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Molecular Characterization of Extended Spectrum Beta-Lactamase Producing Bacteria Isolated from a River Surface Water

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ABSTRACT

The existence and prevalence of multi-drug resistant extended-spectrum β-lactamase (ESBL)-producing bacteria in the river water is a major cause of numerous diseases worldwide. In this study, the molecular characterization of antimicrobial-resistant bacteria producing ESBL encoding genes was investigated for a better understanding of the risk factors and public health issues. The potential ESBL-producing bacterial species were detected using 16S ribose ribonucleic acid (rRNA) polymerase chain reaction (PCR). This served as a screening step to detect potential ESBLs encoding genes which were confirmed by phenotypes (DDST and E-test) and genotypes (PCR) assays with the presence of the* bla *genes; TEM, CTX-M, OXA-1 and SHV. Furthermore, all the confirmed bacterial isolates producing ESBL encoding genes were analysed for antibiotic susceptibility patterns against 10 different classes of antibiotics as a choice of therapy using antibiotic susceptibility testing (AST) by the disc diffusion method. The highest bacterial isolates were confirmed as Enterobacteriaceae (89.5%), predominantly* Escherichia coli *and* Klebsiella pneumoniae*. Among the 20 bacterial isolates, 12 (60 %) bacteria possessed one or more* ESBLs encoding genes*. Relatively high occurrence rates of β-lactamase genes; bla TEM 35%, bla SHV 20%, bla OXA-15% and bla CTX-M 10% were recorded. All the ESBLs encoding isolates showed high resistance to* penicillin's, *third-generation cephalosporins,* monobactams, cephamycins and carbapenems. H*igh occurrences of ESBLs producing bacteria in the environment pose a threat to exposed communities. Therefore, early detection of MDR beta-lactamase mediated resistance genes are essential to avoid numerous diseases due to the dissemination of ESBLs producing strains.

Keywords: Antibiotic resistance, ESBL, blaSHV, blaTEM, blaOXA-1, blaCTX-M

1. Introduction

The extended-spectrum β-lactamases (ESBLs) encoding bacterial isolates are among the most harmful biological contaminants originating from urban surface waters, such as river, lakes, sewage treatments plants and wells [1]. Antibiotic resistance Gram-negative bacteria producing ESBLs and multidrug-resistant have emerged and spread throughout the world in recent decades. Based on the literature review, the occurrence of ESBLs encoding enzymes is more prominent among enteric bacterial species, while environmental bacteria carry the most ESBL trait [2]. However, ESBLs associates clinical diseases have been a serious public health concern across the world.

The main mechanisms of bacterial resistance against different classes of antibiotics are due to beta-lactamase enzymes [3]. Besides, ESBLs enzymes are a branch of β-lactamases, targeting a broader spectrum of antibiotics; penicillin's, monobactams, and cephalosporins. Nevertheless, they frequently remain susceptible to imipenem and meropenem [3]. The dissemination of multi-drug resistant bacteria from humans and animals into water poses a serious health risk to local communities. Besides, many antibiotics residues affect the biological activity of the microbes, although β-lactamase enzymes, TEM, SHV, OXA, CTX-M possess one or more virulent factors within the same bacterial species or different species. In general, *E. coli*, *Klebsiella*, *Pseudomonas sp*., *Shigella sp.* and *Salmonella sp*. are the most widespread bacteria possessing ESBL genes [4].

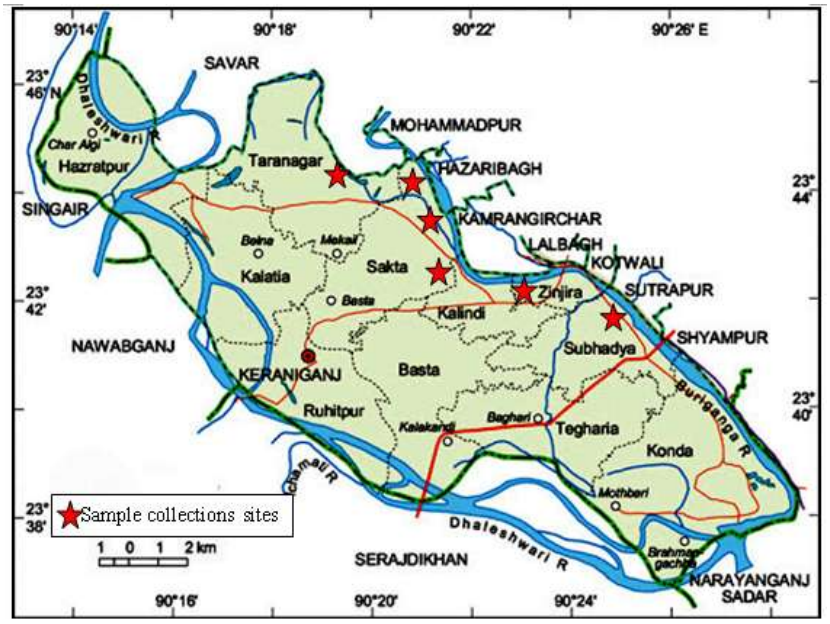
To-date ESBL producing bacteria in Buriganga River water are yet to be known correctly. Besides, antibiotic resistant bacteria are hazardous biological contaminants found in the urban surface area [2]. Therefore, the perseverance of antibiotic-resistant intestinal bacteria in water, particularly ESBLs encoding enzymes producing bacteria pose a serious public health risk and hazards to ecosystem functions. For this reason, a study is needed to assess the present water quality and biological contaminants, which are the root cause for several diseases to the local people who are exposed to this surface water. Besides, infections managements due to these virulent factors is a foremost challenge for health professionals.

This study investigates the role of urban river surface water as a potential source of risky ESBLs encoding enzymes and their multidrug resistant properties which may easily disseminate within the same bacterial species or different species.

2. Materials and methods

Study sampling stations

Water samples were collected from the different part of Buriganga River (Dhaka, Bangladesh) which is exposed to the highly industrialized environment. Sampling was carried out early in the morning, ensuring that it was not a rainy day, in January 2020. The water sampling areas of the Buriganga river were divided into six sampling stations to detect the ESBLs encoding enzymes producing bacteria.



Coordinate place	Buriganga river (latitude)
Sadarghat	23°42'40.2''N90°23'42.6''E
Zinzira	23°42'34.0''N90°23'36.2''E
Showarighat	23°42'40.1''N90°23'38.1''E
Boshila	23°44'54.0"N 90°20'31.9"E
Kamrangirchar	23°42'53.0"N 90°21'39.9"E
Gabtoli	23°47'02.1''N90°20'08.1''E

Figure 1: Sampling stations

Bacterial isolations

Water samples were serially diluted and plated onto the pepton-agar plates and incubated at 30º C under aerobic conditions. Few colonies of bacterial strains were chosen followed by additional grown on nutrient agar (NA) for pure culture strains.

DNA isolation of bacteria

Few colonies of the pure culture bacteria were grown in nutrient broth (NB) medium for overnight followed by isolation of genomic DNA via boiling assays [5]. The desired characteristics of isolated bacteria were identified using 16SrRNA with the universal primers 27f and 1492r [2] as shown in Table 1.

Amplicon/ Target group	Sequence (5'-3')	Size (bp)	Reference
Universal-27F Universal-1492 R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	1500	[2]
bla_{SHV}	CTT TAT CGG CCC TCA CTC AA AGG TGC TCA TCA TGG GAA AG	237	[6]
bla_{TEM}	CGC CGC ATA CAC TAT TCT CAG AAT GA ACG CTC ACC GGC TCC AGA TTT AT	445	[7]
bla_{CTX-M}	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAG CGG	593	[8]

bla_{OXA}	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	813	[9]

Phenotypic ESBLs confirmation assays

In this study, all the bacterial isolates were assessed by the double disk synergy test (DDST) for ESBLs encoding enzymes [10-11]. In the nutrient broth, the bacterial suspensions of 1.5×10^8 cfu/ml (0.5 McFarland standards) concentration were prepared while bacteria were plated on a Muller-Hinton (MH) agar followed by discs of third generation cephalosporins (Ceftazidime-CAZ 30 µg) and amoxicillin/clavulanate (AMC-30 µg) disks (Oxid, UK) were kept 30mm apart from center to center on culture media. The plated disks were also incubated for overnight at 37 °C. The ESBLs encoding enzymes producing bacterial isolates were distinguished by at least 5 mm accumulative of the zone of diameter around Ceftazidime (CAZ 30 µg) and Amoxicillin/clavulanate (AMC 30 µg) disks, respectively. Furthermore, all the bacterial isolates were further investigated using ESBL E-test (AB Biodisk, Sweden) according to the manufacturer's instructions.

Detection of ESBLs genes

The ESBLs producing bacterial colonies were properly mixed in TE buffer, followed by DNA extraction by boiling method [12]. The whole genomic DNA of the ESBLs producing organisms was extracted via the QIAamp Mini Kit (QIAGEN, USA). In this study, commonly occurring ESBL trait among bla genes: TEM, SHV, OXA and CTX-M types were confirmed by polymerase chain reaction (PCR) with specific primers as listed in Table 3. The amplification of ESBLs encoding genes (SHV and TEM) was determined by initial denaturation at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 30 sec at 52 °C and 1 min at 72 °C. Also, 5 min at 72 °C was measured for the final extension. The amplified PCR produced 475 bp and 800 bp for SHV and TEM genes, respectively. Furthermore, OXA and CTX-M genes were amplified using different cycling parameters [5 min at 95°C, 30 cycles (1) min at 94°C, annealing of 1 min at 52°C, final extension of 1.5 min at 72°C] followed by a final extension of 10-min at 72°C]. The PCR mixture consisted of Taq PCR Master mix 12.5 µl, 1 µl of forward and reverse primers, template DNA 7 µl, and H_2O 3.5 µl in 25 µl of PCR reaction. However, all the commercially manufactured oligonucleotide primers were obtained from Apical Scientific Sdn Bhd, Malaysia. The amplified DNA templates were distinguished by 1.5% agarose gel electrophoresis stained with 0.5µg/ml GelRed (Biotium, USA) and examined for DNA under ultraviolet light using gel documentation system (Amersham Imager 680, USA). All the PCR products were purified using the QIAquick PCR purification kit (QIAGEN, USA) following the manufacture's guidelines. After purification, the molecular weight of the DNA was determined and compared with the standard DNA molecular weight (1kb DNA ladder) marker (QIAGEN, USA). The obtained purified DNA was sent for sequencing at Apical Scientific Sdn Bhd Malaysia. All those obtained nucleotide sequences were investigated via the online BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antimicrobial susceptibility testing

All the isolated bacterial species were screened for antibiotic susceptibility patterns by disk diffusion method [11, 13] against 10 clinically relevant antibiotics (Amoxycillin (AML), 10 µg; Amikacin (AK), 30 µg; Amoxicillin/Clavulanic Acid (AMC), 30 µg; Aztreonam

(ATM), 30 µg; Ciprofloxacin (CIP), 5 µg; Cefotaxime (CTX), 30 µg; Ceftazidime (CAZ), 30 µg; Imipenem (IMP), 10 µg; Meropenem (MEM), 10 µg; and Piperacillin/Tazobactam (TZP), 40 µg). Briefly, bacterial colonies were streaked on Muller Hinton agar and antibiotic disks were plated. All these plates were incubated at 37 °C for 24 hours. Finally, inhibition zone measurement was performed using the Clinical and Laboratory Standard Institute (CLSI) guidelines [14].

Statistical analyses

A significance level between different ESBLs encoding bacterial genes was defined by Excel-Megastat software (2007) using Chi-squared test and p-value ($p \leq 0.05$) was considered significant [15].

3. Results

Isolation and identification of bacterial isolates

Twenty different bacterial species were isolated from all the six sampling stations (Figure 1). 90% of the twenty bacterial isolates are Gram negative bacteria from the Enterobacteriaceae family (Table 2).

Table 2 Number of bacterial species

Bacterial species	Number of isolates
Escherichia coli	8
Pseudomonas sp	5
Klebsiella sp.	4
Shigella sp.	2
Salmonella	1

Phenotypic confirmation of ESBL producing bacteria

Among the 20 bacterial isolates, 12 isolates were confirmed for phenotypic detection of ESBL producing bacteria by DDST and E-test (Figure 2).

s on the production of ESBL encoding bacterial isolates. The highest number of ESBL encoding genes were possessed by *E. coli* (50%, 6/12), followed by *Klebsiella* sp (25%, 3/12) and *Pseudomonas* sp (17%, 2/12). The least number of ESBLs genes were detected in *Shigella* sp (8%, 1/12), while *Salmonella* sp isolates were positive for ESBL genes. Among 16 bla genes, TEM (44%) shows the highest prevalence, followed by SHV (25%) and OXA-1

(19%), while bla CTX-M (13%) was the least. Moreover, the existence and prevalence of bla genes detection was significant ($p < 0.01$) as shown in Table 3.

Table 3: Number of ESBLs encoding genes among bacterial isolates

Bacterial isolates	ESBL positive isolates	TEM	SHV	OXA-1	CTX-M	P-value
Escherichia coli	6	4	0	3	0	
Pseudomonas sp	2	1	2	0	1	
Klebsiella sp.	3	2	2	0	0	0.01
Shigella sp	1	0	0	0	1	
Salmonella	0	0	0	0	0	

Genotypic confirmation of ESBL producing bacteria

The presence of ESBL genes was detected by molecular testing. The polymerase chain reaction (PCR) was assessed to determine ESBLs encoding genes: SHV, TEM, CTX-M, and OXA. The genotypic confirmation of ESBLs producing bacteria assays also revealed the same number (60%, 12/20) of positive isolates as of phenotypic detection. The highest prevalence of 7(58.4%) out of 12 isolates was found to possess one or more TEM genes, followed by 33.4% isolates which possessed one or more SHV genes. The least ESBL producing genes (CTX-M - 16.7% and OXA-1 - 12%) were observed too. Among the 5 different bacterial isolates, *E. coli* is the most prominent bacterial isolate, possessed one or more ESBL producing genes with TEM (57%, 4/7) and OXA-1 (100%, 3/3). Besides, *Pseudomonas sp* possessed TEM (14.2%, 1/7), followed by SHV (50%, 2/4) and CTX-M (50%, 1/2), while *Klebsiella sp* was found to possess TEM (28.6%, 2/7) and SHV (50%, 2/4) ESBL genes. Furthermore, *Shigella sp* possessed CTX-M, while *Salmonella* did not contain any ESBLs genes (Figure 3).

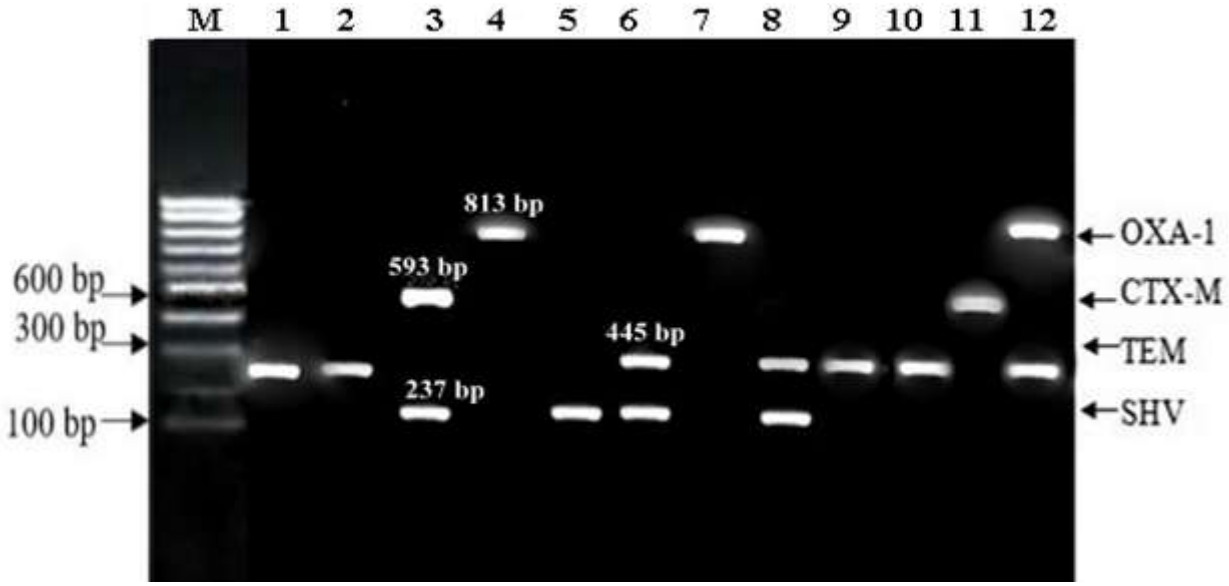


Figure 3 PCR amplification of bla_{SHV}, bla_{TEM}, bla_{CTX-M} and bla_{OXA-1}

Antibiograms of ESBLs producing bacterial isolates

All the 12 ESBL producing bacterial isolates were assessed for the determination of antimicrobial susceptibility patterns. Analysis of antibiotic susceptibility tests revealed that the ESBLs encoding enzymes producing enterobacteria were resistant to all 10 different types of clinical antibiotics tested.

It was found that ESBL encoding genes were 83% resistant to third-generation cephalosporins: amikacin, meropenem, while 75% resistance was recorded against cefotaxime, Piperacillin/Tazobactam and imipenem. Besides, 67% resistance were recorded against aztreonam, amoxicillin/clavulanate and ciprofloxacin, while 58% to ceftazidime. In addition, all the four ESBLs encoding genes producing bacteria (*E. coli*, *Pseudomonas sp, Klebsiella sp,* and *Shigella sp*) were completely resistant (100%) to amoxicillin (Table 4).

Table 4: Antibiogram profile of ESBLs producing bacterial isolates

	***Escherichia Coli* (6)**			***Klebsiella* sp. (3)**			***Shigella* sp. (1)**			***Pseudomonas* sp. (2)**		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
AML	6		-	3	-	-	1	-	-	2	-	-
AK	5		1	2	-	1	1	-	-	2	-	-
AMC	4	1	1	2	-	1	1	-	-	1	1	-
ATM	4	1	1	2	-	-	1	-	-	1-	1	-
CIP	4	1	1	2	-	1	1	-	-	1	1	-
CTX	4	1	1	2	-	1	1	-		2	-	-
CAZ	4	1	1	2	-	1	1		1	-	-	1
IMP	5	1	-	2	-	1	-	-	1	2	-	-
MEM	5	1	1	2	-	1	1	-	-	2		-
TZP	4	1	1	3	-	-	1	-	-	1	1	-

Abbreviation: R = Resistant, I = Intermediate, S = Susceptible, Amoxycillin (AML), 10 µg; Amikacin (AK), 30 µg; Amoxicillin/Clavulanic Acid (AMC), 30 µg; Aztreonam (ATM), 30 µg; Ciprofloxacin (CIP), 5 µg; Cefotaxime (CTX), 30 µg; Ceftazidime (CAZ), 30 µg; Imipenem (IMP), 10 µg; Meropenem (MEM), 10 µg; and Piperacillin/Tazobactam (TZP), 40 µg).

4. Discussion

The existence and prevalence of ESBLs encoding bacteria are common in surface water. This dissemination of virulent factors develops multidrug resistant naturally against commonly available drugs and this trend has become a serious community health concern across the world.

In this study, the dissemination of ESBL encoding genes possessed by multiple bacterial isolates is in accordance with several recent studies [10-11, 16]. Besides, the prevalence rate of ESBLs encoding genes is variable in different regions of the world. Both the phenotypic (DDST and E-test) and genotypic (PCR assays) confirmatory studies were found to be efficient for detection of ESBLs encoding genes, demonstrating an agreement with the results of Abrar et al., 2019 and Khan et al., 2019. Meanwhile, both the phenotypic (DDST and E-test) as a well genotypic test (PCR screening) revealed that among the 20 bacterial isolates present in six different sites in this study, 12 (60%) isolates possessed ESBL encoding one or more genes in accordance with Liu et al., 2018. Furthermore, *E. coli* possessed the highest (6, 50%) prevalence of ESBLs encoding genes, followed by *Klebsiella sp* 3 (25%), and *Pseudomonas sp* 2 (17%), although *Salmonella sp*. did not possess any ESBL gene, providing accordance with a Tissera et al., 2013.

In general, *E. coli* and *Klebsiella sp.* have a higher tendency to possess and transfer bla genes encoding ESBLs. A high rate of ESBLs encoding genes producing *Enterobacteriaceae*; *E. coli* and *Klebsiella* sp was also recorded in infected new-borns [19]. Furthermore, the blaSHV was the most common in pseudomonas sp. in this study, while the most frequently isolated bla enzymes are OXA worldwide [20]. The data obtained from this study showed that the ESBLs genes, SHV were not found in *E. coli*, confirming with several types of research [2, 21], although the ESBLs encoding genes, SHV (52.7%) of *E. coli* were recorded in Turkey [22]. Several studies have demonstrated the possession of ESBLs encoding genes, TEM [23], SHV [24], OXA-1 [25] and CTX-M [26] by *Salmonella sp* across the world. In addition, ESBLs encoding genes possessed by *Salmonella* elevated a serious paediatric infection [2], although *Salmonella* sp. isolates did not harbour any ESBLs encoding gene, agreeing with researches of Tissera & Lee., 2013. *E. coli* isolates harbouring CTX-M were found to be associated with severe illness among human and animals [18]. However, none of the tested *E. coli* possessed CTX-M ESBLs encoding gene in this current study.

The antibiotic resistance among beta-lactams Gram negative bacterial isolates is growing across the world [27]. In this study, the antibiogram of ESBLs producing bacterial isolate of river water was investigated to elucidate their susceptibility patterns which may attribute to efficient treatment therapy by avoiding prolonged or failed treatments. Several studies that revealed that the pathogenic bacteria harbouring ESBL encoding gens have the capability to resist third-generation cephalosporins, other beta-lactams and some non-beta-lactam agent [10, 28] is in agreement with our current study.

Currently, all the antibiotics as a choice of therapy were mostly resistant to all the tested bacterial isolates of the river surface water. Moreover, this present study shows a higher level of resistance compared to several recent studies [10-11, 16, 29]. A higher rate of resistance to amoxicillin, followed by amikacin, imipenem and meropenem in ESBLs encoding genes, reflects the increasing prevalence of resistance. Besides, amoxicillin/clavulanic acid, ciprofloxacin, ceftazidime, and aztreonam were susceptible and may be considered as an appropriate choice of therapy against infections. The high level of resistance to amikacin and meropenem was reported in this study, although these antibiotics were highly sensitive as reported in a recent study by Mohammed et al., 2016. ESBLs encoding genes possessed by *Pseudomonas sp.* revealed the highest resistance (100%) to imipenem, although imipenem was the most suitable choice of therapy [31]. However, the prevalence of ESBL-resistant bacterial genes has been increasing in recent years [10, 16].

The dissemination of ESBLs encoding genes may rise over time and the transfer of bla genes may occur by conjugation, transposons and integrons. Moreover, high contaminated river water containing ESBLs-producing pathogens could be harmful to communities and poses a potential risk of drug resistant infections. However, further studies (monitoring, surveillance and accurate detection) are required for observing the ESBLs encoding genes among different organisms. A review of the current guidelines for ESBL-producing bacterial isolates as a choice of therapy is required in order to ensure effective and appropriate antibiogram therapy to save lives. It is important to note that limited selections of surface waters were involved in this report. However, this study emphasises the necessity of the frequent evaluation of local antimicrobial resistance rates for specific pathogens.

5. Conclusions

A higher prevalence of ESBLs encoding genes by bacterial isolates has been noticed, posing a threat to public health across the world. The dissemination of ESBLs encoding genes, blaTEM, blaSHV, blaCTX-M and blaOXA-1 from the surface waters could be a crucial and potential health risk to communities. Therefore, regular monitoring and urgent water quality control measures and practices are necessary to avoid infections due to ESBLs producing organisms. Moreover, further studies including different surface waters across the highly industrial areas are necessary to scrutinize the emergence of ESBLs encoding genes resistance among bacterial species.

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