, S_9, and S12. The isolate S_{13} showed the lowest MIC value (300 mg/L (Table 4). Likewise, Cr (VI) and Ni (II) isolates were also tolerant to different concentrations of Cd (II). The highest MIC value (800 mg/L) for Cd (II) was observed for S_{12} followed by S_2, S_4(600 mg/L) and S_1, S_3, S_{10}, S_{13}isolates (100 mg/L) (Table 4). For Co, MIC of S_1, S_3, S_4, S_6, S_7, S_{10}, S_{11}, S_{12}, S_{13} isolates was 200 mg/L while it was 600 mg/L for S_2, S_5, S_8, S_9 (Table 4). We observed the highest metal tolerance among the isolates for Pb (II). All the isolates except S_{13} were found to display the maximum level of tolerance (MIC value 3000 mg/L). It exhibited tolerance to Pb (II) at 2500 mg/L concentration. The MHMR value was very high for all thirteen bacterial strains (0.83) (Table 5). They shared the same resistance pattern with no exception. In our study, the MHMR index was very high for all thirteen bacterial strains (0.83). MHMR index indicated that the water environments, such as rivers around Dhaka and Chattogram city, are industrially polluted with metals. The MHMR value was greatest for all the strains and consequently, it is evident that the river water samples around Dhaka and Chattogram City are exposed to serious heavy metal pollution.

3.6. Antibiotic susceptibility of the isolates and their MAR index

Antibiotic resistance of the thirteen bacterial strains was determined by the Kirby-Bauer disc diffusion method with the following antibiotic discs such as ciprofloxacin (CIP), amoxicillin (AMX), ampicillin (AMC), streptomycin (STRP), gentamycin (GEN), azithromycin (AZM), vancomycin (VAN), tetracycline (TET), chloramphenicol (CLMP), ceftriaxone (CTR) and cefotaxime (CTX). The isolates from the studied river exhibited different levels of resistance to multiple antibiotics, as presented in Table S1, Fig. 4 and Fig. 5. Five bacterial strains (S_5, S_6, S_9, S_2 and S_4) showed almost similar resistance patterns where they were resistant to AMX, CTX, AMC, and CTR. The isolates S_5 and S_6 were from the Balu river, whereas S_9, S_2 and S_4 were isolated from the Buriganga, Shitalakhya and Dhaleswari rivers, respectively. The other isolates of the Buriganga, known as S7, were resistant to only STRP, while the S_1 isolate of the

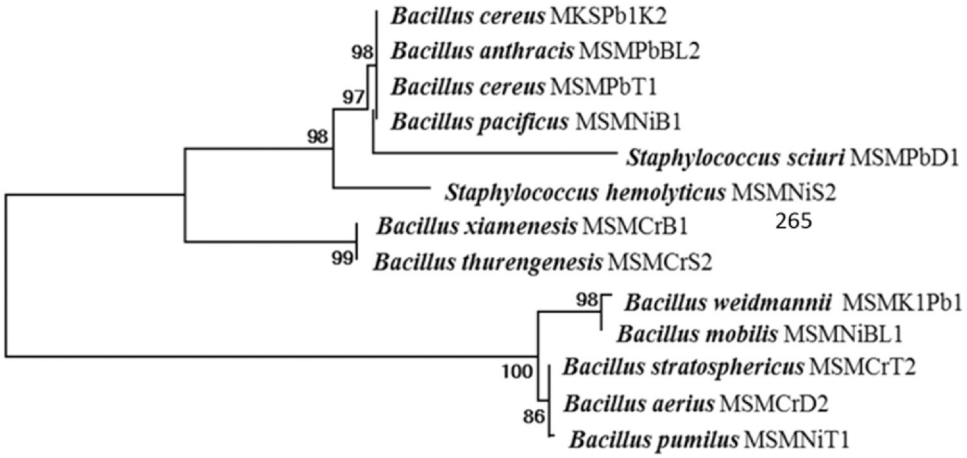

Fig. 3. The phylogenetic trees of the bacterial strains constructed by the maximum likelihood method.

Table 4
Heavy metal tolerance of bacterial strains.

Concentrations of heavy metals (mg/L)		Bacterial Strains												
		S1	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*	*S9*	*S10*	*S11*	*S12*	*S13*
Cr	100	+	+	+	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+	+	+	MIC
	300	+	+	+	+	MIC	+	MIC	+	MIC	+	+	+	-
	400	MIC	+	MIC	+	-	MIC	-	+	-	+	+	+	-
	500	-	MIC	-	+	-	-	-	MIC	-	MIC	MIC	MIC	-
	1000	-	-	-	+	-	-	-	-	-	-	-	-	-
	1500	-	-	-	+	-	-	-	-	-	-	-	-	-
	2000	-	-	-	+	-	-	-	-	-	-	-	-	-
	2500	-	-	-	MIC	-	-	-	-	-	-	-	-	-
Ni	100	+	+	+	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+	+	+	+
	300	+	+	+	+	MIC	+	MIC	+	+	+	+	+	MIC
	400	+	+	+	+	-	MIC	-	+	+	MIC	+	+	-
	500	+	MIC	+	+	-	-	-	+	+	-	MIC	+	-
	600	MIC	-	MIC	MIC	-	-	-	MIC	MIC	-	-	MIC	-
Cd	100	MIC	+	MIC	+	+	MIC	+	MIC	+	MIC	+	+	MIC
	200	-	+	-	+	+	-	MIC	-	+	-	+	+	-
	300	-	+	-	+	+	-	-	-	+	-	+	+	-
	400	-	+	-	+	MIC	-	-	-	MIC	-	MIC	+	-
	500	-	+	-	+	-	-	-	-	-	-	-	+	-
	600	-	MIC	-	MIC	-	-	-	-	-	-	-	+	-
	700	-	-	-	-	-	-	-	-	-	-	-	+	-
	800	-	-	-	-	-	-	-	-	-	-	-	MIC	-
Co	100	+	+	+	+	+	+	+	+	+	+	+	+	+
	200	MIC	+	MIC	MIC	+	MIC	MIC	+	+	MIC	MIC	MIC	MIC
	300	-	+	-	-	+	-	-	+	+	-	-	-	-
	400	-	+	-	-	+	-	-	+	+	-	-	-	-
	500	-	+	-	-	+	-	-	+	+	-	-	-	-
	600	-	MIC	-	-	MIC	-	-	MIC	MIC	-	-	-	-
Pb	100	+	+	+	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+	+	+	+
	500	+	+	+	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+
	1500	+	+	+	+	+	+	+	+	+	+	+	+	+
	2000	+	+	+	+	+	+	+	+	+	+	+	+	MIC
	3000	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	-

Dhaleshwari river was resistant to CTX and CTR. However, S_8, another isolate from the Shitalakhya river, exhibited resistance to CTR, AZM, and TET, thereby showing a multidrug resistance mechanism. The isolates (S_3, S_{10}, S_{11}) from the Turag river also displayed antibiotic susceptibility. The isolate S_{11} was multidrug-resistant, which showed resistance to five antibiotics such as AMX, STRP, CTX, AMC and CTR. The isolate S_{10} was found to be resistant to only CLMP and susceptible to seven antibiotics (AMX, TET, AZM, GEN, STRP, CIP and AMC). The other isolate, S_3, was also multidrug-resistant, exhibiting resistance to CLMP, STRP, CIP and AMC.

In the Karnaphuli river, we also found antibiotic-resistant isolates (S_{12}, S_{13}), of which S_{12} was multidrug-resistant, showing resistance to AZM, GEN, AMC, and CTX. Another isolate, S_{13}, showed resistance to only STRP and CTR. In summary, out of 13 bacterial strains, 69.23% of the isolates were resistant to CTR, 61.54% to AMC, 53.85% to CTX,46.15% to AMX, 30.77% to STRP, 15.38% to AZM, 15.38% to CLMP, 7.69% to TET, 7.69% to GEN and 7.69% to CIP. No VAN-resistant bacteria were recorded in the present study. The antibiotic resistance trend was as follows: CTR > AMC > CTX > AMX > STRP > AZM > CLMP > TET > GEN > CIP > VAN. The MAR value was very high in S_{11} (0.45), while it was low

Table 5
Determination of MHMR index.

Name of bacterial Strains	Isolates ID	MHMR index	Number of heavy metals resistant to bacterial strain	Total number of heavy metals
Bacillus pacificus MSMNiB1	S_1	0.83	5	6
Bacillus xiamenensis MSMCrB1	S_2	0.83	5	6
Bacillus thuringiensis MSMCrS2	S_3	0.83	5	6
Staphylococcus hemolyticus MSMNiS2	S_4	0.83	5	6
Bacillus aerius MSMCrD2	S_5	0.83	5	6
Bacillus cereus MSMPbT1	S_6	0.83	5	6
Bacillus pumilus MSMNiT1	S_7	0.83	5	6
Bacillus stratosphericus MSMCrT2	S_8	0.83	5	6
Bacillus mobilis MSMNiBL1	S_9	0.83	5	6
Bacillus anthracis MSMPbBL2	S_{10}	0.83	5	6
Bacillus wiedmannii MSMPb1K1	S_{11}	0.83	5	6
Bacillus cereus MSMK2Cd	S_{12}	0.83	5	6
Staphylococcus sciuri MSMPbD1	S_{13}	0.83	5	6

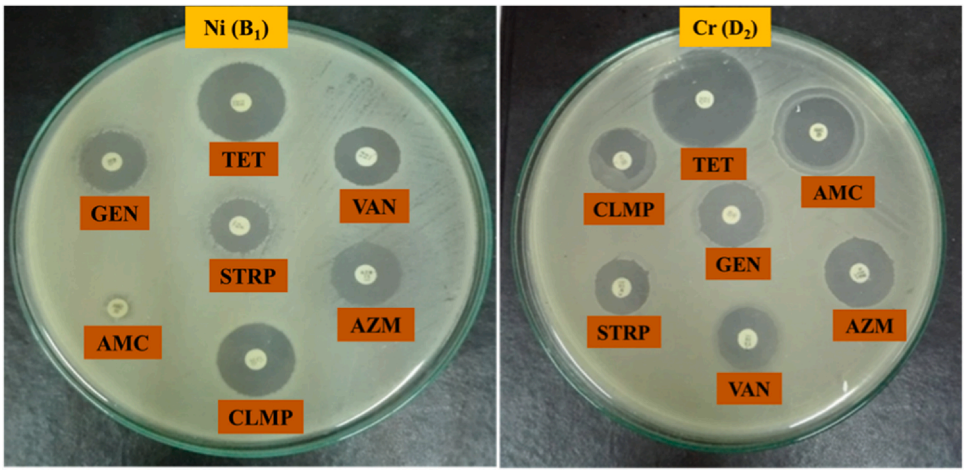

Fig. 4. Antibiotic susceptibility testing of two isolates, S9 (left) and S1 (right).

in S_7 and S_{10} (0.09) (Fig. 5). A similar MAR value (0.36) was observed in S_9, S_4, S_5, S_{12}, S_3 and S_6. The MAR value of S_8 was 0.27.

3.7. Correlation between heavy metal resistance and antibiotic resistance of the bacterial isolates

The correlation between heavy metal resistance and antibiotic resistance of the bacterial isolates was checked in our study. As almost all the isolates were resistant to the heavy metals used in the study, they were tested for their susceptibility to antibiotics. It was found that a high percentage of the isolates were also multidrug-resistant, with some exceptions. The resistance to AMX, AMC, CTR and CTX was common among the multidrug-resistant isolates. The isolates were not resistant to VAN. Moreover, the isolates that were tolerant to high concentrations of heavy metals were comparatively more resistant to antibiotics than others. The isolate S_4, which treated the highest concentrations of Cr^{+6} (2500 mg/L), Ni^{2+} (600 mg/L), and Pb^{2+} (3000 mg/L), did exhibit resistance to four antibiotics. Similarly, S_{12} showed resistance to four antibiotics when they were tolerant to the highest concentration of Cd^{2+} (800 mg/L), Ni^{2+} (600 mg/L) and Pb^{2+} (3000 mg/L). But S_{11} was exceptional, which exhibited resistance to five antibiotics, although it was not tolerant to the highest concentration of metals like others.

We also examined whether there was any association between multiple antibiotics and heavy metal resistances among the isolates by Pearson correlation value. However, we did not find any significant correlation between them. Only there was a significant relationship (negative correlation) between the concentration of Ni^{2+} and AMX ($r = -0.581$), Cd^{2+} and GEN ($r = -0.693$), Cd^{2+} and CIP ($r = -0.789$) as well as Cr^{+6} and CTR sensitivity ($r = -0.593$ (Table 6). The highest tolerance of Ni^{2+}, Cd^{2+}, Pb^{2+} and Cr^{+6}, with the lowest zone of inhibition to AMC, GEN, CIP and CTR, indicating the resistance level observed by the isolates.

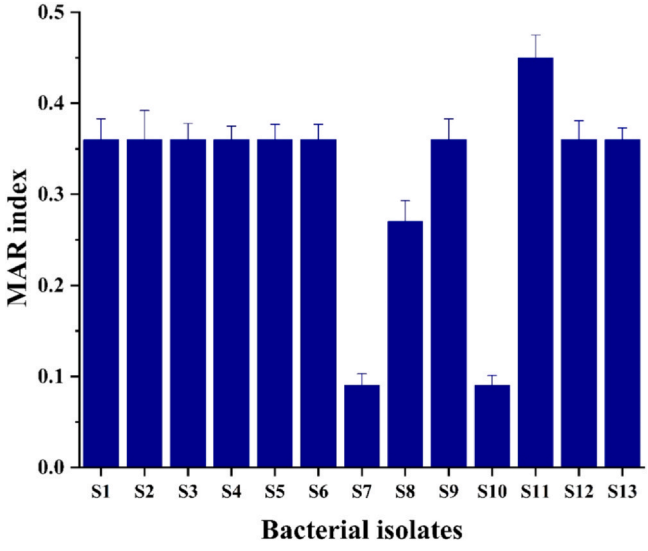

Fig. 5. MAR index of the bacterial isolates.

3.8. Hemolytic activity of the isolates

The hemolytic activity of thirteen bacterial strains was investigated. We found eleven α-hemolytic bacterial strains (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{11}, S_{13}). These eleven bacterial strains showed a dark green zone around their colony on blood agar media supplemented with 5% sheep RBC (Red Blood Cell). Only S_8 exhibited β-hemolysis, whereas no hemolysis occurred in the case of S_{12} (Table 7 and Fig. 6).

4. Discussion

In the present study, the mean bacterial load ranged from 1.2×10^4 to 1.7×10^9 CFU/mL in Turag river, Dhaka and Karnaphuli river water at Chittagong, respectively, indicating a high level of pollution of the river water, which is higher than the acceptable microbial load for drinking purpose [29]. In Dhaka city, most of the industries are situated along the riverbanks for the easy disposal of the effluents that contain heavy metals [24,26] and other toxic chemicals. These toxic chemicals may kill or inhibit the growth of microorganisms, so the present study found the lowest total microbial count in the Turag river and the other 4 peripheral rivers around Dhaka city rather than the Karnaphuli river in Chittagong. The present investigation found the highest total microbial load in Karnaphuli river because Chittagong city is not congested with so many industries like Dhaka city, and the number of industrial effluents discharged in Karnaphuli river is lower than the rivers around Dhaka city. The quality of river water is greatly influenced by industry, agriculture and a plethora of human activities [35,36]. The continuous discharge of treated and untreated wastes from the city's drainage also plays a key role in contaminating the river water. They can harbor microorganisms that are resistant to heavy metals and antibiotics [15]. Therefore, the main urban rivers of two major cities, including Dhaka and Chattogram, may have the possibility to be affected by this.

We isolated 13 bacterial isolates resistant to heavy metals and antibiotics from six major urban rivers and identified them by 16 S rDNA sequencing, of which were mostly *Bacillus* spp (84%), and the rest were *Staphylococcus* spp. Shammi and Ahmed studied the river water of

Table 6
Correlation between heavy metal resistance and antibiotic resistance of the bacterial isolates.

Antibiotics	No. of resistant isolates (N = 13)	Tolerance to heavy metals (%)					Pearson Correlation value (r) with significance < 0.05
		Cr^{+6} **** (100-2500 mg/L)	Ni^{2+} * (100-600 mg/L)	Pb^{2+} (100-3000 mg/L)	Cd^{2+} ***** (100-800 mg/L)	Co^{2+} (100-600 mg/L)	
AMX*	6	100	100	100	100	100	*r = −0.581
AMC	7	100	100	100	100	100	
TET	1	100	100	100	100	100	
AZM	2	100	100	100	100	100	
CHLMP	2	100	100	100	100	100	
GEN**	1	100	100	100	100	100	**r = −0.693
STRP	4	100	100	100	100	100	
CIP***	0	100	100	100	100	100	***r = −0.789
CTR****	11	100	100	100	100	100	*** *r = −0.593
CTX	8	100	100	100	100	100	
VAN	1	100	100	100	100	100	

Buriganga and Shitalakhya surrounding Dhaka city, and they reported 25 *Bacillus* spp. strains [36–38]. Another study by Nupur et al. (2020) on the Buriganga river found *Proteus* spp. and *Pseudomonas* spp. [24].

The isolates were tested for their tolerance against heavy metals, including Cr (VI), Pd (II), Cd (II), Co (II), Hg (II), and Ni (II). Initially, the isolates were tolerant to 100 mg/L of Cr^{+6}, Ni^{2+}, Cd^{2+}, Co^{2+} and Pb^{2+}. However, when further investigated, 7.2% of isolates could tolerate 2500 mg/L of $Cr+^{6}$, while 46.15% were tolerant to 600 mg/L of Ni^{2+}. However, 7.2% showed tolerance to 800 mg/L of Cd^{2+} and 30.76% to 600 mg/L of Co^{2+}. Most of the isolates (92.30%) could grow at 3000 mg/L of Pb^{2+}, which was higher than the bacterial isolates from the soil samples of Egypt and India [38,39]. These isolates could be tested for the bioremediation of lead-contaminated environments. Similarly, *B. spp* isolated by Samanta et al. (2012) were able to grow at different concentrations of heavy metals like nickel, cadmium, chromium and cobalt, but their tolerance level was lower than that of isolates of the present study against $Cr^{6+}$and Co^{2+} except for $Cd^{2+}$and Ni^{2+} [41,69]. *B. spp.* tolerant to mercury, lead, cadmium, chromium, nickel and cobalt was also isolated from agricultural soil in Egypt by Bahig et al. [39,40]. Generally, metal resistance is observed among the bacteria when they are exposed to metals in a variety of habitats and environments [42,43]. Studies revealed that metal resistance genes exist in both the genomes of bacteria as well as mobile genetic elements (MGEs) of bacteria isolated from ecosystems that are contaminated by anthropogenic activities [1]. Multiple heavy metal resistance is important to indicate whether the water environments are industrially polluted [44,70]. Therefore, the MHMR index was assessed. In the present study, the MHMR value was high for all bacteria, indicating that the examined river water samples around Dhaka and Chittagong city may be exposed to serious heavy metal pollution.

Antibiotic-resistant bacteria in river water might be found when municipal and industrial wastewater is discharged to the river with antibiotic residues [45–48]. However, antibiotic-resistance genes have been reported in antibiotic-free environments [49]. All the isolates from the studied river water were also tested for antibiotic resistance by the disc diffusion method. We observed a high level of resistance to CTR in our 69.23% isolates, and no resistance was found in the case of VAN. The absence of VAN resistance may indicate that the rivers were not contaminated with VAN. The use of VAN in that area could be very low. Antibiotic-resistant bacteria were isolated from rivers in Terengganu, Malaysia, by Salikhan et al. (2020), and they found that most of the bacterial isolates were highly resistant towards AMC, rifampicin (RIMP), GEN, TET and CHLMP, but low or no resistant to VAN [50]. However, the lowest resistance was shown to TET by 7.69% of isolates, which contrasted with the findings of Shammi et al. (2013), reported that most of their *B. spp.* isolates from the Buriganga and Sitalakshya river water of Bangladesh were resistant to TET (100%) [50].

A greater percentage of our isolates were multidrug-resistant (69.23%). The MAR value of the isolates was also calculated. The MAR value for most of the bacterial strains (69.23%) was > 0.2 [51–55]. We noticed high MAR values among the isolates from river water samples of Shitalakkha, Buriganga,Turag, Dhaleshwari, Balu and Karnaphuli. This may be due to a large amount of effluent from hospitals, pharmaceutical companies, feed mills, animal farms, and municipal waste that goes into the rivers [56,57,66]. However, smaller MAR values were found from some of the isolates of the river water samples of Dhaleshwari, Karnaphuli, Turag and Buriganga. The study of more water samples and their isolates from these rivers may strengthen this finding.

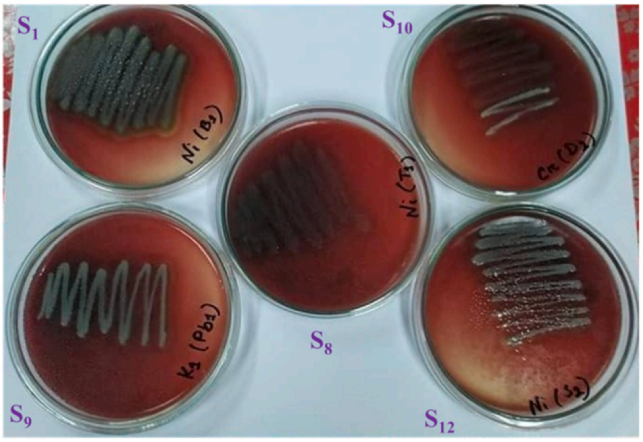

Fig. 6. Hemolytic activity of the bacterial isolates in blood agar media, S_9 (β-hemolysis), S_{12} (β -hemolysis), S_{10} (α-hemolysis), S_1 (α-hemolysis) and S_8 (α-hemolysis).

Table 7
Hemolytic activity of the bacterial strains.

Bacterial strains	Isolates	Hemolysis
Bacillus pacificus MSMNiB1	S_1	α-hemolysis
Bacillus xiamenensis MSMCrB1	S_2	α-hemolysis
Bacillus thuringiensis MSMCrS2	S_3	α-hemolysis
Staphylococcus hemolyticus MSMNiS2	S_4	α-hemolysis
Bacillus aerius MSMCrD2	S_5	α-hemolysis
Bacillus cereus MSMPbT1	S_6	α-hemolysis
Bacillus pumilus MSMNiT1	S_7	α-hemolysis
Bacillus stratosphericus MSMCrT2	S_8	β- hemolysis
Bacillus mobilis MSMNiBL1	S_9	β-hemolysis
Bacillus anthracis MSMPbBL2	S_{10}	α-hemolysis
Bacillus wiedmannii MSMPb1K1	S_{11}	α-hemolysis
Bacillus cereus MSMK2Cd	S_{12}	β- hemolysis
Staphylococcus sciuri MSMPbD1	S_{13}	α-hemolysis

The association between heavy metal and multidrug resistance among the various bacterial species is evident in numerous studies [58–61]. In the present study, the isolates that were resistant to various heavy metals demonstrated their resistance abilities to multiple antibiotics, although some did not exhibit this capability. This might occur due to genes encoding heavy metals located on plasmids together with antibiotic resistance genes where selective pressure by similar compounds may select the whole set of resistance indirectly, or bacteria might have unspecific resistance mechanisms widespread to various substances, including heavy metals, antibiotics, biocides, to name but a few [62,63,71]. Most of the findings revealed that zinc and cadmium are closely associated with resistance to antibiotics [64]. However, our isolates were tolerant to chromium, lead, nickel, cobalt, and cadmium and mostly resistant to AMX, AMC, CTX and CTR. We did not find any mercury-resistant bacteria in our study. Kamala-Kannan and Lee (2008) isolated *B. cereus* from Sunchon Bay, South Korea and reported that it was resistant to mercury, cobalt, chromium, zinc, manganese, and several antibiotics, including AMC, kanamycin, STRP and TET [62]. Moreover, a significant correlation was determined between chromium to CTX, cadmium to GEN, CIP and nickel to AZM resistance among the isolates. Though almost all the isolates were sensitive to AZM, GEN and CIP, there is the possibility that they would be resistant to these antibiotics. The hemolysis assay was also conducted in our study to understand whether the bacterial isolates were pathogenic or not. Approximately 92.30% of the isolates were hemolytic in nature. Rahman and Singh investigated bacteria from surface water of different locations in India that were both heavy metal and antibiotics and revealed that 25% of the isolates showed α and β hemolysis [65].

5. Conclusion and outlook

The current study provides significant information about the major urban river water of Bangladesh, where heavy metals and multidrug-resistant organisms were found. Almost all the isolated organisms were pathogenic in nature, which triggers a public health concern. The discharge of industrial effluents without treatment, antibiotics originating from hospitals and farms into the river, and antibiotics-resistant bacterial loads in wastewater may be responsible for this. Furthermore, the untreated sewage water carrying pathogenic bacteria from the municipality may also adversely affect the river bacterial communities, leading to antibiotic resistance. The government of Bangladesh should take proper steps to ensure wastewater treatment in industries as well as sewage treatment in city municipalities to reduce the dissemination of pathogenic metal and antibiotic-resistant bacterial strains. The industry can play a key role here by optimizing their disinfection procedures. The proper management of wastewater, sewage water and manure is also very necessary. The number of samples and isolates was low in the current study, but the considerable number of samples and bacterial isolates could depict a clearer picture of these rivers. The plasmids of the bacterial isolates could be investigated to figure out the heavy metals and antibiotic resistance genes in bacteria. However, this investigation could be a baseline study for other rivers of Bangladesh to understand the environmental dissemination of metals and antibiotics-resistant bacteria throughout the country.

CRediT authorship contribution statement

Mohammad Mahbub Kabir: Conceptualization, supervision, project administration investigation, designing the experiments, methodology, validation, and writing of the original draft. **Sadia Mahbub Maleha**: Investigation, designing the experiments, methodology, validation, and writing of the original draft. **Md. Saddam Hossain:** Supervision, investigation, designing the experiments, methodology, validation, and writing of the original draft. **Nazmin Sultana:** Investigation, designing the experiments, methodology, validation, and writing of the original draft. **Rashedul Islam:** Investigation, designing the experiments, methodology, validation, and writing of the original draft. **Saiful Islam:** Supervision, data curation, data interpretation, and statistical analysis. **Firoz Ahmed:** Review, discussion, writing-review, and editing. **Newaz Mohammed Bahadur**: Review, discussion, writing-review, and editing. **Tasrina Rabia Choudhury:** Validation, writing, review, and editing. **Md Didar-ul-Alam**: Review, discussion, writing-review, and editing. **Nasima Kabir:** Review, discussion, writing-review, and editing. **Leonard Tijing:** Validation, writing, review, and editing. **Ho Kyong Shon:** Discussion, writing, review, and editing.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dwt.2024.100298.

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