

Molecular characterization of extended spectrum β -lactamase (ESBL) producing *Shigella* species isolated from patients with bacillary dysentery in Iraq.

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Summary:

Background: Extended-spectrum β -lactamases (ESBLs), including cefotaximases (CTX-M), mediate resistance to extended spectrum cephalosporins and significantly compromise the treatment tools for Shigellosis.

Objective: To determine the ESBLs production by *Shigella* spp. and its role in the resistance to third generation cephalosporins and to determine the occurrence of plasmid-borne blaCTX genes in ESBLs *Shigella* isolates by Multiplex PCR.

Methods: Susceptibility of 59 clinical *Shigella* isolates was tested by disk diffusion method to six antimicrobial agents. Presence of ESBLs was established by the combination disk method. Minimum inhibitory concentration of β -lactams was determined by agar dilution method for resistant isolates, and then ESBL producers were subjected to plasmid extraction and PCR experiments.

Results: Overall isolates had high rate of resistance to Ampicillin (84.7%), Tetracycline (84.7%), Cotrimoxazole(72.9%) and Cefotaxime(69.5%). Moderate to Ceftriaxone (52.5%) and low rate of resistance to Ceftazidime(30.5%). The MIC value of resistant isolates of *S. sonnei* and *S. flexneri* for CTX and CRO ranged from (64-512 μ g/ml) for both, and (32-256 μ g/ml) for CAZ, whereas *S. dysenteriae* exhibit only high level resistance MIC (256 μ g/ml) for CTX, low level resistance (64 μ g/ml) for CRO and complete susceptibility to CAZ. 31 of 41 (75.6 %) of *Shigella* spp. were β -lactamase producers, of these (82.6%) were *S. flexneri*, (68.8%) *S. sonnei*, and one *S. dysenteriae* isolate. The results of PCR amplification of bla CTX gene groups showed that blaCTX-M genes were identified in (74.19%) ESBLs isolates distributed as 14(45.16%) *S. flexneri* isolates, 9(29.03%) *S. sonnei*, while no gene was detected in ESBLs *S. dysenteriae* isolate. The prevalence of bla CTX-M I, bla CTX-M II (TOHO 1) and blaCTX-M III, were (54.8%, 16.1% and 3.2%) respectively. Non of the isolates carried more than one type.

Conclusion: The occurrence of plasmid-borne blaCTX genes in ESBLs *Shigella* isolates are increasing in Iraq with co-resistance to some other classes of antibiotics

Key words: Cephalosporines Resistance, *Shigella* spp., Plasmid; ESBLs, CTX-M.

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Introduction:

β -lactamases are located in the periplasmic space between the outer and cytoplasmic membranes of gram-negative bacteria (1). These enzymes can be carried on bacterial chromosomes, that is, inherent to the organism, or may be plasmid-mediated with the potential to move between bacterial populations (2). Plasmid-mediated extended-spectrum β -lactamases (ESBLs) among the most important resistance determinants capable of degrading the expanded-spectrum cephalosporins and emerging worldwide in Enterobacteriaceae (3). The first occurrence of third generation cephalosporin resistant *Shigella flexneri* was from the stool sample of Algerian child in 1995 (4). Since that time strains of *Shigella* harboring different types of ESBLs have been reported in Plasmids are playing a major role in the diffusions of ESBLs genes such

as CTX-M. The name CTX reflects the potent hydrolytic activity of these β -lactamases against cefotaxime. CTX-M β -lactamases hydrolyze cefotaxime and ceftriaxone better than they do ceftazidime. Five main clusters are classified to : CTX-M-1 cluster: CTX-M-1, -3, -10, -12, -15, -22, -23; CTX-M-2 cluster: CTX-M-2, -4, -5, -6, -7, -20, -76, -77; CTX-M-8 cluster: CTX-M-8, -40, -63; CTX-M-9 cluster: CTX-M-9, -14, -15, -16, -17, -18, -19; CTX-M-25 cluster: CTX-M-25, -26, -39, -41, -91. Members within a group have >94% amino acid relatedness and \leq 90% relatedness across the groups (7). Surveys that were done in Canada, Greece, United Kingdom and Italy showed an association between the CTX-M type ESBL and resistance to other antimicrobial agents (8). This study is designed to determine the susceptibility of *Shigella* species to six antimicrobial and the occurrence of plasmid-borne blaCTX genes in ESBLs *Shigella* isolates by PCR.

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Patients and Methods:

Disk diffusion test: Fifty nine Shigella spp. were tested for their susceptibility to Ampiciline(10µg), Cefotaxime (30 µg), Ceftriaxone (30 µg) Ceftazidime(30 µg), Tetracycline(30 µg), Co-trimoxazole(25µg) according to CLSI recommendations(9). Double-disc synergy test (DDST): Production of extended-spectrum β-lactamase (ESBL) was detected by the double-disc synergy test (DDST) using Cefotaxime (30 µg), Ceftriaxone (30 µg), and Ceftazidime (30 µg) discs placed 20 mm (centre to centre) from the Cefotaxime (20 µg)/Clavulanate (10 µg) disc. An increase in diameter of zone of inhibition by the synergy of Clavulanate indicated production of ESBL. Minimum inhibitory concentration: The MIC was determined for shigella spp. resistant to one or more β-lactams by a

standard agar dilution method (10) and according to the CLSI recommendations(9). Plasmid extraction: Modified alkaline lysis method was used to extract plasmid DNA from Shigella species (11). The supernatant contains plasmids were subjected to PCR study. Multiplex PCR-based screening for CTX-M genes: Multiplex PCR was done by modification of previously described PCR protocol (12) for amplification of plasmid born CTX-M I, II, III genes. The amplification was performed using GoTaq Green Master Mix, specific primers sequences (1.5 forward and 1.5 reverse) for each gene (Table1) and plasmid DNA of Shigella isolates resistant to TGCs as a template for PCR experiments. Sterile distilled water was used instead of DNA template as negative control to ensure absence of contaminants in the reaction preparations.

Table 1: The sequences and products of bla CTX-M genes.

blaCTX-M genes		Nucleotide Sequences (5'> 3'>)	Products bp	Reference
CTX-MI	$\frac{F^*}{R^*}$	GACGATGTCACCTGGCTGAGC AGC CGC CGACGCTAATAC A	499	
CTX-MII (TOH01)	$\frac{F}{R}$	GCGACCTGGTAACTACAATCC CGGTAGTATTGCCCTTAAGCC	351	Mirzaee et al., 2009
CTX-MIII	$\frac{F}{R}$	CGCTTTGCCATGTGCAGCACC GCTCAG TACGATCGAGCC	307	

* Forward and reverse

In a microcentrifuge reaction tube, 25 µl master mix contained the following components was used according to the manufacturer instruction (Promiga USA) with a little modification:

Go-Taq green master mix	12.5 µl
3 sets primer (for each forward and reverse) (15picomol\µl)	1.5 µl
Plasmid DNA template	3µl
Nuclease free distilled water	0.5 µl
Total	25 µl

Amplification was carried out with the following thermal cycling profile: 5min at 94°C and 30 cycles of amplification consisting of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C and 7min at 72°C for the final extension. 100bp DNA ladder was used to analyze DNA fragments by electrophoresis in

1% agarose gel dissolved in 1x TAE (40 mM Tris-HCl (pH 8.3), 2 mM acetate and 1 mM EDTA) and ethidium bromide (0.05 mg/L) at 7 v/cm2 for 1.5hrs and visualized under UV transilluminator using digital camera (Sony-Japan) . The significance of differences in proportions was analyzed by the chi-square test with SPSS version 15. Fisher's exact test was used when there was a cell with a number less than 5 and P values equal or less than 0.05 were considered statistically significant.

Results:

Shigella isolates had high rate of resistance to Ampicillin, Tetracycline, Co-trimoxazole and Cefotaxime, moderate to low rate of resistance to Ceftriaxone and Ceftazidime (Table 2).

Table 2: Number and percentage of resistant Shigella isolates to different antibiotics. AMP,

AB	S. flexneri (32)		S. sonnei (22)		S. dysenteriae (5)		Total (59)	%	
	No.	%	No.	%	No.	%		R	S
AMP	29	90.6	18	81.8	3	60	50	84.7	15.3
CTX	23	71.9	16	72.7	2	40	41	69.5	30.5
CRO	19	59.4	11	50	1	20	31	52.5	47.5
CAZ	11	34.4	7	31.8	0	0	18	30.5	69.5
Tet	28	87.5	19	86.4	3	60	50	84.7	15.3
SXT	23	71.9	18	81.8	2	40	43	72.9	27.1

Ampicillin; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; Tet, Tetracycline; SXT, Co-trimoxazole. The MIC value of resistant isolates of *S. sonnei* and *S. flexneri* for CTX and CRO ranged from (64-512 μg/ml) for

both, and (32-256 μg/ml) for CAZ, whereas *S. dysenteriae* exhibit only high level resistance MIC (256 μg/ml) for CTX, low level resistance (64 μg/ml) for CRO and complete susceptibility to CAZ (Table 3).

Table 3: Numbers of Shigella isolates with MIC values (μg/ ml) of β-lactams

strains	CTX				CRO				CAZ				
	512	256	128	64	512	256	128	64	512	256	128	64	32
<i>S. flexneri</i>	7	3	9	4	1	8	5	5	0	2	5	1	2
<i>S. sonnei</i>	2	4	5	5	0	3	2	6	0	2	3	0	2
<i>S.dysenteriae</i>	0	2	0	0	0	0	0	1	0	0	0	0	0

Resistance phenotypes of ESBL-producing isolates

According to the current study, 31 of total 41 isolates resistant to one or more TGCs were β-lactamase producers and were able to produce the ESBLs in a percentage reached to (75.6 %). The results showed that *S. flexneri* accounted for the majority of the β-lactamases producer (82.6 %, 19/23) followed by *S. sonnei* (68.8%, 11/16), and one (50%) *S. dysenteriae* isolate. Figure (1) shows the positive result for *S.flexneri* by observing the enlargement of inhibition zone of Cefotaxime /Clavulanate towards the three tested antibiotics Cefotaxime, Ceftriaxone and Ceftazidime. There is significant association between ESBLs productions and resistance to CTX, CRO, CAZ (p<0.001) in *S. flexneri* and resistance to CTX (p=0.012), CRO (p<0.001), CAZ (p=0.013) in *S. sonnei*, while no significant association observed in *S. dysenteriae*. (p=0.400).

Genetic basis of β-lactamase enzymes in Shigella isolates The results of PCR amplification of bla CTX gene groups showed that, blaCTX-M genes were identified in (74.19%, 23of 31) ESBLs isolates distributed as 14(45.16%) *S.flexneri* isolates, 9(29.03%) *S.sonnei*, while

Table4: Distribution of plasmid mediated cephalosporines resistant genes (bla CTX-M) in the ESBLs Shigella spp.

Genes	<i>S.flexneri</i> (19)	<i>S.sonnei</i> (11)	<i>S.dysenteiae</i> (1)	Total (31)	%
blaCTX I	10	7	0	17	54.8
blaCTXII (TOHO1)	4	1	0	5	16.1
blaCTX III	0	1	0	1	3.2
Total	14	9	0	23	74.19

lane1(MW):100bp DNA ladder; lane(2-6,8,10,12-14): *S.flexneri* positive isolates for blaCTX-MI(499bp); lane(7,9,11,15): *S.flexneri* positive isolates for blaCTX-MII(351bp); lane 16 (NC): Negative control DW.

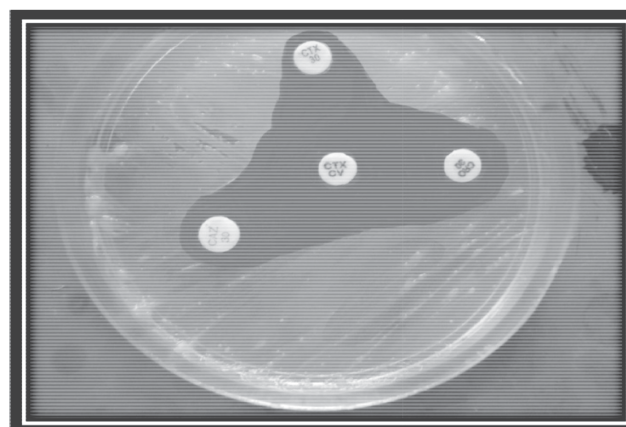


Figure 1: The positive result of ESBLs S.flexneri by double-disc synergy test.

no gene was detected in ESBLs *S. dysenteriae* isolate. The prevalence of bla CTX-M I was (54.8%, 17 of 31), bla CTX-M II (16.1%, 5 of 31) and bla CTX-M III (3.2%, 1 of 31) (Table 4). The product's size of bla CTX-M I, bla CTX-M II and bla CTX-M III were 499bp, 351 bp and 307 bp respectively (Figure 2, 3).

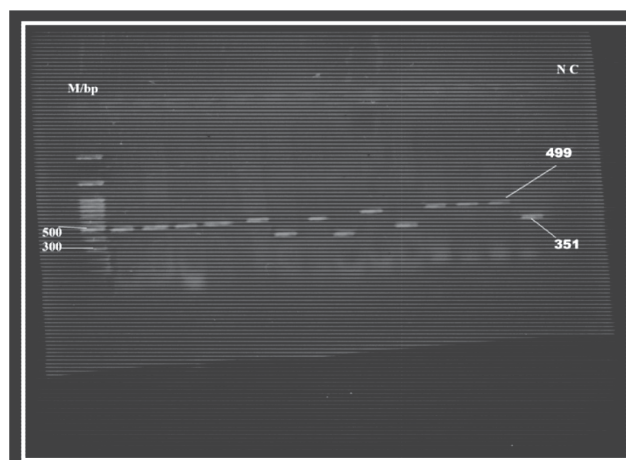


Figure 2: Gel electrophoresis (1% agarose, 7 v/cm², 1.5hrs) of Multiplex PCR positive products.

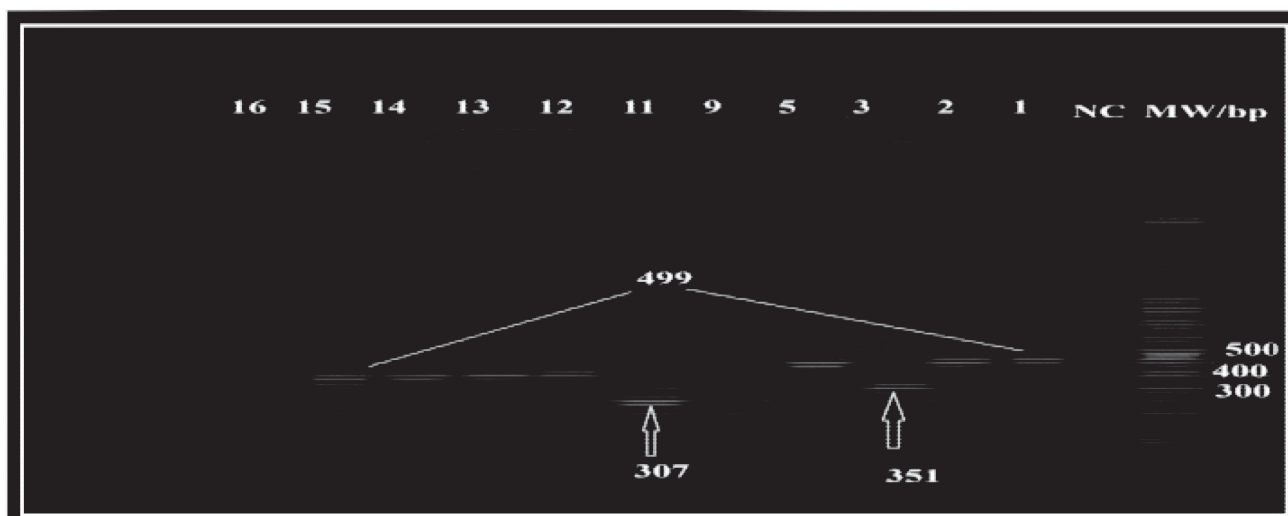


Figure 3: Gel electrophoresis (1% agarose, 7 v/cm², 1.5hrs) of Multiplex PCR positive products

Lane MW: 100bp DNA ladder; lane NC: Negative control (DW); lane (1,2,5,12,13,14,15): *S. sonnei* positive isolates for blaCTX-MI(499bp); lane 3: *S. sonnei* positive isolate for blaCTX-MII(351bp); lane 11: *S. sonnei* positive isolate for blaCTX-MIII(307bp); lane(9,16): *S. sonnei* negative isolate.

Discussion:

Emergence of multidrug-resistant strains of *Shigella* is a growing concern across the globe. In the present study, overall of *Shigella* isolates had high resistance to Ampicillin, Tetracycline (84.7%) Co-trimoxazole(72.9%). These results are very close to those documented in Brazil that reported a high Tet (93.4%), Amp (83.3%) and SXT(63.1%) level of resistance(13). One of the main factors of the high level of resistance to Amp, Tet, SXT antibiotics might be due to the cheap price, broad-spectrum activities and widespread use of these antibiotics as an empirical treatment of inflammatory diarrhea in Iraq, thereby ensuring strong selection pressure for the maintenance of resistance to these antibiotics (14). Extended-spectrum cephalosporins are generally

used for the treatment of infections caused by MDR *Shigella*. However, the development of resistance to cephalosporins in *Shigella* spp. was strongly suggested. Our study revealed that 69.5%, 52.5% and 30.5% of the isolates were resistant to Cefotaxime, Ceftriaxone and Ceftazidime respectively. These results are comparable with the results of a previous study carried out in Iraq where they found that the resistant rates to Cefotaxime, Cefepime, Cefixime and Ceftazidime were uniform (54.54%) in all *Shigella* spp.(15). Other studies reported that all tested *S. flexneri* isolates(100%) were resistant to Cefotaxime, Ceftazidime, Ceftriaxone,

and Cefepime in the United States(16). Resistance to TGCs is mostly due to indiscriminate use of the drugs and emergence of chromosomal or plasmid-mediated extended-spectrum β -lactamases (ESBLs), which confirm resistance to all β -lactams except Cephams and Carbapenems(17). The MIC values of resistant isolates of *S. sonnei* and *S. flexneri* for CTX and CRO ranged from (64-512) μ g/ml for both, and (32-256) μ g/ml for CAZ, whereas *S. dysenteriae* exhibits only high level resistance MIC (256 μ g/ml) for CTX, low level resistance (64 μ g/ml) for CRO and complete susceptibility to CAZ. A recent study in India reports that MIC of CRO (30 to >256) μ g/ml, CTX MIC (5 to >256) μ g/ml and CAZ MIC (5 to >256) μ g/ml for all *Shigella* spp. (18). The current study reported an increase in β -lactamase production in a percentage that reached to (75.6%) of *Shigella* spp. resistant to one or more cephalosporins ($p < 0.001$). Several studies described strains of *Shigella* spp. that produced the characteristic ESBL pattern that are resistant to third-generation cephalosporins in Asia (4, 19). The intestinal flora may be a considerable reservoir of ESBL-encoding genes and the genetic elements they circulate on, permitting potential transmission to their pathogenic counterparts (20). Most of the β -lactamase genes are located on self-transferable genetic factors, and the high level of antibiotic resistance is frequently conferred through the transfer of such plasmids which mediate numerous resistance genes, including genes for multiple β -lactamases from different functional classes (21). In the current study, the amplified blaCTX-M genes by Multiplex PCR were identified in (74.19%, 23 of 31) of ESBL-producing *Shigella* isolates and there is a significant association between the occurrence of these plasmid-borne genes and ESBLs. The blaCTX-M I, blaCTX-M II (TOHO1), blaCTX-M III

were present in (54.8%, 16.1%, 3. 2%) of the isolates respectively. MDR Shigella species producing blaCTX-M have been frequently identified from different regions of the globe. The blaCTX-M 1 and blaCTX-M2 were detected by PCR and blaCTX-M-14 by sequence analysis in Sh. sonnei isolates in Korea (22). TOHO1 and blaCTX-M-3 genes were also detected in Argentina and Turkey in Sh. flexneri and MDR Sh. sonnei isolates producing ESBL from successive pediatric bacillary dysentery patients. (23,24). Most of the studies showed that bla CTX-M-15 which belongs to the blaCTXI cluster was the most prevalent variants in the world(25). These results are very closely related to the current results, that we found the high occurrence of blaCTX-M I (54.8%) than other type. Eight of ESBLs isolates were negative for plasmid borne blaCTX-M genes and this may show that some of the ESBLs genes may be coded potentially by chromosomal DNA (26). The sources of blaCTX-M determinants are chromosomal genes resident in members of the genus Kluverella with no pathogenic activity against humans. The CTX-M-encoding genes have been captured from the chromosome on genetic elements like conjugative plasmids that mediate β -lactam selective force such as that exerted by Cefotaxime and Ceftazidime has fueled mutational events underscoring diversification of different clusters led to rapid dissemination among pathogenic enterobacteria(7). In conclusion the blaCTX-M occurrence is associated with the resistant to TGCs especially CTX and CRO and these genes are plasmid-borne mediates the increasing in dissemination of extended spectrum β -lactamases in Iraq with co-resistance to some other classes of antibiotics. So, administration of TGCs combined with β -lactamase inhibitor is recommended for treatment of Shigellosis.

Author's Contribution

Dr. Thanaa R. Abd Al-Rahman: Preparation, performing and doing the tests of the research. Dr. Sabah A. Belal: Interpretation the results of the research. Dr. Kifah A. Jasim: Certify that this paper is prepared under her supervision. However it is derived from Ph.D. thesis.

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