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ORIGINAL ARTICLE

Prevalence of the $bla_{CTX-M-1}$ group and their transferability in resistant clinical isolates of *Salmonella* serogroups from several hospitals of Tehran

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ABSTRACT

Background and Objectives: *Salmonella* is an important food-borne pathogen in humans. Strains of *Salmonella* spp. that producing extended-spectrum β-Lactamases have become a concern in medicine regarding both antimicrobial treatment and infection control program. The objective of this study was to describe the antibiotic susceptibility, ESBL production and determining the prevalence of the $bla_{CTX-M-1}$ group among clinical isolates of Salmonella spp.
Materials and Methods: A total of 110 *Salmonella* isolates collected from four Tehran hospitals during May 2012 and April 2013. The specific monovalan *Salmonella* antisera were used for serogrouping of *Salmonella* isolates. Antibacterial susceptibility was determined by disk diffusion and ESBL phenotype was confirmed by combination disk method. The $bla_{CTX-M-1}$ group was identified by PCR with specific primers. The transferability of the bla_{CTX-1} group was tested by conjugation with broth matting method.
Results: The prevalence of *Salmonella* serogroups consist of 56.4% serogroup D, 13.6 % serogroup C, 10 % serogroup B, and 1.8 % serogroup A and 18.2% other serogroups. Maximal resistance in *Salmonella* isolates was noticed against trimethoprim–sulfamethoxazole (63.6%) and nalidixic-acid (47/3%). All isolates were susceptible to imipenem and ciprofloxacin. Four isolates (3.6%) showed ESBLs phenotype. All *Salmonella* spp. that produce ESBls have bla_{CTX-1} genes group. A conjugative plasmid containing bla_{CTX-1} group was found in one *Salmonella* isolate.
Conclusion: This study demonstrates the predominant presence of the gene encoding CTX-M-1 group among ESBLs producing of *Salmonella* spp. They can transmit to bacteria of this genus or even other genera of enteric bacteria.

Keywords: *Salmonella spp*., $bla_{CTX-M-1}$ group, antibiotic resistance, conjugation, broth matting

INTRODUCTION

Salmonella is enteropathogenic Gram-negative bacterium that infects humans and animals and causing each year about 1.3 billion cases of human disease ranging from diarrhea to systemic typhoid fever (1). After the first report of resistance in *Salmonella*, nowadays, developing resistance in *Salmonella* is an important issue in salmonellosis (2). ESBLs (Extended-Spectrum Beta-Lactamases) are typically inhibitor-susceptible ß-lactamases that hydrolyze penicillins, cephalosporins and aztreonam and are mostly associated with mobile genetic elements. The most frequently encountered ESBLs belong to

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the CTX-M, SHV, and TEM families (3). In clinical strains, CTX-M-encoding genes have commonly been located on plasmids which vary in size from 7-200 kb (4). Many of these plasmids are conjugative and have transfer frequencies ranging from $10^{-2} – 10^{-7}$ (5). To date, more than 60 types of CTX-M ESBLs belonging to 5 evolutionary groups have been described. In most clinical isolates CTX-M-1 group is the most frequent CTX-M type, and has been reported in *Enterobacteriaceae* isolates from many regions of world (6-8). It is necessary to know the frequency of strains carrying genes encoding ESBLs in hospitals in order to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher (7-8). The aim of this study was to describe the antibiotic susceptibility, ESBL production and determining the prevalence of the $bla_{CTX\text{-}M\text{-}1}$ group among clinical isolates of *Salmonella* spp. and determining their transferability by broth matting.

MATERIALS AND METHODS

Bacterial isolates and identification. A total of 110 isolates of *Salmonella* collected from four hospitals in Tehran, Iran during May 2012 and April 2013. They were mostly isolated from the stool culture (n=105), and blood culture (n=5). Identification was based on the routine biochemical tests. The specific monovalan *Salmonella* antisera (Bahar-afshan (were used for serogrouping of *Salmonella* isolates by slide agglutination method (9).

Antibiotic susceptibility testing. The antibiotic susceptibility was determined by disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar plates (Merck, Darmstadt, Germany) based on CLSI guidelines (10). The disks containing the following antibiotics (Mast, UK) were used: cefotaxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), imipenem (10µg), aztreonam (30µg), ciprofloxacin (5µg), trimethoprim-sulfamethoxazole (25µg), tetracycline (30µg), ofloxacin (5µg), ampicillin (25µg), chloramphenicol (30µg), nalidixic acid (30µg), cefoxitin (30µg), tobramycin (10µg), amikacin (30µg), Gentamicin (10µg). *E. coli* ATCC 25922 was used as quality control for antimicrobial susceptibility testing.

ESBL screening and confirmation by phenotypic method. The isolates showing reduced susceptibility to ceftazidime or cefotaxime were tested for ESBLs production by the combination disk method according to CLSI guidelines. Combination disk method was performed using four disks: cefotaxime (CTX) (30µg), cefotaxime (30µg) + clavulanic acid (10µg), ceftazidime (CAZ) (30µg), and ceftazidime (30µg) + clavulanic acid (10µg). A ≥5 mm increase in a zone diameter for antimicrobial agent tested (CAZ or CTX) in combination with clavulanic acid versus its zone when tested alone was considered as a ESBLs positive. Quality control for the production of ESBL was performed using *E.coli* ATCC 25922 as negative control. Minimum inhibitory concentration (MIC) of ceftazidime and cefotaxime was determined for ESBLs isolates by the E-test (AB Biodisk, Solna, Sweden) according to the guidelines of CLSI.

PCR Analysis. The DNA from ESBL-producing isolates were extracted by boiling method and used as template in PCR assay. For the PCR reactions we used the ctx-m-1 (F): 5′-AGAATAAGGAATCCCATGGTT and ctx-m-1 (R): 5'-GCAAGACCTCAACCT TTT CC specific primers generating an 850-bp fragment (11). Cycling conditions were as follows: Initial denaturation at 94°C for 5min; 35 cycles of 94°C for 1min, 55°C for 45 seconds, and 72°C for 1 min followed by a final extension at 72°C for 7 min. *K. pneumoniae* TMU4 was used as positive control.

Conjugation experiments. The isolates with $bla_{CTX\text{-}M\text{-}1}$ group were used as donor strains in conjugation experiments. Conjugation transfer assay was performed in broth culture with *E. coli* $15AR^r$ (cefotaxime sensitive and rifampicin resistant) as the recipient. Before conjugation transfer assay, donor strains are tested for sensitivity to rifampicin and resistance to cefotaxime on nutrient agar containing rifampicin (50mg/ml) and cefotaxime (100mg/ml). Donor and recipient cells were mixed at a ratio of 1:10. The transconjugants were selected on nutrient agar containing cefotaxime (100mg/ml) supplemented with rifampicin (50mg/ml) (12, 20-21). Transconjugants and recipients were counted after growth on selective agar media.

Conjugation frequency. Conjugation frequency also was expressed as the percentage number of transconjugants per added donor cell in 1 ml (12, 20-21). We used cfu per ml (colony forming units/

ml) instead of the number of cells. We counted the CFU of donors and transconjugants from the dilution plates with selective antibiotics (cefotaxime and rifampicin) (12, 20-21).

We determined donor number by plating 10^{-4}, 10^{-5} and 10^{-6} dilutions. For transconjugants we plated all dilutions (from 1 to 10^{-6}).

Antibiotic susceptibility testing of transconjugant strain. The antibiotic susceptibility profile of transconjugant strain was determined by disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar plates (Merck, Darmstadt, Germany) based on CLSI guidelines (10).

PCR Analysis and determination of MIC of transconjugants. DNA of transconjugants were obtained by the plasmid extraction kit (BIONEER) and screened for $bla_{\text{CTX-M-}}1$ group. Minimum inhibitory concentration (MIC) of ceftazidime and cefotaxime was determined for transconjugants by the E-test (AB Biodisk, Solna, Sweden) according to the guidelines of CLSI.

RESULTS

The prevalence of *Salmonella* serogroups consist of 56.4% serogroup D, 13.6 % serogroup C, 10% serogroup B, and 1.8 % serogroup A and 18.2% other serogroups. Analysis of the antimicrobial susceptibility profile of the isolates showed that all were susceptible to imipenem and ciprofloxacin. Of 110 isolates, 63.6 % of the isolates were resistant to trimethoprim-sulfamethoxazole, 47.3 % were resistant to nalidixic acid, 6.4 % were resistant to ceftriaxone and ceftazidime, and 2.7 % were resistant to cefotaxime (Table 1). Of 110 *Salmonella* isolates, 16 (14.5%) were susceptible to all antimicrobials tested and 39 (35.5%) were multidrug-resistant and showed resistance to more than two antimicrobial families. Combined disc test was performed for 7 isolates. Four isolates of *Salmonella* showed ESBL phenotype. All of four isolates of *Salmonella* were in the $bla_{\text{CTX-M-1}}$ group. The transferability of the $bla_{\text{CTX-M-1}}$ was tested by conjugation. A conjugative plasmid containing of $bla_{\text{CTX-M-1}}$ group was found in one *Salmonella* isolates. These results were confirmed by PCR. Antibiotic susceptibility profile of transconjugant strain and donor strain was showed in Table 2. Conjugation frequency was calculated by the number of transconjugants in 1 ml per the number of donor cells in 1 ml and it was 0.9×10^{-5}.

The MIC of parental isolates and transconjugants were similar and included cefotaxime ≥256 µg/ml and for ceftazidime 2 µg/ml.

Table1. Antibiotic resistance observed in *Salmonella* collection was determined by disk diffusion assay.

Antibiotic	Resistant no. (%)	Antibiotic	Resistant no. (%)
NA	52 (47.3)	CAZ	7 (6.4)
SXT	70(63.6)	ATM	6 (5.5)
OFX	2 (1.8)	FOX	6 (5.5)
AMP	27 (24.5)	AK	2 (1.81)
CHL	30 (27.3)	GM	1 (0.9)
CIP	0 (0)	TN	1 (0.9)
IPM	0 (0)	CTX	3 (2.7)
T	37(33.6)	CRO	7 (6.4)

Abbreviations: NA, nalidixic acid; SXT, trimethoprim-sulfamethoxazole; OFX ,ofloxacin; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem; T, tetracycline; CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FOX, cefoxitin; GM, gentamicin; AK, amikacin; TN, tobramycin.

Table 2. Antibiotic susceptibility profile of donor and transconjugant strains

Antibiotic Susceptibility profile (donor strain)	Antibiotic Susceptibility profile (transconjugant strain)
NAR, SXTR, OFXS, AMPR, CHLS, CIPs IMP^{S} GM^{S}, TN^{S}, T^{R}, CRO^{R}, CAZ^{R}, ATM^{R}, FOX^{S}, AN^{S}, CTX^{R}	NA^{R}, SXT^{S}, OFX^{S}, AMP^{R}, CHL^{S}, CIP^{s}, IMP^{S} GM^{S}, TN^{S}, T^{R}, CRO^{R}, CAZ^{R}, ATM^{R}, FOX^{S}, AN^{S}, CTX^{R}

DISCUSSION

Diseases caused by *Salmonella* spp. are increasing in many countries including Iran (13). Data from the present study indicated that the highest clinical *Salmonella* serogroup is serogroup D and so the highest resistance in the collected *Salmonella* isolates was to trimethoprim-sulfamethoxazole (63.6%), followed by nalidixic acid (47.3%), tetracycline (33.6%), chloramphenicol (27.3%), and ampicillin (24.5%). All isolates were susceptible to ciprofloxacin and imipenem. Resistance of *Salmonella* strains to amoxicillin, trimethoprim-sulfamethoxazole (co-trimoxazole) and chloramphenicol has posed an issue in treatment of systemic salmonellosis (14).

Problems associated with ESBL producing isolates include multidrug resistance, difficulty in detection and treatment, and increased mortality of patients (16). For treatment of infections caused by ESBL producer and MDR Gram-negative bacteria e.g., *Salmonella* carbapenems (e.g., imipenem) are the first drugs that used (14, 17). The use of third-generation cephalosporins is an important risk factor for the development of ESBL-producing organisms. Similar to previous studies, all isolates of *Salmonella* in our study were susceptible to imipenem. This resulted from restricted prescription of carbapenems in Iran (18, 19). This study demonstrates the predominant presence of CTX-M-1 ESBL-producing *Salmonella*, commonly with a large plasmid, in our setting. Dissemination of the ESBL phenotype is linked to the lateral transfer of conjugative plasmid. Based on these findings, larger multi-center studies to determine the molecular epidemiology of *Salmonella* isolates, the distribution of CTX-M ESBL as well as the presence of conjugative plasmids among *Enterobacteriaceae* in hospital populations are warranted. Our results show that the MIC of the transconjugants and parental strains to CAZ and CTX were similar and can say the resistance determinants to CAZ and CTX were transferred on a conjugative large plasmid. As a result, third-generation cephalosporin, fluoroquinolones and imipenem are suggested to be used as frontline remedial antibiotics in treatment of *Salmonella* infections. Careful monitoring and use of appropriate infection control policy are necessary in preventing further emergence and spread of resistant organisms in our hospitals.

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