

THE PREVALENCE OF CTX-M-15 EXTENDED-SPECTRUM β -LACTAMASES AMONG *SALMONELLA* SPP. AND *SHIGELLA* SPP. ISOLATED FROM THREE IRANIAN HOSPITALS

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Bacterial antimicrobial resistance mediated by the production of extended-spectrum β -lactamases (ESBLs) is considered a major threat for treatment of *Salmonella* and *Shigella* infections. This study aimed to investigate antibiotic resistance patterns of *Salmonella* and *Shigella* spp. and presence of CTX-M from three teaching hospitals in Iran. In the present study, 58 clinical *Shigella* and 91 *Salmonella* isolates were recovered between 2009 and 2013 from 3 teaching hospitals in Iran. After culture and antimicrobial susceptibility testing, ESBL-positive isolates were subjected to further investigations. These included polymerase chain reaction (PCR) amplification and DNA sequencing of *bla*_{CTX-M-15} encoding plasmid. In both genera, high sensitivity to gentamicin and amikacin, but high resistance to ampicillin, tetracycline, and trimethoprim–sulfamethoxazole, was found. Molecular investigation showed that 31.8% isolates of *Salmonella* spp. and 34.48% isolates of *Shigella* spp. were CTX-M positive and all of them were also positive for ISEcpI. Protein translation, comparing with reference sequences, showed that all CTX-M isolates belong to CTX-M-15. The present study suggests that the resistance of ESBLs-producing *Salmonella* and *Shigella* spp. in Iran hospitals is very serious. Therefore, strategies to minimize the spread of ESBL-producing isolates should be implemented.

Keywords: *Salmonella*, *Shigella*, antimicrobial resistance, ESBL, CTX-M-15, Iran

Introduction

Emergence of extended-spectrum- β -lactamases (ESBLs) in *Enterobacteriaceae* family, especially in *Salmonella* and *Shigella* spp., is a global major health threat, affecting both developed and developing countries [1, 2]. Although ESBLs-producing strains of *Salmonella* and *Shigella* spp. were relatively rare during the last decades compared to other enteric species [3, 4], increasing numbers of these organisms are known to be resistant to various antibiotics.

Several ESBLs have been reported in this genus. Although most ESBLs are derivatives of TEM and SHV β -lactamase families, *Salmonella* and *Shigella* spp. have

also the capability to express CTX-M family, which have emerged worldwide [5, 6]. Among them, the CTX-M type β -lactamases are a growing family that comprises at least 40 enzymes [7]. These enzymes are characterized by selective hydrolysis of ceftriaxone and cefotaxime and even more distinctly of ceftazidime, although some CTX-M types, such as CTX-M-15, may actually hydrolyze ceftazidime [5, 8].

A dramatic increase in the number of CTX-M producing variants has been reported since 1995, especially among enterobacterial isolates of hospitalized patients. Plasmid-mediated CTX-M-15 ESBL was first reported in *Klebsiella pneumoniae*, *Escherichia coli*, and human

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isolates of *Enterobacter aerogenes* from India [9] and in Japan [3]. Since then, CTX-M-15 has been identified in *Enterobacteriaceae* isolates from various countries across the world, including the United Kingdom, Bulgaria, Canada, Russia, Poland, Turkey, and France [10]. Most of the research in this area has been conducted in industrialized countries, where organisms such as *E. coli* and *Klebsiella* spp. are the most common cause of urinary tract infections [11–13]. Surveys performed in different countries revealed that, once a CTX-M β -lactamase penetrates in a specific geographic area, it becomes prevalent with displacement or super-imposition over TEM and SHV ESBL variants.

Relatively little is known about the distribution of such genes in organisms found in developing or countries undergoing an economic transition, where the circulating pathogens may differ [14]. According to the studies that were carried out in Iran, the prevalence of ESBL producing *Salmonella* was lower than in neighboring countries [15], while the prevalence of ESBL-producing *Shigella* isolates is higher than detection rates observed in many other countries [16, 17]. In this study, we aimed to investigate antibiotic resistance patterns of *Salmonella* and *Shigella* spp. and presence of CTX-M as responsible gene of β -lactamase from three teaching hospitals in Iran between 2009 and 2013.

Materials and methods

Study design

In this cross-sectional study that was conducted between March 2009 and September 2013, a total of 58 clinical *Shigella* spp. isolates and 91 clinical isolates of *Salmonella* were collected from three teaching hospitals in Iran (all isolates were derived from stool samples of inpatients). Two of the hospitals were a general and one a pediatric hospital. This study was approved by the ethical committee of regional Medical Research of Tabriz University of Medical Science and all patients provided written informed consent for this research. Identification of isolates as *Salmonella* spp. and *Shigella* spp. was done by using conventional standard biochemical tests, including plating in MacConkey agar, *Salmonella*–*Shigella* agar (Merck, Darmstadt, Germany), triple sugar iron agar, urea agar, SIM medium, and IMViC (indole, methyl red, Voges-Proskauer, and citrate) tests [18]. Serotyping by polyvalent and monovalent antisera was done according to the manufacturer's instructions (Baharafshan, Iran; MAST Diagnostics, UK and Denkafeiken, Japan).

Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion methodology according to Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Antibiogram was done for tetracycline (TET: 30 μ g), ampicillin (AMP: 10 μ g), gentamicin (GEN: 10 μ g), amikacin (AMK: 30 μ g), chloramphenicol (CHL: 30 μ g), ceftizoxime (ZOX: 30 μ g), trimethoprim–sulfamethoxa-

zole (SXT: 25 μ g), and cefotaxime (CTX: 30 μ g) antibiotics (Mast, Merseyside, United Kingdom).

To confirm ESBL production, those strains that were identified as ceftriaxone-resistant were further subjected to the combination disc method [20, 21]. In this method, a set of discs including cefotaxime (CTX) (30 mg), ceftazidime (CAZ) (30 mg), and both antimicrobials combined with clavulanic acid (CLA) (10 mg) were placed onto agar plates. In ESBL-producing strains, increase in the zone diameters of combined antimicrobials was 5 mm greater than single antibiotics [22].

DNA extraction

DNA extraction was done according to tissue buffer boiling method. First, 20 μ l of tissue buffer (0.25% SDS + 0.05 M NaOH) was mixed with a single colony of bacterial isolate and the mixture was incubated for 10 min in 95 °C. Then, the mixture was centrifuged for 1 min with 13,000g, and finally, 180 μ l of Milli-Q water was added [23, 24].

Polymerase chain reaction (PCR)

Multiplex PCR was used for serotyping of isolates to identify *Salmonella enterica* serovars Typhimurium (*S. typhimurium*) and Enteritidis (*S. enteritidis*) as described previously [25]. In the case of *S. enteritidis*, amplification was carried out in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany) as follows: 35 cycles of 30 s for denaturation at 94 °C, 90 s for annealing at 56 °C, and 30 s for primer extension at 72 °C, followed by a terminal extension at 72 °C for 10 min. Target genes for *S. typhimurium* were amplified using the same thermocycler, as follows: 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, and primer extension at 72 °C for 30 s, followed by 7 min at 72 °C for terminal extension. PCR was done by CINNAGEN Master Mix (SinaClon, Tehran, Iran), which was performed with oligonucleotide primers as detailed in a recent study [26]. Initial denaturation at 95 °C for 5 min was used for both amplifications. Electrophoresis of PCR products was performed on 1.2% agarose gel for *S. typhimurium* and *S. enteritidis* isolates. The gel staining was performed in ethidium bromide for 15 min and visualized in gel document system (Bio-Rad, Hercules, United States of America). *E. coli* CTX-M standard strains were provided for quality control from Tarbiat Modares University, Tehran, Iran.

PCR and DNA sequencing were performed for the detection of CTX-M-15 gene in all ampicillin-resistant isolates with previously described oligonucleotide primers [27]. All amplicon sequences were compared with those in the GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast/), and all CTX-M-positive isolates were sequenced with blasting sequences in PubMed and Lahey Clinic databases (<http://www.lahey.org/Studies>). PCR mapping experiments using combinations of the ISEcp1 forward

primers and the *bla*_{CTX-M-1} group or *bla*_{CTX-M-9} group reverse primers were performed to detect the flanking regions of *bla*_{CTX-M-1}-group and *bla*_{CTX-M-9}-group genes.

Sequencing

Sequencing of PCR products of CTX-M was done by Takapozist Company-Iran (on behalf of Bioneer, Daejeon, South Korea). All sequencing results were interpreted by LaserGene Software (DNASTAR, Madison) including SeqMan, MegAlign, and EditSeq Softwares.

Ethics statement

This research was approved by the ethical committee of regional Medical Research of Tabriz University of Medical Science and all patients provided written informed consent for this study.

Results

In total, 91 *Salmonella* spp. and 58 *Shigella* spp. were isolated from stool of patients referred to three teaching hospitals. Patients' age ranged from 3 to 70 years. Of 58 *Shigella* isolates, 45 (77.6%) isolates were *S. sonnei*, 7 (12%) were *S. flexneri*, and 6 (10.3%) were *S. dysenteriae*. In *Salmonella* spp., 73 (80.2%) were *Salmonella enterica* (*S. enterica*) serovar enteritidis, 10 (11%) were *S. enterica* serovar typhimurium, and eight (8.8%) belonged to other serotypes. Almost all isolates were resistant to at least three classes of antimicrobial agents. *S. sonnei* isolates were the most resistant ones. Rate of resistance in *Shigella* spp. and *Salmonella* spp. isolates is shown in Table 1. Molecular investigation demonstrated the presence of CTX-M in 29 (31.8%) isolates of *Salmonella* spp. and 20 (34.48%)

isolates of *Shigella* spp. Moreover, all CTX-M-positive isolates were positive for ISEcPI. Protein translation comparing with reference sequences (CTX-M-15 AY044436) showed that all CTX-M isolates belong to CTX-M 15.

Discussion

Though ESBLs might be produced by several members of the Enterobacteriaceae family, the present study focused on the prevalence of ESBL-producing clinical *Salmonella* spp. and *Shigella* spp. isolates. This study also described the presence of *Salmonella* and *Shigella bla*_{CTX-M}-producing isolates in three teaching hospitals in Iran. In *Salmonella* spp., more than 60% of isolates were susceptible to gentamicin, amikacin, ceftizoxime, and cefotaxime. Conversely, more than 60% of the strains were resistant to other antibiotics, which was significantly higher than in a study carried out by Hamidian et al. in 2009 [28]. In the case of *Shigella* spp., more than 75% of isolates were susceptible to gentamicin and amikacin, while more than 85% of isolates were resistant to tetracycline, ampicillin, and trimethoprim-sulfamethoxazole. This resistance rate was relatively higher than in a study conducted by Tajbakhsh et al. in 2012 [29], but it is in agreement with those reported in another study showing the same trends of resistance [30]. This antimicrobial finding points towards a very serious epidemiological situation. In the present study, CTX-M ESBLs have been found in 29 (31.8%) isolates of *Salmonella* spp. and 20 (34.48%) isolates of *Shigella* spp., whereas sequencing results showed that all CTX-M isolates belong to CTX-M 15. The understanding of the various reports worldwide is that CTX-M-15 is the most important type, posing a threat to human health [31].

The first characterization of ESBL-producing *S. enterica* harboring *bla*_{CTX-M-15} from Iran was described in 2009 [28]. This study reports the first known description of ESBL producers (harboring *bla*_{CTX-M-15}), in which two of three

Table 1. Rate of antibiotic resistance in *Shigella* and *Salmonella* spp. isolates

Antibiotic(s) tested	<i>Salmonella</i> spp.		<i>Shigella</i> spp.	
	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)
TET	9 (8.9)	82 (91.1)	3 (5.2)	55 (94.8)
AMP	13 (14.29)	78 (85.71)	2 (3.8)	56 (96.2)
GEN	71 (78.03)	20 (21.97)	48 (82.76)	10 (17.24)
AMK	76 (72.53)	25 (27.47)	44 (75.78)	14 (24.13)
CHL	35 (38.5)	56 (61.5)	29 (50)	29 (50)
ZOX	56 (61.54)	35 (38.46)	34 (58.7)	24 (41.3)
SXT	6 (6.6)	85 (93.4)	8 (13.8)	50 (86.2)
CTX	56 (61.54)	35 (38.46)	34 (58.7)	24 (41.3)

AMK: amikacin (30 µg), AMP: ampicillin (10 µg), CHL: chloramphenicol (30 µg), CTX: cefotaxime (30 µg), GEN: gentamicin (10 µg), SXT: trimethoprim-sulfamethoxazole (25 µg), TET: Tetracycline (30 µg), ZOX: ceftizoxime (30 µg)

isolates harbored both *bla*_{CTX-M-15} and *bla*_{TEM} gene, while the third one carried only *bla*_{CTX-M-15} gene. Because of data from the mentioned study, two thirds of isolates harbored both *bla*_{CTX-M-15} and *bla*_{TEM} genes, while one third carried only *bla*_{CTX-M-15}. The emergence of *bla*_{CTX-M-15}-positive *S. sonnei* and *S. flexneri* may compromise the treatment of shigellosis, which was firstly reported from Iran in 2010 [29]. A study in 2013 by Ranjbar et al. described that 7.3% of all *Shigella* isolates were ESBL positive. Notably, this rate was higher than those reported from many other countries [16].

The distribution of *bla*_{CTX-M} genes in other genera of Enterobacteriaceae in Iran seems to be different than *Salmonella* and *Shigella* spp. In 2008, Mehrgan and Rahbar [32] reported that around 11% of *E. coli* isolated in a tertiary care hospital in Tehran were phenotypic CTX-M-producer. This prevalence in Enterobacteriaceae excluding *Salmonellae* has been corroborated by the findings of Ramazanzadeh et al. [33], who found an incidence of 31.7% of *bla*_{CTX-M-1}-type genes. In another study, Peerayeh et al. [34] detected 36.0% ESBL-positive *K. pneumoniae*, in which the *bla*_{CTX-M-15} was dominant (62.5%). Relatively similar distributions of *bla*_{CTX-M-15} have been identified in adjacent countries, such as Lebanon, Kuwait, United Arab Emirates, and Turkey [34–36].

In conclusion, the presence of CTX-M-producing *Salmonella* and *Shigella* species is significant challenge to infection control management in Iran hospitals and exposure by such isolates may lead to further spread of antimicrobial drug resistance. It demonstrates further that the ongoing flow of ESBL-producing bacteria between different regions, including their constant supply from high-prevalence areas, makes the control of their wide spread enormously difficult. Continuous surveillance and monitoring of this entity are needed, because the co-dissemination of multiple drug-resistant genes with *Salmonella* and *Shigella* spp. may become a serious therapeutic problem. Furthermore, this study is important for strict antibiotic policy implementation in hospitals to estimate the effect of higher drug resistance in bacteria and to take steps in reducing such resistance.

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Conflict of interest

The authors declare no conflict of interest.

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