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Isolation of *CTX-M1*, *CTX-M2*, *CTX-M3* Genes Producing Extended-Spectrum Beta-Lactamase in Clinical Samples of *Salmonella typhimurium*

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Abstract

Objectives: The purpose of this study was to isolate *CTX-M1* and *CTX-M3* genes producing extended-spectrum beta-lactamase (ESBL) from clinical samples of *Salmonella typhimurium* isolated from poultry in Zabol, Iran.
Methods: All the strains were cultured and identified in a clinical microbiology laboratory and were recovered from blood and urine cultures. The in vitro presence of ESBL was confirmed with Clinical and Laboratory Standard Institute double disc and PCR methods for *CTX-M1* and *CTX-M3*.
Results: The results of this study showed that *Salmonella typhimurium* samples were resistant to ampicillin (41.66%), gentamycin (0%), cefazolin (0%), amoxi-clav (10%), and azithromycin (5%) antibiotics.
Conclusions: The widespread use of ESBL antibiotics has increased the spread of ESBL enzymes in Iran and throughout the world and the use of these antibiotics is becoming more and more limited. Therefore, the complete identification of ESBL by experiments, the restriction of the use of beta-lactam antibiotics and the use of beta-lactamase inhibitors can maintain the efficacy of beta-lactam antibiotics as much as possible.

Keywords: ESBL, *Salmonella typhimurium*, Zabol, Iran

1. Background

Salmonella is one of the most important genera in Enterobacteriaceae family, which was first identified by Daniel Salmon (1). Many of the bacteria that constitute this genus can be pathogenic to humans and animals (2). These bacteria are among the most commonly transmitted bacteria from animals to humans. Due to their numerous animal reservoirs, this genus is one of the most important causes of foodborne diseases and is a health problem worldwide (3).

There is usually no cure for *Salmonella* infection in cases of acute and invasive disease, typhoid fever and immunodeficiency (4). According to the Center for Disease Control (CDC), currently 50% of *Salmonella* species are resistant to multiple drugs. Despite reports on resistance to various fluoroquinolones and cephalosporins in most parts of the world, these two drugs are nowadays the most effective drugs in the treatment of stem cells (5).

Recently, resistance to extended-spectrum cephalosporins such as cefotaxime, ceftriaxone, ceftazidime and ceftizoxime, which are mainly produced by β-lactamase, is increasing. Probably the excessive use of new antibiotics for treatment and selective pressure on bacteria has caused the production of new beta-lactamases by bacteria (6).

The extended spectrum of cephalosporin resistance is mainly related to the production of an enzyme called extended-spectrum beta-lactamase (ESBL). The ESBL enzyme is capable of hydrolyzing and disabling a wide range of β-lactams, including third-generation cephalosporins, penicillin, and aztreonam. This enzyme is the result of mutations in *TEM-1*, *TEM-2*, and *SHV-1*. All these beta-lactams are found in the Enterobacteriaceae family (7).

2. Objectives

This enzyme mediates resistance to extended spectrum cephalosporins and monobactams, such as aztreonam, but there is no detectable activity against cefixime and imipenem. Due to the extended range of substrates,

these were called ESBL enzymes. The purpose of this study was to isolate *CTX-M1* and *CTX-M3* genes producing ESBL in clinical samples of *Salmonella typhimurium* isolated from poultry in Zabol, Iran.

3. Methods

3.1. Isolation of Bacteria

Samples of poultry feces were collected from Zabol, southeastern Iran, from September 2010 to March 2011. In brief, 1 mL of the sample from the transport swab was inoculated in 9 mL of buffered peptone water (Hi Media) and incubated at 37°C for 18 hours for pre-enrichment. Further, for selective enrichment 0.1 mL of the pre-enriched inoculum was transferred to 10 mL of Rappaport-Vassiliadis broth (Hi Media) and incubated at 42°C for 24 hours. After enrichment, a loopful (10 μL) of inoculums was then streaked on xylose lysine desoxycholate (XLD) agar (Hi Media) and incubated at 37°C for 24 hours. The presumptive *Salmonella* colonies (4 - 5 colonies/plate) appearing slightly transparent red halo with a black center surrounded by a pink-red zone on XLD agar were screened further for its biochemical characterization. The presumptive colonies of *Salmonella* were further subjected to biochemical tests *viz*., triple sugar iron (TSI), ortho-nitrophenyl galactosidase (ONPG), urease broth, indole, methyl red, Voges-Proskauer and Citrate test (IMViC) as per the standard test protocol described in bacteriological analytical manual FDA.

3.2. Preparation of 0.5 McFarland Suspension

A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4) (8).

In order to prepare a microbial suspension, 24 hours before the experiment, bacterial culture was inoculated onto a sloped agar culture medium. After the growth of bacterial colonies, the culture medium was washed with normal saline solution and a concentrated microbial suspension was obtained. Then, some of the bacterial suspension was poured into a sterile tube containing normal saline solution and its opacity was measured with a spectrophotometer (Unico, US) at a wavelength of 630 nm. Afterwards, it was diluted with normal saline solution until the opacity of the solution was equal to 0.5 McFarland. Thus, bacterial suspension was prepared at a concentration of 1×10^8 CFU/mL.

3.3. Determining the Sensitivity of Bacteria to Conventional Antibiotics

The susceptibility of 24 bacterial strains to ampicillin (AM), gentamycin , cefazolin (CZ), amoxi-clav (AMC), and azithromycin (AZM) antibiotics (Antibody Medicine, Iran) was evaluated using standard kirby-bauer diffusion method. To this end, all bacterial strains with a concentration of 0.5 McFarland were prepared in a Mueller-Hinton agar. Antibiotic discs were placed at a proper distance from each other. The plates were incubated for 24 hours at 37°C and the diameter of the inhibitory zones was measured to determine the resistance and sensitivity of the strains to the desired antibiotics.

3.4. PCR Reaction

In order to detect the *CTX-M1* and *CTX-M3* genes of β-lactamase, genomic DNA of the isolates was identified using phenol-chloroform method. The identification of beta-lactamase genes, *SHV*, *CTX-M1* and *CTX-M3*, was performed using the primers mentioned in Table 1 (9). Finally, the PCR reaction was carried out in a volume of 25 μL containing 12 μL Master Mix, 1 μL of each primer, 3 μL of DNA and 8 μL of sterilized deionized water, according to the primer temperatures presented in Table 2. The PCR product was electrophoresed in the presence of the gene on 2% agarose gel. Finally, the PCR product was stained with ethidium bromide and photographed and captured with ultraviolet (UV).

Table 1. Primers for Each Gene

Gene Name	Primer
CTX-M1	
F	GACGATGTCACTGGCTGAGC
R	AGCCGCCGACGCTAATACA
CTX-M3	
F	CGCTTTGCCATGTGCAGCACC
R	GCTCAGTACGATCGAGCC

4. Results

The results of this study showed that *Salmonella typhimurium* strains were resistant to AM (41.66%), GM (0%), CZ (0%), AMC (10%) and AZM (5%) antibiotics (Table 3).

The results of this study showed that of the 24 samples tested, 8 (28.57%) were positive for the *CTX-M1* gene (Figure 1) and 12 (42.85%) were positive for *CTX-M3* gene (Figure 2). These results indicate the low incidence of *CTX-M1* and *CTX-M3* genes in *S. typhimurium*.

5. Discussion

Salmonella species are considered as one of the most important food and gastroenteritis contaminants in humans. In *Salmonella*, there are more than 2600 serotypes,

Table 2. The Temperatures of the PCR Reaction for Each Gene

Primer	PCR Reaction Steps			Number of Cycles	Reaction Product
	Denaturation	Annealing	Extension		
CTX-M1				35	499
	94	94	55		
	180 s	60 s	30 s		
CTX-M3				35	307
	94	94	55		
	180 s	60 s	30 s		

Table 3. The Antibiotic Resistant Patents

	AM	GM	CZ	AMC	AZM
S	25	100	58.33	75	83.0
I	33.33	0	41.66	15	16.0
R	41.66	0	0	10	5

Abbreviations: AM, Ampicillin; AMC, amoxi-clav; AZM, azithromycin; CZ, cefazolin; GM, gentamycin.

many of which are important pathogens for humans and animals (10, 11).

The World Health Organization has reported an annual *Salmonella* infection rate of 16 to 33 million patients and 500 to 600,000 deaths from *Salmonella*. It is a major health problem in the developing countries, including Iran (12).

On the other hand, the presence of carriers, which are usually difficult to detect and identify with the usual laboratory methods, in humans and animals plays a special role in the epidemiology of the disease. In Iran, the second cause of diarrhea in humans after *Shigella* is *Salmonella* (13).

Resistance to β-lactam antibiotics is mainly mediated by a large number of β-lactamases (14). Beta-lactamase enzymes have been identified in *Salmonella*, which are coded by different genes. Approximately, 10 genes such as *TEM*, *SHV*, *PSE*, and *OXA* have been identified as beta-lactamases (15).

The results of this study showed that *S. typhimurium* samples were resistant to AM (41.66%), GM (0%), CZ (0%), AMC (10%), and AZM (5%) antibiotics. The results of this study showed that of the 12 samples tested, 6 (42.85%) were positive for the *CTX-M1* gene and 4 (28.57%) were positive for the *CTX-M3* gene. These results indicate the low incidence of *CTX-M1* and *CTX-M3* genes in *S. typhimurium*.

In a study by Ziech et al. who tested *S. typhimurium* resistance patterns, the results showed that of 98 species, 84 were multidrug resistant. The highest resistance rates were observed to nalidixic acid (95%), tetracycline (91%), ampicillin (45%), streptomycin 19%, and gentamycin 15% (16).

In a study by Akinyemi et al., 35 (25.9%) *Salmonella* strains were isolated and identified, 74.3% of which were *S. typhimurium* and 22.9% were *S. parathypha*. A total of 24 strains produced beta-lactamases. Meanwhile, *CTX-M1* gene was detected in about 45.8% of *Salmonella* specimens. They reported that 81.8% of *S. typhimurium* strains carry the *CTX-M1* gene, while 18.2% of *S. enteritysis* strains carry the *CTX-M1* gene (17).

Warren et al. observed that 33.3% of the *E. coli* isolated from poultry in Britain had ESBL genes (18). Jahantigh and Ordoni examined the prevalence of β-lactamase genes in *E. coli* isolated from poultry with Coli-septicaemia. The results showed that the prevalence of CTX gene in *E. coli* isolated from the chickens was 20%, which had an equal prevalence of 10% in the liver and kidneys. In TEM gene examination, the frequency of 24.28% was observed using PCT on *E. coli* isolated from the liver and kidneys (19).

Asadi et al. conducted a study on 56 *E. coli* isolates from poultry fecal samples in Urmia, Iran. They found that 26 (46.4%) isolates had *CTX-M* gene and 15 (26.7%) isolates had *TEM* gene (20).

In the study of Hasannejad et al., *blaTEM*, *blaCTX-M* and *blaSHV* were detected in *E. coli* isolated from poultry by multiplex-PCR and their antibiotic susceptibility profiles were examined. The antibiotic susceptibility test results showed that the lowest and highest resistance rates were related to imipenem (0%) and aztreonam (77%), respectively, and 41 (68.3%), 28 (46.6%) and 0 (0.0%) strains were positive for *CTX-M*, *TEM* and *SHV*, respectively. Also, 16 (26%) isolates carried both *TEM* and *CTX-M* genes (21).

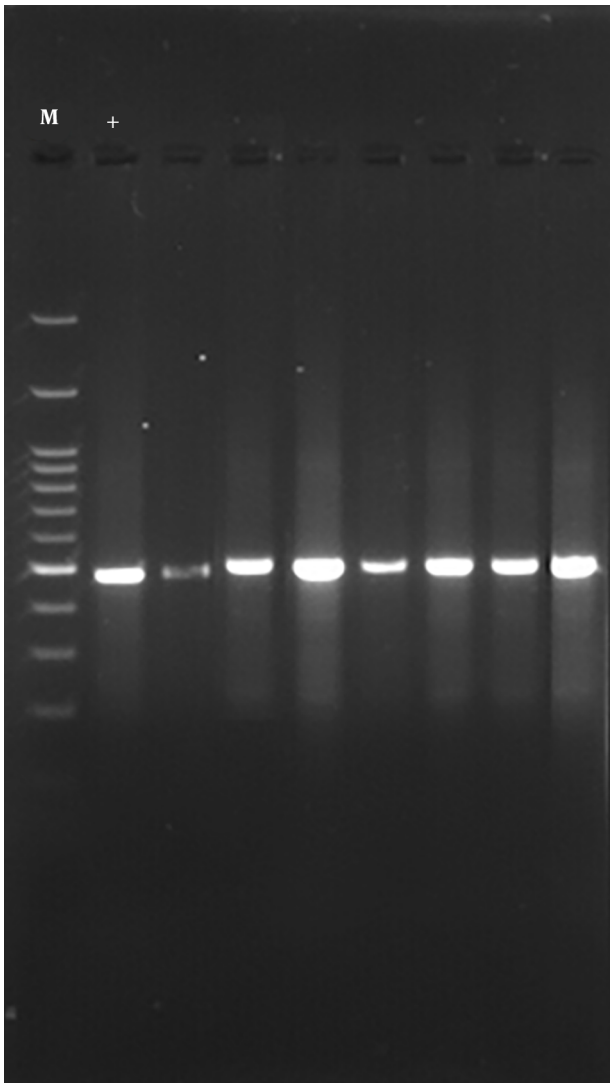

Figure 1. The *CTX-M1* gene fragment, the first band is the 100 bp ladder

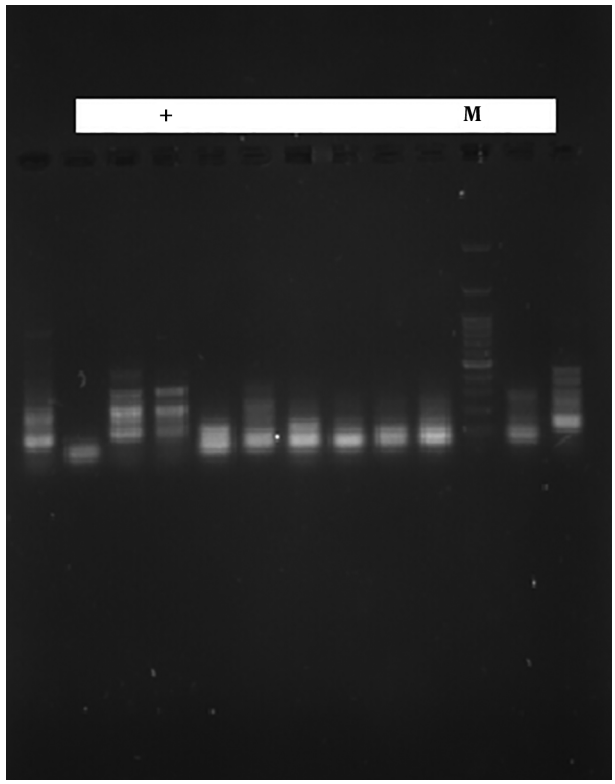

Figure 2. The *CTX-M3* gene fragment. The first and second bands are related to the desired gene, the third band is 100 bp ladder

5.1. Conclusions

The widespread use of beta-lactam antibiotics has increased the spread of ESBL enzymes in Iran and throughout the world and the use of these antibiotics is becoming more and more limited. Therefore, the complete identification of ESBL by experiments, the restriction of the use of beta-lactam antibiotics can maintain the efficacy of beta-lactam antibiotics as much as possible.

Footnotes

Authors' Contribution: All the authors had an equal role in design, work, statistical, analysis and manuscript writing.

Conflict of Interests: The authors declare no conflict of interest.

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