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Extended-Spectrum β-Lactamase-Producing *Salmonella* Species Isolated from Diarrhoeal Patients in Bangladesh: Characterization and Their Dissemination through Conjugation

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AP and MMH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Author MRH managed the analyses of the study and literature searches. Authors KAT and IJA supervised the experiment and manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Objective: Infections with *Salmonella* resistance to extended-spectrum cephalosporins threaten the efficacy of drugs for treating samonellosis. So the aim of this study was to characterize the ESBL (Extended Spectrum β-lactamase)-producing *Salmonella* species.
Methods: The ESBL-producers were extensively characterized using antibiogram, double disc

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diffusion synergy test, plasmid profiling, PCR, pulsed-field gel electrophoresis (PFGE) and conjugation experiments.

Results: Of the 200 *Salmonella* strains, n=8 were found to be ESBL-producers and these belonged to only two serogroups, *Salmonella* Group B (n=4) and *Salmonella* Group G (n=4). Most of the ESBL-positive strains were found to be resistant to third and fourth-generation cephalosporins and monobactams. Plasmid profiling indicated that n=6 and n=2 of the ESBL-producing strains harbored a 62-MDa and a 90-MDa plasmid, respectively. The PCR analysis revealed that *bla*TEM (β-lactamase Enzyme TEM Producing Gene) (n=6) was most predominant gene, followed by the *bla*OXA (β-lactamase Enzyme OXA Producing Gene) (n=4), *bla*SHV (β-lactamase Enzyme SHV Producing Gene) (n=2) and *bla*CTX-M-1 (β-lactamase Enzyme CTX-M-1 Producing Gene) (n=2) genes. Fifty percent (n=4) of the strains were positive for the *int1* gene. The PFGE analysis revealed that almost similar clones were disseminated within the ESBL-producing strains and the non-ESBL-producing strains. The conjugation study revealed that the 62-MDa plasmid was transferred to *E. coli* K-12 and contained the *bla*TEM, *bla*OXA, *bla*CTX-M-1 and *int1* genes.

Conclusion: The emergence of ESBL-producing *Salmonella* is of great concern and horizontal gene transfer plays an important role in the spread of ESBL.

Keywords: ESBL; blaTEM; blaSHV; blaOXA; blaCTX-M; plasmid; conjugation.

INTRODUCTION

In developing countries, diarrhoea is a major factor in infant mortality [1]. Salmonellosis caused by non-typhoid *Salmonella* is one of the most common human gastroenteritis worldwide and improper handling and digestion of inadequately cooked food are the risk factors for the infection [2]. Although human outbreaks of *Salmonella* Livingstone are relatively uncommon [3], the death rate due to salmonellosis is higher in infants and among the very old [1].

In case of infectious diarrhoeal episodes, antimicrobial agents are the most effective treatment strategy. Infections caused by resistant pathogens have higher rates of morbidity and mortality associated with them due to potential treatment failure than do infections caused by susceptible pathogens [4]. In this context, routine screening for antibiotic susceptibility of gram-negative bacteria is of great importance.

Antimicrobial-resistance to extended spectrum cephalosporins (third-generation), *e.g.*, ceftazidime, ceftriaxone, cefotaxime and monobactams, *e.g.*, aztreonam is inhibited by clavulanic acid (CA) [5,6] and does not affect second-generation cephalosporins, *e.g.,* cefoxitin, cefotetan [7]. More than 340 β-lactamases have been described and many of these proteins have been identified in *Salmonella* [6]. ESBL-producing *Salmonella* has been reported in a nosocomial outbreak in a neonatal ward in Tunisia [8].

There were a limited number of studies showing the prevalence of ESBLs in Bangladesh. It has been reported that ESBL–producing *Klebsiella* species were predominant (57.89%) followed by *Proteus* species (50.0%), *Escherichia coli* (47.83%) and *Pseudomonas* species (31.35%) in 2010 in Bangladesh [9]. Till now there was no report about the prevalence and characterization of ESBL-producing *Salmonella* species in Bangladesh.

In India, TEM and SHV β-lactamases were found in 30% of *Escherichia coli* isolates and in 38% of *Klebsiella* species, respectively, in 2010 [10]. Carbapenem (metallo-β-lactam), *e.g.*, meropenem and imipenem resistant-*Escherichia coli* (producing a new type of metallo-β-lactamase called NDM-1) was isolated in 2009 [11]. In 2011, Manoharan and his colleagues found TEM and CTX-M in *Escherichia coli* (39.2%) and TEM, SHV and CTX-M in *Klebsiella* species (42.6%) [12].

These newly emerged enzymes are encoded by genes that are usually present in plasmids [13]. Bacteria exposed to antimicrobial agents may have had their populations selected for a resistance trait and thus become a reservoir for potentially mobile resistance genes [13]. These genes are capable of being transferred to pathogenic non-clonal strains through horizontal gene transfer (HGT). Particularly conjugation [13] is thought to be the main mechanism of HGT, because transformation and transduction are notably rare in the environment [14]. The dissemination of resistance genes by conjugation

has led to the rapid emergence of antibiotic resistance among clinical bacterial isolates [14].

In this study, we have characterized ESBL-producing *Salmonella* species regarding their ESBL-gene, plasmid and PFGE profiles, and investigated the transfer by conjugation of plasmids possibly carrying ESBL–genes. The aforementioned characterization would allow the investigation of resistant clonal strains that have been circulating in Bangladesh in the last years.

2. MATERIALS AND METHODS

2.1 Bacterial strains

From a total of 2,502 *Salmonella* strains, isolated between 2007-2013, from children and adults at the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Among these, 200 multi-drug resistant (MDR) strains (all were resistant to ampicillin, some were resistant to sulfomethoxazole-trimethoprim, cholaramphenicol, tetracycline, nalidixic acid and ciprofloxacine), were selected for ESBL screening. This group was formed by 60 (30%) *Salmonella* Typhi, 42 (21%) *Salmonella* Paratyphi, 36 (18%) *Salmonella* Group C1, 32 (16%) *Salmonella* Group B and 30 (15%) *Salmonella* Group G. After *Salmonella* serogroup confirmation (by anti-sera kit, Denka Seiken Company, Japan), strains were grown in trypticase soy broth with 0.3% yeast extract and stored at -70°C after the addition of 15% glycerol. It is important to note that 100 species were isolated from adult and another 100 from children patient's stool sample.

Escherichia coli (ATCC 25922) was used as negative strain for quality control of the disc diffusion tests and PCR. The *Escherichia coli* strains PDK-9, V-517, R1 and RP_4 were used as plasmid molecular mass standards [15]. *Escherichia coli* K-12 was used as the recipient in the conjugation experiments. *Salmonella* Braenderup (1962) was obtained from the Enteric and Food Microbiology Laboratory, International Center for Diarrhoeal Disease Research, Bangladesh and used as the Pulsed Field Gel Electrophoresis molecular weight standard [16].

2.2 Antimicrobial susceptibility test

The 200 strains were re-screened for resistance to 13 antimicrobials, on Mueller-Hinton (MH) agar by the disc diffusion method according to the guidelines of CLSI (2013) [17]. The following antimicrobials (Bio-Rad) were tested: ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg) (third-generation cephalosporin), cefepime (FEP, 30 µg, fourth-generation cephalosporin), cefoxitin (FOX, 30 µg, second-generation cephalosporin), mecillinam (MEC, 25 µg), aztreonam (ATM, 30 µg, monobactam), imipenem (IMP, 10 µg, carbapenem), ampicillin (AM, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg) and nalidixic acid (NA, 30 µg).

2.3 Double disk diffusion test

Third-generation cephalosporin-resistant *Salmonella* strains were screened for the production of ESBLs using the double disc diffusion test as described previously [18] with some modifications [19]. Briefly, a central disc of amoxicillin-clavulanic acid containing 10 µg of the latter compound was surrounded by disks with 30 µg of one of the antibiotics, such as ceftazidime, ceftriaxone, aztreonam and cefoxitin, at a distance of 15 mm (edge to edge) on a MH agar plate, which was inoculated according to standard procedures [19]. After overnight incubation at 37ºC, any enhancement of the zone of inhibition between a β-lactam disc and the central disc was considered as the indication of the presence of ESBL [19].

2.4 Plasmid analysis

The plasmid DNA was prepared according to the simplified alkaline lysis method developed by Kado and Liu (1981) and subjected to agarose gel electrophoresis (0.8% agarose) [15,20]. The molecular mass of the plasmids were determined through comparison with the reference molecular mass (PDK-9, 140, 2.7, 2.1 MDa; R1, 62 MDa; RP4, 36 MDa and V517, 35.6, 4.8, 3.7, 3.4, 3.1, 2, 1.8, 1.4 MDa) [15,21].

2.5 Detection of ß-lactamase genes and integrons by PCR

The primers and the parameters used for the detection of the ESBL-producing genes, class 1 integron (*int*1) and class 2 integron (*int* 2) through the PCR method are presented in Table 1. The template DNA was prepared using the standard method [15] and the PCR reaction conditions used were as described previously [22-25].

2.6 PFGE

Intact agarose-embedded chromosomal DNA from the *Salmonella* strains were prepared and the PFGE was performed using a contour-clamped homogeneous electric field (CHEF-Mapper) apparatus from Bio-Rad Laboratories (Richmond, CA, USA) according to procedures described earlier [15]. The genomic DNA was digested with the *Xba*-I restriction enzyme (Gibco-BRL). The restriction fragments were separated using a CHEF Mapper system apparatus in 1% pulsed-field certified agarose in 0.5X TBE buffer. The electrophoresis was conducted for 20 h at 6 volts and 14ºC with an initial switch time of 2.16 s and a final switch time of 54.17 s. The gel was stained with ethidium bromide, destained and photographed on a gel documentation system according to procedures described earlier [15]. The DNA size standards used were the *Salmonella* serotype Braenderup (H9812), which ranged from 20.5 to 1,135 kb [16]. Isolates with one band difference were considered to be of distinct pulsetypes.

2.7 Resistance transfer determination by conjugation

The conjugation between eight ESBL-producing *Salmonella* species containing 62- and 90-MDa plasmids as donors and *E. coli* K-12 (Nal^R, lactose-fermenting, fertility factor-negative) as the recipient was conducted using the plate matting procedure that was described by Munshi et al. in 1987 [26]. The transconjugants were selected on MacConkey agar plates supplemented with and without nalidixic acid (1 mg/liter) and ampicillin (1 mg/liter).

3. RESULTS

3.1 Antimicrobial susceptibility test

Of 200 *Salmonella* strains, 100% (n=200) were resistant to ampicillin (AM), 65% (n=130) were resistant to nalidixic acid (NA), 80% (n=160) were to chloramphenicol (C), 95% (n=190) were to tetracycline (TE), 97.5% (n=195) were to sulfamethoxazole-trimethoprim (SXT) and 15% (n=30) were to ciprofloxacine (CIP). Eight strains exhibited resistance to third-generation cephalosporins such as ceftriaxone and cefotaxime, which were used as surrogate markers to detect ESBL-producers. So, within these eight strains, ceftriaxone and cefotaxime showed the highest (n=8) number of resistance, followed by cefepime (n=6), aztreonam (n=4) and ceftazidime (n=4). All of these strains were susceptible to second-generation cephalosporin (cefoxitin) and also to mecillinam and imipenem. Four strains were resistant to nalidixic acid (NA) and tetracycline (TE), six strains were resistant to chloramphenicol (C) and all were resistant to sulfamethoxazole-trimethoprim (SXT) and ampicillin (AM). S5 and S10 were susceptible to all of the antibiotics tested (Table 2). The double disc diffusion test confirmed the presence of ESBL within these eight strains.

3.2 Plasmid analysis

These representative strains exhibited three plasmid patterns, which were designated P1 through P3. Of these, P3 was predominant, followed by P1 and P2. Six strains harbored a 62-MDa plasmid and two strains harbored a 90-MDa plasmid. The ESBL-negative strains exhibited different patterns, which were designated P4 and P5 (Table 3, Fig. 1).

3.3 PCR amplification of ß-lactamase genes and class I integrons

Seventy five percent (n=6) and fifty percent (n=4) of these eight *Salmonella* strains possessed the *bla*TEM and *bla*OXA genes, respectively. In addition, 25% of the strains contained the *bla*SHV and *bla*CTX-M-1 genes and none of the ESBL-producing *Salmonella* strains was positive for the *bla*CTX-M-2, *bla*CTX-M-8, *bla*CTX-M-9 and *int2* genes. In contrast, 50% (n=4) of the ESBL-producing *Salmonella* strains possessed the *int1* gene (Table 3).

3.4 PFGE

The PFGE analysis of the *Xba*-I digested chromosomal DNA of the ESBL-producing *Salmonella* strains yielded 12 to 16 reproducible DNA fragments that ranged in size from approximately 20.5 to 1135 kb (Fig. 2).

Three PFGE patterns (A, B, and C) were found in the eight ESBL-producing strains in this study. The banding patterns of these strains were also compared with the non-ESBL-producing *Salmonella* strains (banding patterns D and E).

Table 1. Primers and the PCR parameters for the detection of ESBL-producing genes and *int* 1 and *int* 2

Genes	Primer	Oligonucleotide Sequence (5´ to 3´)	Annealing Temperature (ºC)	Size of Amplicon (bp)	Reference
*bla*TEM	TEM-F	5' TCG GGG AAA TGT GCG CG 3'	50	971	23
	TEM-R	5' TGC TTA ATC AGT GAG GAC CC 3'			
*bla*SHV	SHV-F	5' CAC TCA AGG ATG TAT TGT G 3'	50	885	23
	SHV-R	5' TTA GCG TTG CCA GTG CTC G 3'			
*bla*OXA	OXA-F	5' ACCAGATTCAACTTTCAA 3'	55	598	24
	OXA-R	5' TCTTGGCTTTTATGCTTG 3'			
*bla*CTX -M-1	CTXM1- F	5' GGA CGT ACA GCA AAA ACT TGC 3'	57	200	22
	CTXM1-R	5' CGG TTC GCT TTC ACT TTT CTT 3'			
*bla*CTX -M-2	CTXM2 –F	5' CGG YGC TTA AAC AGA GCG AG 3'	59	891	22
	CTXM2-R	5' CCA TGA ATA AGC AGC TGA TTG CCC 3'			
*bla*CTX -M-8	CTXM8 –F	5' ACG CTC AAC ACC GCG ATC 3'	57	490	22
	CTXM8-R	5' CGT GGG TTC TCG GGG ATA 3'			
*bla*CTX -M-9	CTXM9-F	5' GAT TGA CCG TAT TGG GAG TTT 3'	57	947	22
	CTXM9-R	5' CGG CTG GGT AAA ATA GGT CA 3'			
int 1	int 1 F	5' ACA TGT GAT GGC GAC GCA CGA 3'	60	569	25
	int 1 R	5' ATT TCT GTC CTG GCT GGC GA 3'			
int 2	int 2 F	5' GTA GCA AAC GAG TGA CGA AAT G 3'	58	789	25
	int 2 R	5' CAC GGA TAT GCG ACA AAA AGG T 3'			

Table 2. Characteristics of eight ESBL-producing and two non-ESBL-producing *Salmonella* strains isolated from human patients in Bangladesh

Strain ID	Lab ID	Serogroup	Year	Category	General Antimicrobial Resistance Pattern[a]	ESC-Resistance Pattern[a]
S1	K-5609	*S*. Gr. G	2008	Adult	(AM, SXT, C)^{R}, NAL^{I}	(CRO, CAZ, CTX, FEP, ATM)^{R}
S2	K-5659	*S*. Gr. G	2009	Adult	(AM, SXT)^{R}, NAL^{I}	(CRO, CAZ, CTX, ATM)^{R}
S3	K-5665	*S*. Gr. G	2011	Children	(AM, SXT)^{R}, NAL^{I}	(CRO, CAZ, CTX, ATM)^{R}
S4	K-6650	*S*. Gr. G	2012	Children	(AM, SXT, C)^{R}, NAL^{I}	(CRO, CAZ, CTX, FEP, ATM)^{R}
S5	K-7223	*S*. Gr. G	2012	Adult	All Susceptible	All Susceptible
S6	K-33	*S*. Gr. B	2007	Children	(AM, SXT, NAL, C, TE)^{R}	(CRO, CTX, FEP)^{R}, ATM^{I}
S7	K-35	*S*. Gr. B	2008	Adult	(AM, SXT, NAL, C, TE)^{R}	(CRO, CTX, FEP)^{R}, ATM^{I}
S8	K-37	*S*. Gr. B	2008	Adult	(AM, SXT, NAL, C, TE)^{R}	(CRO, CTX, FEP)^{R}, ATM^{I}
S9	K-42	*S*. Gr. B	2010	Adult	(AM, SXT, NAL, C, TE)^{R}	(CRO, CTX, FEP)^{R}, ATM^{I}
S10	KD-1554	*S*. Gr. B	2012	Adult	All Susceptible	All Susceptible

[a]*Abbreviations: S. Gr. B, Salmonella Group B and S. Gr. G, Salmonella Group G; ESC, Extended-spectrum cephalosporin; AM, ampicilin; SXT, Sulfamethoxazole-Trimethoprim; NAL, nalidixic acid; C, chloramphenicol; TE, tetracycline; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; CAZ, ceftazidime ; ATM, aztreonam; R, Resistant and I: Intermediary Resistant*

Table 3. Characteristics of the *Salmonella* species used in this study

Strain ID	Approximate Molecular Weight (MDa)	Plasmid Pattern (P)	*bla* Genes	Integrons	PFGE Banding Pattern
S1	90, 35, 1	P1	*bla*TEM, *bla*SHV	None	A
S2	62, 35, 1	P2	*bla*TEM, *bla*CTX-M-1	None	B
S3	62, 35,1	P2	*bla*TEM, *bla*CTX-M-1	None	B
S4	90, 35, 1	P1	*bla*TEM, *bla*SHV	None	A
S5	35, 1	P4	None	None	D
S6	62, 36, 4.8, 2.7, 1, 0.5	P3	*bla*OXA	*int*1	C
S7	62, 36, 4.8, 2.7, 1, 0.5	P3	*bla*OXA	*int*1	C
S8	62, 36, 4.8, 2.7, 1, 0.5	P3	*bla*TEM, *bla*OXA	*int*1	C
S9	62, 36, 4.8, 2.7, 1, 0.5	P3	*bla*TEM, *bla*OXA	*int*1	C
S10	62, 2.7, 2.1	P5	None	None	E

[a]*Abbreviations: S. Gr. B, Salmonella Group B and S. Gr. G, Salmonella Group G*

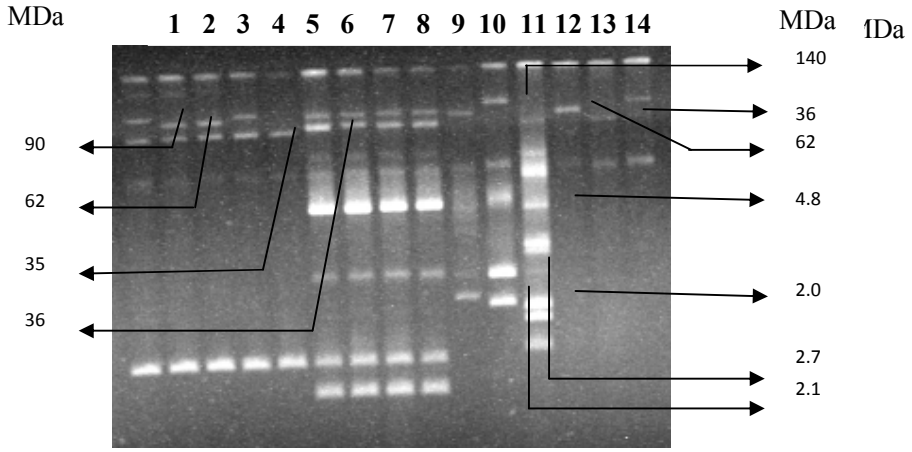

Fig. 1. Agarose gel electrophoresis of plasmid DNA of ESBL-positive and negative strains showing representative patterns of different *Salmonella* strains

Lanes: 1: S1 (P1) 2: S2 (P2); 3: S3 (P2); 4: S4 (P1); 5: S5 (P4) (non-ESBL Salmonella strain); 6: S6 (P3); 7: S7 (P3); 8: S8 (P3); 9: S9 (P3); 10: S10 (P5) (non-ESBL Salmonella strain); 11: E. coli PDK-9 (marker); 12: E. coli V-517 (marker); 13: E. coli R1 (marker); 14: E. coli RP4 (marker)

3.5 Resistance Transfer Determination by Conjugation

Among the eight ESBL-producing *Salmonella* strains, successful conjugation was achieved in six cases. The transconjugants had the 62-MDa plasmid and similar ESBL characteristics as the donors, as detected by the antibiotic susceptibility test and PCR (Table 4) (Figs. 3 and 4).

4. DISCUSSION

Bacterial antibiotic resistance has become a major clinical concern worldwide including Bangladesh. In adults, fluoroquinolones are the drugs of choice. In children, the use of fluoroquinolones is contraindicated due to their potential adverse effects on cartilage development. For this reason and due to their low incidence of resistance, extended spectrum cephalosporins are commonly used in children with salmonellosis [27]. In this study, we already found three strains obtained from children. These patients were unable to use ciprofloxacine, ESCs and other drugs as these were multidrug resistant. So, this is going to be a growing concern in the future.

Xiong et al. in 2002 explained that ESBL-producers are also resistant to other commonly used antibiotics, such as nalidixic acid, chloramphenicol, tetracycline and sulfamethoxazole-trimethoprim, which implied that ESBL-producers are multidrug resistant [28]. The eight strains which we found are all multidrug resistant. Thus this character severely limits the therapeutic options of patients infected with these organisms.

Recently, the use of third generation cephalosporins has led to the selection of Gram-negative organism's resistance to β-lactamase stable cephalosporins. This resistance is attributed to the production of extended spectrum β-lactamases [29]. In our study, we had eight ceftriaxone and cefotaxime resistant strains, which might be due to the overuse of these antibiotics by the patients [5,29,30].

On the other hand, Coudron et al. [19] suggested that the combination of an antibiotic and an augmenting agent is helpful for the treatment of infections by resistant strains, because the combinations of β-lactam antibiotics and β-lactam inhibitors are associated with a lower incidence of colonization of an ESBL-producing strain [31]. So, for maximum efficacy, several oxyimino-cephalosporins and amoxicillin-clavulanic acid should be used simultaneously.

The plasmid profile analysis revealed that most of these strains contained a multiple number of plasmids ranging from 0.5 to 90 MDa in size (Table 3). Previous studies have shown that plasmids responsible for ESBL ranged in size

from 53 to 200 MDa (80 to 300 Kb) [32]. In the present study, we found 90 and 62-MDa plasmids containing MDR-*Salmonella* species. The ESBL-producers often harbor a multidrug resistant plasmid that confers resistance to both β-lactams and non-β-lactams [28].

The PCR analysis revealed that the *bla*TEM, *bla*SHV, *bla*OXA, *bla*CTX-M-1 and *int1*genes were present either alone or together in the ESBL-producing strains (Table 3). CTX-M-ESBLs confer greater activity against cefotaxime than against ceftazidime. However, some hydrolyze ceftazidime more rapidly than cefotaxime [33]. OXA-ESBLs confer greater resistance to cefotaxime and cefepime than to ceftazidime [34]. SHV- ESBLs show higher activity against cefotaxime, ceftazidime and cefepime. Class 1 integrons are the most commonly found integrons in nosocomial and community environments, followed by class 2 integrons; the other integron classes have been scarcely reported [35]. Previous findings were in good agreement with the genes detected in this study.

In PFGE study, in case of *Salmonella* Group G, two patterns were found, A and B. Interestingly, pattern A was similar to pattern D, though it didn't have any ESBL-gene (non-ESBL-producer), but pattern B had some dissimilarity. This investigation revealed a common origin compared with the non-ESBL producer in *Salmonella* Group G though they were isolated from different specimens and in different years. On the other hand, in case of *Salmonella* Group B, only one pattern C was observed for ESBL-producers, though they had some genetic discrimination and this pattern had some discrepancies to E pattern (non-ESBL-producer) unlike *Salmonella* Group G.

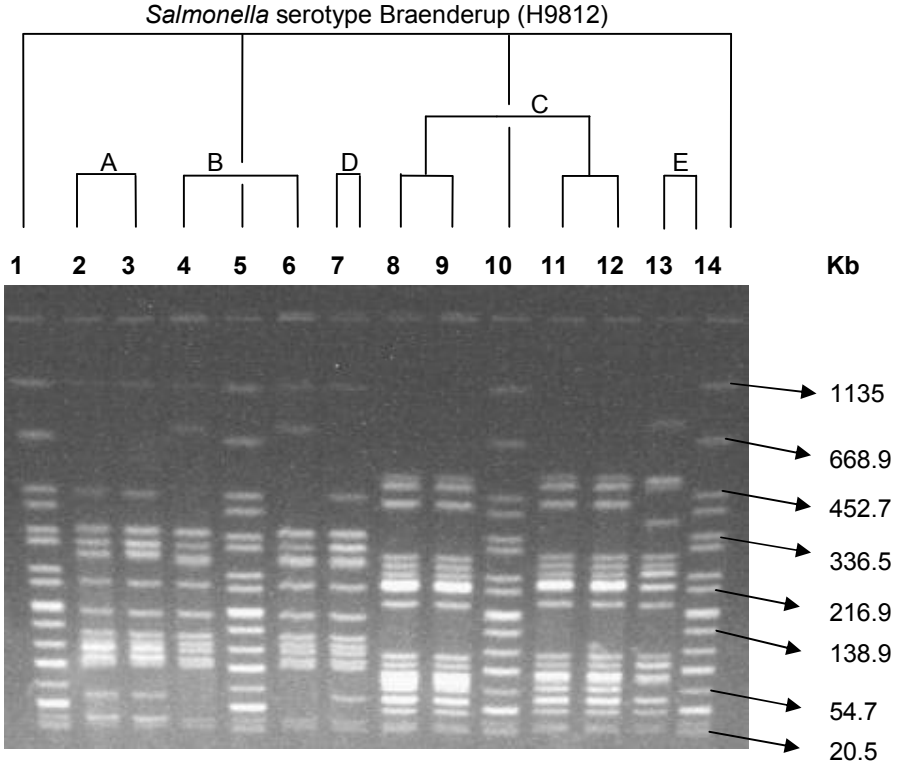

Fig. 2. PFGE banding patterns of X*ba*-I digested chromosomal DNA of representative *Salmonella* strains of ESBL-positive and negative

Lanes: 1, 5, 10, 14: Salmonella serotype Braenderup (H9812) (marker); 2: S1, 3: S4, 4: S2, 6: S3, 7: S5 (non-ESBL) (S. Gr. G) and 8: S6, 9: S7, 11: S8, 12: S9 and 13: S10 (non-ESBL) (S. Gr. B)

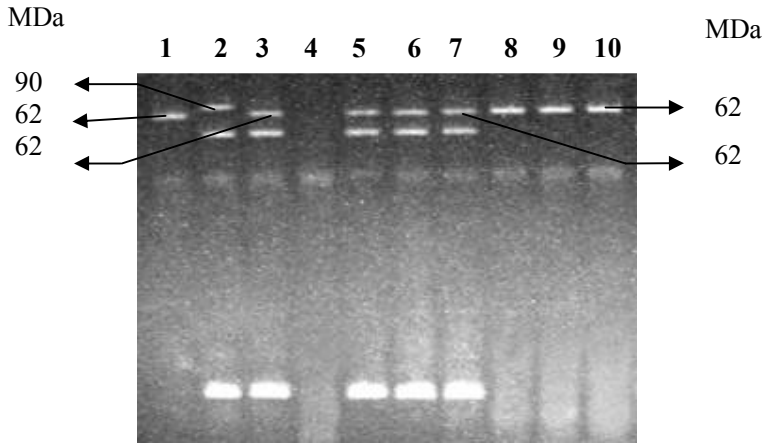

Fig. 3. Agarose gel electrophoresis of plasmid DNA of the tested strains and transconjugants
Lanes: 1: R1 (marker= 62 MDa plasmid); 2: S1 having 90 MDa plasmid; 3: S2 plasmid DNA of donor strain having 62 MDa plasmid; 4: Recipient K-12 having no plasmid; 5, 6, 7: S2 plasmid DNA of donor strain; 8, 9, 10: Plasmid DNA of transconjugants having the molecular weight of 62 MDa plasmid

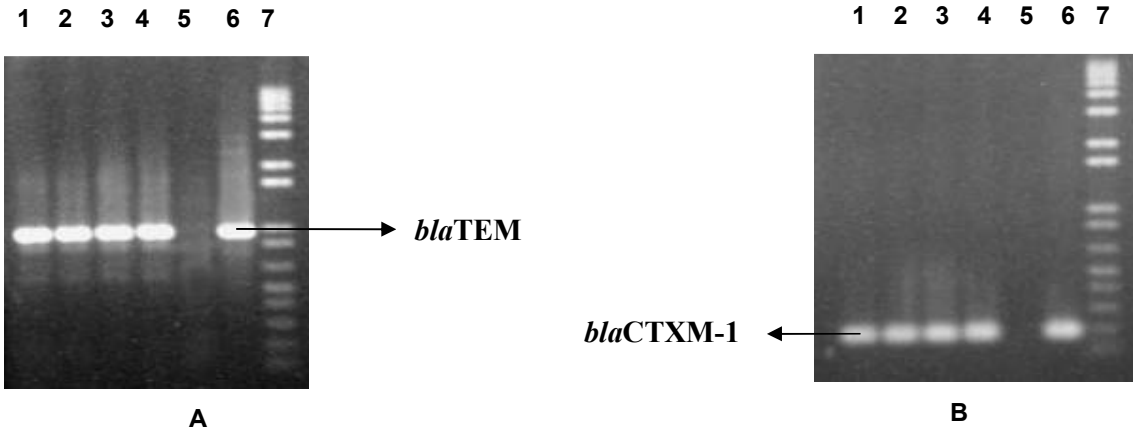

Fig. 4. Agarose gel electrophoresis showing representative PCR amplification products of A: *bla*TEM and B: *bla*CTX–M-1 Genes of transconjugant K-12, recipient K-12 and donors S2. (A)
Lanes: 1, 2: Representative transconjugants (E. coli K-12); 5: Representative recipient E. coli strain (K-12); 3, 4, 6: Representative donor Salmonella strains (S2); 7: DNA molecular size marker (1 kb DNA ladder from GIBCO-BRL). ***(B)*** *Lanes: 1, 2: Representative transconjugants (E. coli K-12); 5: Representative recipient E. coli strain (K-12); 3, 4, 6: Representative donor Salmonella strains (S2); 7: DNA molecular size marker (1 kb DNA ladder from GIBCO-BRL)*

Conjugation was unsuccessful in two cases containing 90-MDa plasmid, which reveals that the ESBL-genes in these strains may be either chromosomally encoded or 90-MDa plasmid may be non-transferable due to the absence of some mechanisms through which the plasmids can transfer, such as the absence of *tra* genes encoding the transferase enzyme [36]. The transconjugants containing the 62-MDa plasmid showed similar resistant-patterns to ampicillin, cephalosporins, and monobactam as the donors but did not expressed resistance towards other antibiotics, such as nalidixic acid, tetracycline, sulfamethoxazole-trimethoprim and chloramphenicol. Thus, this study suggests that the 62-MDa plasmid contains only the ESBL-genes, but not other genes responsible for resistance to nalidixic acid, tetracycline, sulfamethoxazole-trimethoprim and chloramphenicol, which is contradictory to the results reported by Xiong et al. in 2002 (Table 4) [28].

Table 4. Characters of transconjugants comparing with donors and recipient *E. coli* K-12

Strain ID		Recipient K-12	Donor Group G S2, S3	Transconjugants G	Recipient K-12	Donor Group B S6, S7, S8, S9	Transconjugants B
General Antibiotic Sensitivity Pattern[a]	AM	I	R	R	I	R	R
	CIP	S	S	S	S	S	S
	C	S	S	S	S	S	S
	SXT	S	R	S	S	R	R
	NA	R	I	R	R	R	R
	TE	S	R	S	S	R	S
Plasmid Pattern		None	62, 35, 1	62	None	62, 36, 4.8, 2.7, 1.2, 1	62
ESC-Antibiotic Sensitivity Pattern[a]	CRO	S	R	R	S	R	R
	CAZ	S	R	R	S	S	S
	CTX	S	R	R	S	R	R
	FOX	S	S	S	S	≥S	≥S
	FEP	S	S	S	S	R	R
	ATM	S	R	R	S	I	I
Genes	*bla*TEM	_	+	+	_	+	+
	*bla*CTXM-1	_	+	+	_	_	_
	*bla*OXA	_	_	_	_	+	+
	int1	_	_	_	_	+	+

[a]*Abbreviations: AM, ampicilin; CIP, Ciprofloxacine; SXT, Sulfamethoxazole-Trimethoprim; NAL, nalidixic acid; C, chloramphenicol; TE, tetracycline; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FOX, Cefoxitin; FEP, cefepime; ATM, aztreonam; R, Resistant, I: Intermediary Resistant and S: Sensitive; (+): Positive and (-): Negative*

The plasmids were stable, as was further confirmed by plasmid extraction and antibiotic susceptibility test from the strains that were cultured in both selective and non-selective medium consecutively for 28 days. This nature of the conjugative plasmid implies that the use of any β-lactam antibiotic to which the strain exhibits resistance would favor the maintenance of the plasmid [37]. The PCR analysis of transconjugants showed the presence of the *bla*CTX-M-1, *bla*TEM, *bla*OXA and *int*1 genes, similar to the donor. The *bla*TEM, *bla*CTX-M-1, *bla*OXA and *int*1 genes were carried by the 62-MDa plasmid and were transferable. So, the presence of the *bla*TEM, *bla*SHV, *bla*OXA, and *bla*CTX-M-1 genes in these eight strains was caused by a combination of both vertical and horizontal spread. Here, we found four *bla*OXA and four *bla*TEM genes located in the class 1 integrons. Generally, integrons are significantly associated by the genetic linkage between conjugative plasmids and transposons [35].

This result also suggests the association of transposable elements with resistance to certain antibiotics, such as ampicillin, ceftriaxone, cefotaxime and cefepime but not sulfafurazole-trimethoprim, chloramphenicol, tetracycline and nalidixic acid which is partially opposite to the report given by White PA et al. 2001 [35]. According to his suggestion, transposable elements are associated with the both the β-lactams and non β-lactams [37]. This overall study reveals that the carrying of such transmissible plasmids by *Salmonella* may facilitate the spread of β-lactam resistance determinants to other community-acquired pathogens. In addition, further dissemination of such strains may markedly reduce the therapeutic options for severe *Salmonella* infections [38].

It is interesting to note that Antibiotic susceptibility, plasmid profiling, PCR and PFGE suggest that two groups of isolates of *Salmonella* Group G showed similar pattern though these were found from different specimen and from different years. On the other hand, all four *Salmonella* Group B strains isolated from 2007, 2008 and 2010 (first one from child and last three from adults) showed similar pattern except two in PCR result, maintaining one more *bla*TEM gene. These analyses reveal that some isolates in different provinces were indistinguishable, which indicates a common source and person-to-person dissemination.

5. CONCLUSION

Although the number of ESBL-producing *Salmonella* strains was not notably high, from this study it is clear that ESBL-producers are disseminating carrying different types of β-lactamases, which are primarily being spread through replication and then conjugation. So, there is a high chance of spreading resistant genes in different bacteria through conjugation. We should avoid bacterial contamination as far as possible. Besides, imipenems and cefoxitins are the β-lactam drugs that are active against organisms that produce β-lactamases, the appropriate monitoring of the usage of these drugs for the treatment of patients should be handled carefully. Otherwise, infection with these organisms will cause more detrimental effects especially in children in Bangladesh in near future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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