Molecular Investigation of Extended Spectrum Beta-Lactamase (ESBL) among *Shigella* spp. in Al-Diwaniyah city, Iraq

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Shigella species are among the common bacterial causes of diarrheal diseases. *Shigella* species caused Shigellosis with low infectious dose, which is an acute enteric infection that is characterized by a liquid and mucoid bloody diarrhea, also known as bacillary dysentery. The aim of present study is to determine the occurrence of specific plasmid-mediated genes that responsible for antimicrobial drug resistant *Shigella* species in Al-Diwaniyah city/Iraq. Stool samples were obtained from diarrheal patients who were admitted to Al-Hussain children hospital , Feminine and children teaching hospital and Al-Diwaniyah teaching hospital during the period from November 2017 to May 2018 . A total of 282 stool samples were collected from different patients ages (adults, teenagers and children) and included males and females. Four selective and differential media were used for identification of *Shigella* species to increase the chance of isolation, which are Xylose Lysine Deoxycholate agar, MacConkey agar, Hektoen Enteric agar and Salmonella Shigella agar. The suspected colonies identified by Api20 E system and by Vitek2 system to confirm the diagnosis. *Shigella* isolation rate was (6.73%, 19/282) in this study. The higher infection rate (78.9%, 15/19) was appeared in ≤ 10 years age group, while ages of 11 to 20 years showed lower rate (15.7%, 3/19) and ages of 41 to 50 years showed (5.2%, 1/19). *Shigella* isolates were differentiated molecularly to 10 (52.63%) *Shigella flexneri*, 8 (42.10%) *Shigella sonnei*, 1 (5.26%) *Shigella boydii* and no *Shigella dysenteria* was detected by using specific target genes: *rfc* (537 bp), *wbgZ* (430 bp), conserved hypothetical protein (248 bp) and *rfpB* (211 bp), respectively.The *Shigella* isolates were tested for ESBL production by disk approximation test. ESBL-positive isolates were *S. flexneri* (7/10, 70%), *S. sonnei* (7/8, 87%), *S. boydii* (1/1, 100%). *Shigella* isolates were investigated genotypically for harboring β-lactamases genes including bla_{AMPC} (670bp), bla_{CTX-M} (247bp), bla_{TEM} (531bp) and bla_{SHV} (410bp) by PCR technique. The distribution of β-lactamases genes was as follow: *S. flexneri* isolates harbored bla_{CTX-M} (30%, 3/10), bla_{ampC} (60%, 6/10) and bla_{TEM} (20%, 2/10), *S. sonnei* isolates harbored bla_{CTX-M} (75%, 6/8), bla_{ampC} (62.5%, 5/8) and *S. boydii* isolate harbored only bla_{TEM} (100%, 1/1), while bla_{SHV} was not detected in this study.

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Introduction

Shigella genus are strict human pathogens that target the gastrointestinal tract and induce acute bacillary dysentery that known as shigellosis. *Shigella* are Gram-negative, facultative anaerobic, non-motile and facultative intracellular pathogens that are firmly related to *Escherichia coli* but have improved particular features of physiology, pathogenicity and serology [(1)].

There are four species of *Shigella: Shigella dysenteriae, S. flexneri, S. boydii* and *S. sonnei*, each possess their own global burdens and epidemiological form and all of which are capable of inducing disease in human beings. *Shigella* infection is highly contagious can transmits by the fecal-oral way, individual to individual contact or ingestion of polluted food or water [(2)]. Diarrhea is an first symptom of shigellosis and might be begun as the pathogen attack the small bowel leading to inflammatory colitis [(3)].

Shigellosis keeps on being a noteworthy medical issue in numerous parts of the world, especially in developing nations. In 2013, mortality risk data, suggesting between 28,000 and 48,000 deaths annually amongst children under 5 years due to Shigellosis [(4,5)]

The development of antibiotic resistance in bacteria, as a result of evolutionary process in them has now been revealed as a matter of global emergency by the World Health Organization [(6)]. It has led to increased decline rates of treatment of infectious diseases triggered by bacteria, for example, therapies to which they were previously sensitive no more act. Bacteria have intrinsically resistance to its inherent characteristics or gain this resistance ability during mutations and gene transfer.

Different strategies of antibiotics resistance involve deficient drug penetration into the cell, outflow of antibiotics through efflux pumps, target alteration by mutation and hydrolysis of antibiotics [(7)]. Antibiotics resistance has been recorded in both Gram positive bacteria and Gram negative bacteria [(8,7)]. One such truth is that emergence of multidrug resistance *Shigella* species [(9)].

Development of *Shigella* antibiotic resistance

There are multiple mechanisms by which the antimicrobial resistance may occur. In *Shigella* species, antimicrobial resistance is frequently due to class 1 and class 2 integrons which include resistance gene cassettes. Integrons are movable and transferrable from one bacterial cell to

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another, supplying a flexible access for bacteria to harmonize to the environmental tension caused by antibiotics. This mechanism of action may explain the spreading of resistance genes and the emergence of MDR strains, and explain why *Shigella* resistance patterns vary worldwide [10].

Extended-spectrum β-Lactamases as a cause of antimicrobial-drug resistance

Even if there is a several kinds of strategies of bacterial resistance to β-lactam antibiotics, the most considerable are the β-lactamases, which are enzymes able to hydrolyze the β-lactam ring of penicillins, cephalosporins, and related antimicrobial medications, making them inactive. There are numerous of β-lactamases, which differ in substrate specificity and host extent [11,12]. Much of the motivation to develop new β-lactam antibiotics has been failed because the emergence of bacteria that develop β-lactamases able to damage present antibiotics. The previous cephalosporins (such as, cephalothin) are liable to cleavage by a different of β-lactamases frequently present in Gram-negative rods, involving the chromosomal cephalosporinases of pseudomonas, enterobacter, and other genera, in addition to the main plasmid-produced enzymes of Enterobacteriaceae. The former enzymes additionally hydrolyze a variety of penicillins and in contrast to the chromosomal cephalosporinases, are generally in activated by β-lactamase inhibitors like clavulanic acid [11].

Several Iraqi studies were performed to investigate the spreading of ESBLs among Enterobacteriacea. In Al-Diwaniyah, Habeeb and Al-Azawi (2014)[13] who investigate ESBLs occurrence in *Klebseilla pneumoniae* isolated from urinary tract infections, Al-Mayahi (2014) [14] who detects the incidence rate of *Escherichia coli* ESBLs producers in urinary tract infection patients. In Al- Najaf, Abdul-Hadi *et al*. (2010) [15] were determined the *Klebseilla pneumonia* resistance to third generation cephalosporins in urinary tract infections that mediated by ESBLs.

The encoding genes of the extended spectrum β-lactamases were perfectly kept on self-transportable plasmids that usually loaded other determinants of antibiotic resistance [16,17]. Due to these genes may be carried on transposable components, they may transfer into different plasmids, allowing the spreading of extended-spectrum β-lactamases within Gram-negative rods [18]. The dominant β-

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lactamases in Gram-negative bacteria are TEM-, OXA-, SHV- and CTX-M [19].

Patients and methods

The study was conducted over a period of seven months from November 2017 to May 2018. A total of 282 stool samples were collected from different ages of clinical diarrhoeal patients who were visited different hospitals in Al-Diwaniyah city, Iraq, including Al-Hussain children hospital , Feminine and children teaching hospital and Al-Diwaniyah teaching hospital. The ages of patients ranged from four days to 7o years old. The patients group covered 144 females and 138 males.

All stool samples were cultured on XLD, MacConkey, Hektoen and SS agars at (37^0C) for (18-24) hours to identify and isolate *Shigella* spp. The suspected colonies identified by Api20 E system. Further confirmatory test was done by Vitek2 system. Genomic DNA was extracted from obtained *Shigella* isolates according to manufacturer instructions of Genomic DNA purification kit (Geneaid, USA). The purity and concentration of DNA for each isolate were measured by Nonodrop instrument (THERMO, USA).

Specific target genes were used to differentiate *Shigella* species, whereas *rfc* gene, *wbgZ* gene, *rfpB* gene and conserved hypothetical protein gene for *S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*, respectively.

Table 1. Programs of PCR thermocycling conditions

Target gene	Primer sequences (5' – 3')	Size(bp)	Target identity	Conditions Tempreature^{0}C/time	Reference
rfc	F TTT ATG GCT TCT TTG TCG GC R CTG CGT GAT CCG ACC ATG	537	*S. flexnrei*	95/2min/1cycle 95/30sec/15cycle 69.8/30 sec decrease 0.5^{0}C per cycle 72/50 sec 95/30sec/20cycle 53.8/30 sec 72/50 sec 72/5 min 4/forever	(Ojha, Yean Yean et al. 2013)

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wbgZ	F TCT GAA TAT GCC CTC TAC GCT R GAC AGA GCC CGA ACC G	430	*S. sonnei*	95/2min/1cycle 95/30sec/15cycle 63.4/30 sec decrease 0.5^{0}C per cycle 72/50 sec 95/30sec/20cycle 56.4/30 sec 72/50 sec 72/5 min 4/forever	(20)
rfpB	F TCT CAA TAA TAG GGA ACA CAG C R CAT AAA TCA CCA GCA AGG TT	211	*S. dysentriae*	95/2min/1cycle 95/30sec/15cycle 58.7/30 sec decrease 0.5^{0}C per cycle 72/50 sec 95/30sec/20cycle 51.7/30 sec 72/50 sec 72/5 min 4/forever	(20)
Conserved hypothetical protin	F TCT GAT GTC ACT CTT TGC GAT T R GAA TCC GGT ACC CGT AAG GT	248	*S. boydii*	95/5.0 min/ 30 cycles 95/0.45 sec 56/0.35 sec 72/0.45 sec 72/5.0 min.	(21)

Table 2. Polymerase chain reaction master mix preparation

PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol/µL)	1.5µL
Reveres primer (10pmol/µL)	1.5µL
PCR water	12µL
Total volume	20 µL

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Phenotypic detection of ESBL was done by using Disk Approximation Method. In this test, a disk containing amoxicillin clavulanate is placed in proximity to disks containing oxyimino-β-lactam and aztreonam antibiotics. The enhancement of the zone of inhibition of the oxyimino-β-lactam is a positive result [22].

In this study the test was performed by putting cefotaxime, ceftazidime, ceftriaxone, and aztreonam antibiotic discs (20 µg for each one) on Muller Hinton agar at a similar distances (30 mm from center to center) from the amoxicillin/clavulanic acid (Augmetin) disc which put in the center of the agar plate.

All the *Shigella* isolates were investigated genotypically for harboring β-lactamases genes including bla_{AMPC}, bla_{CTX-M}, bla_{TEM}, and bla_{SHV} by PCR technique. The amplicon size were 670bp, 247bp, 531bp and 410bp, respectively. The PCR conditions are as follows: Initial denaturation at 95 ^{0}C for 3.0 min., 30 cycles of denatuaration at 95 ^{0}C for 0.30 sec., anneling at 58.3^{0}C for 0.30 sec., extension at 72^{0}C for 1.30 sec., final extension at 72^{0}C for 5.0 min. and finally hold at 4^{0}C.

The specific primers for β-lactamases genes used in this study were designed by using NCBI Gene Bank .Table (3).

Table 3. β-lactamases primers

Primer	Primer sequences (5' _ 3')		Amplicon size (bp)	Target gene	Gene bank
CTX-M	F	ACG GAT AAC GTG GCG ATG AA	247	bla_{CTX-M}	JN411912.1
	R	TCA TCC ATG TCA CCA GCT GC			
TEM	F	GGT GCA CGA GTG GGT TAC AT	531	bla_{TEM}	JN037848.1
	R	TGC AAC TTT ATC CGC CTC CA			
AMPC	F	AAA CGA CGC TCT GCA CCT TA	670	Bla_{AMPC}	AY533245.1
	R	TGT ACT GCC TTA CCT TCG CG			
SHV	F	CCG CCA TTA CCA TGA GCG AT	410	bla_{SHV}	FJ668798
	R	AAT CAC CAC AAT GCG CTC TG			

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Results and discussion

In this study out of 282 patients only 19(6.73%) of them were confirmed diagnosed with Shigellosis. According to the obtained results in current study showed the most common spp. among *Shigella* isolates was *S. flexneri* (52.63%) and followed by *S.sonnei* (42.10%), then *S.boydii* (5.26%) and no *S.dysenteriae* was detected. In developing countries, the predominant species is *S. flexneri*, which is characterized by long-term persistence of sublineages in shigellosis-endemic regions with inadequate hygienic conditions and unsafe water supplies [(23)]. More rarely isolated are *S. dysenteriae*, responsible for large epidemics in the past, and *S. boydii* [(2)]. Shigellosis occurs predominantly in developing countries due to overcrowding and poor sanitation conditions. Infants, non-breast fed children, children recovering from measles, malnourished children, and adults older than 50 years are more susceptible and have a more severe illness and a greater risk of death [(24)]. The observation of this study agreed with other previous results reported by local study by Mohammed (2009) [(25)] who reported that the predominant species was *S. flexneri* (66.6%, 6/9) followed by *S. sonnei* (33.3%, 3 / 9). Additionally, the present results was closely similar to results gained by Qiu, Xu et al. (2015) [(26)] who showed the most common species isolated from diarrhoeal patients in China was *S. flexneri* (55.3%), followed by *S. sonnei* (44.1%).

All the 19 *Shigella* isolates were tested for ESBL production by disk approximation test. This method was the first detection test described in 1980's; it is also known as double-disk test [(27)]. The results revealed that the three obtained *Shigella* spp. had ESBL-positive phenotype, whereas *S. flexneri* (7/10, 70%), *S. sonnei* (7/8, 87%), *S. boydii* (1/1, 100%). Fig (1).

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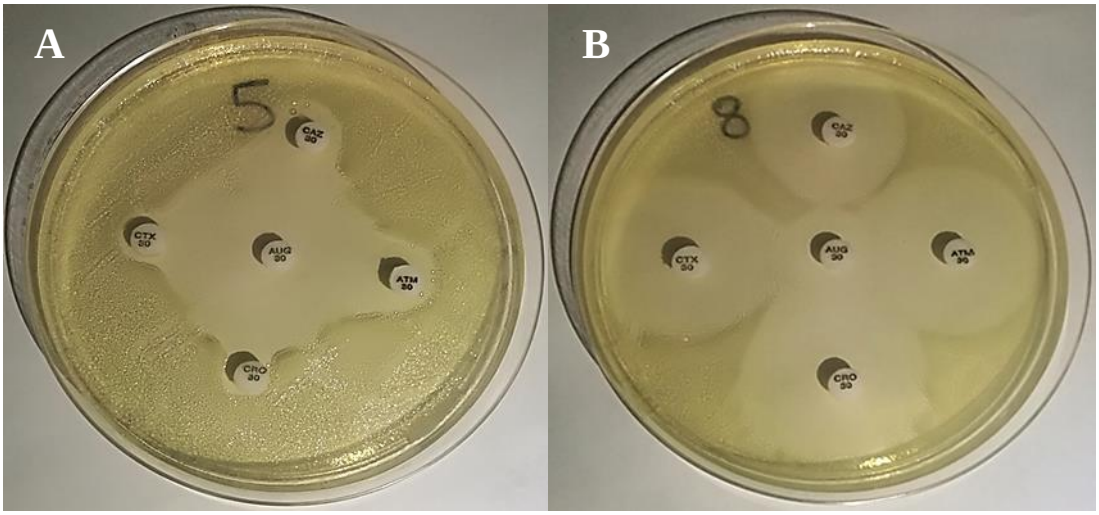

Fig(1) Disk approximation test for detection of ESBL in *Shigella* spp.(AUG, Augmentin; ATM, Aztreonam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime); A: Positive result ; B: Negative results.

The clavulanic acid is separated and diffused around disc, this component can penetrate the bacterial cell walls and can inactivate extracellular enzymes [28,29]. The synergy effect seen depended on the diffusion from side to side the agar plate. An obvious enhancement of the inhibition zone was seen on sides of the amoxicillin/clavulanic acid disc against other discs, these obvious enhancement revealed some synergistic effect between the clavulanic acid and other an oxyimino-cephalosporin (third generation cephalosporins) and monobactam antibiotics which denote that these bacteria are ESBL producers, while the strains without synergistic effect considered non-ESBL producers [30] .

Although the β-lactamases absolutely play a crucial role in the resistance to β-lactam antibiotics, the high rate of resistance to ampicillin was not only attributable to the production of β-lactamase enzymes. The other mechanism conferring resistance to these compounds is caused by lowering of the activity of β-lactam antibiotics in a resistant cell due to several factors such as; the sensitivity of the antibiotic to β-lactamases, the penetration through the outer membrane, the affinity for the target (PBPs), the amount of β-lactamase, and the affinity of the antibiotic for the β-lactamase [31,32,33].

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In this study, the rates of ESBL production among *Shigella* spp. were high if compare with results achieved in Iran by Ranjbar, Ghazi et al. (2013) [34] who reported that 4 out of 55 *Shigella* isolates, including three *S. sonnei* and one *S. flexneri*, showed an ESBL-positive phenotype.

The best efficiency method for β-lactamaes detection is PCR because it is more rapid than phenotypic detection method and also detect the presence of inadequately or non-expressed genes difficult to determine by phenotype [35]. Moreover, plasmid of a pathogen may harbor more than one gene that may encode for different types of ESBL enzyme. Additionally, the spread of most broad-spectrum β-lactamases is facilitated by transferable and trans-conjugable plasmids, which often convey other resistance genes by means of their integrin design [33]. In this study, the β-lactamases genes distributed as follow: *S. flexneri* isolates harbored bla_{CTX-M} (30%, 3/10), bla_{ampC} (60%, 6/10) and bla_{TEM} (20%, 2/10), *S. sonnei* isolates harbored bla_{CTX-M} (75%, 6/8), bla_{ampC} (62.5%, 5/8) and *S. boydii* isolate harbored only bla_{TEM} (100%, 1/1), while bla_{SHV} was not detected in this study.

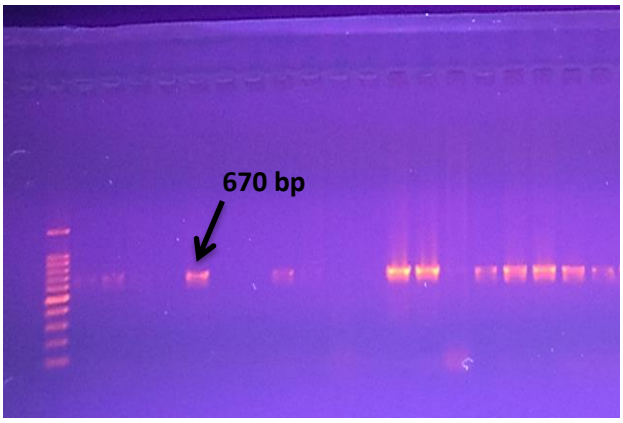

Fig (2) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of bla_{AMPC} gene (670bp) for 1 hours at 70 volts.

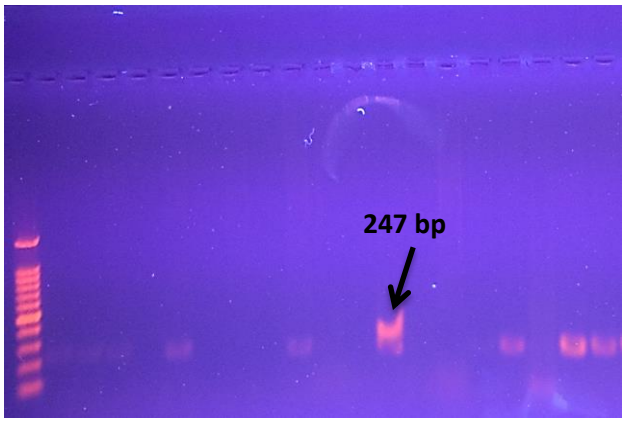

Fig (3) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR of bla_{CTX-M} gene (247bp) for 1 hours at 70 volts.

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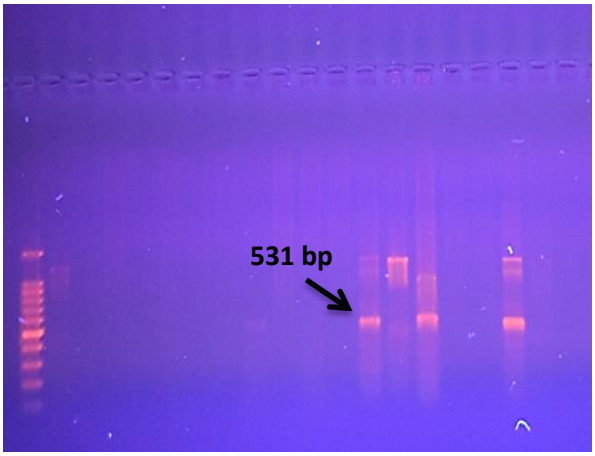

Fig (4) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *bla*$_{TEM}$ gene (531bp) for 1 hours at 70 volts.

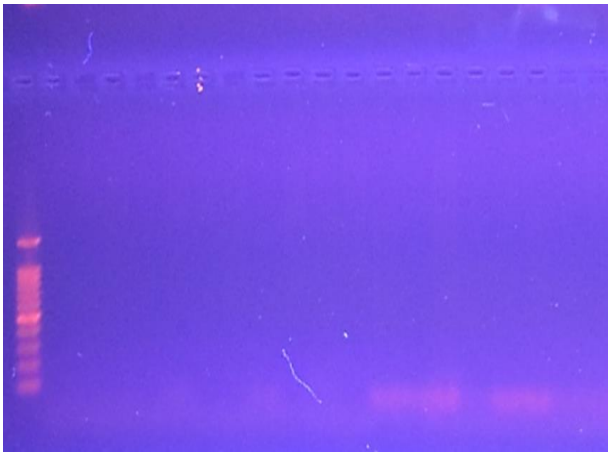

Fig (5) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *bla*$_{SHV}$ *gene* (410bp) for 1 hours at 70 volts.

An increasing concern around the world is the bacterial resistance to broad spectrum β-lactams which mediated by extended spectrum β-lactamase (ESBL), and AmpC beta-lactamases (AmpC) [36]. These enzymes often cause inappropriate treatments rising to increased infections and mortality, medical cost and prolonged residence in hospital [37]. Bacterial strains that have such genes are usually resistant to various antimicrobial agents and can confront treatment, as therapeutic choices are few.

The ESBLs are frequently locate on plasmids and the common members are CTX, TEM and SHV families. Plasmids involving genes encoding for ESBLs usually accommodate resistance determinants for different classes of antimicrobial drugs by possessing different resistance mechanisms and are easily transmitted from strain to strain and between distinctive species of enteric Gram-negative rods [38]. Another resistance mechanism includes overproduction of chromosomal or plasmid-derived *AmpC* beta-lactamases [39].

In this study, regarding PCR results showed that *bla*$_{AMPC}$ gene and *bla*$_{CTX-M}$ gene were the most present in studying bacterial isolates from than *bla*$_{TEM}$ gene, while *bla*$_{SHV}$ was not detected. *S. flexneri* isolates differentiated into (6/10) harbored *bla*$_{AMPC}$ gene, (3/10) harbored *bla*$_{CTX-M}$ gene and (2/10) harbored *bla*$_{TEM}$ gene. *S. sonnei* isolates differentiated into (5/8) harbored *bla*$_{AMPC}$ gene and (6/8) harbored *bla*$_{CTX-M}$

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gene. *S. boydii* (1/1) was harbored bla_{TEM} gene only.

The CTX-M types ESBLs are plasmid-mediated β-lactamases having higher hydrolytic action against cefotaxime. CTX-M-15 has been observed as common genotype of ESBL among *Shigella* isolates [(40)].

The SHV (sulfydryl variable) and TEM enzymes are another family of β-lactamases. The originator of the SHV enzymes, SHV-1, was first described in *Klebsiella pneumoniae*. SHV confers resistance to broad- spectrum penicillins. TEM-1 was first manifested in 1965 in an *Escherichia coli* isolate from Athenian patient, named Temoneira (designation TEM) [(41)]. Mutants of the TEM derivatives (called CMT-1, CMT-2, CMT-3 and CMT-4) have been identified that have the ability to hydrolyse both third-generation cephalosporins and β-lactamase inhibitors [(42,43)].

Organisms producing enough AmpC β-lactamase will typically give a positive ESBL screening test but fail the confirmatory test involving increased sensitivity with clavulanic acid [(44,45)].

In Iraq, Al-Rahman (2013) [(46)] who observed that bla_{CTX-M} genes were identified in (74.19%) *Shigella* isolates whereas (45.16%) of *S. flexneri* and (29.03%) of *S. sonnei*, while no gene was detected in ESBLs *S. dysenteria* isolates. Nevertheless, this study reported high occurrence rate of bla_{CTX-M} genes harboring *S.sonnei* (75%) and lower rate (30%) in *S. flexneri*.

The present results were approximate to results of Chinese study by Zhang, Liu et al. (2014) [(10)] that observed the majority of *Shigella* isolates carried bla_{CTX-M} genes and with less number of isolate carried bla_{AMPC} genes and bla_{TEM}, while no isolate carreied bla_{SHV}, but Akhi, Bialvaei et al. (2016) [(47)] showed different results that bla_{SHV} and bla_{TEM} genes were the most dominant in *Shigella* isolates from than bla_{CTX-M} and AmpC plasmid mediate (*cmy*) gene. Of all the isolates, (25.9%) isolates had only one gene, which (18.5%) were bla_{SHV} and (7.4%) were bla_{TEM}.

The CTX-M enzymes appear to have a superior ability to diffuse and result in outbreaks. There are more than 50 variants of CTX-M to date, and they have been related to several outbreaks of infections in the hospitals and in the community [(48).] The clinical and commercial tension to use β-lactams, as well as the global traveling of humans, animals and food products ensure

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the continuous spreading of β-lactamase genes .

Conclusion

Alarming occurrence rate of β-lactamases producing *Shigella* isolates was revealed, especially CTX-M and AMPC which are the most common among investigated isolates.

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