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## Prevalence of ESBL types TEM, SHV, CTX in isolates of *Salmonella*, *Citrobacter* and *Enterobacter spp.* from patients feces in Tabriz

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### ABSTRACT

Extended-spectrum  $\beta$ -lactamase-producing bacteria are a worldwide healthcare problem exacerbated by antibiotic use and transmission of resistant bacteria by carrier. This study analyzed stool carriage of ESBL producing *Salmonella*, *Citrobacter* and *Enterobacter spp.* in the outpatients and inpatients in order to assessment entrance of these bacteria into hospitals. During the period from April 2015 to July 2016 stool samples were obtained from 200 out patients at beginning of admission and 200 inpatients after 48 hours hospitalization. Specimens were cultured in CTX-MacConkey agar and selenite F broth for salmonella enrichment. All cefotaxim resistant bacteria were identified by biochemical tests and ESBL-producing bacteria were detected using double-disk synergy test (DDST). Susceptibility of ESBL-producing isolates was also determined by disk diffusion method. TEM, SHV and CTX genes were investigated by PCR. Of the 400 stool samples tested, 9 (2.25%) ESBL producing bacteria including 2 (1%) from outpatients and 7 (3.5%) from inpatients were isolated. *Citrobacter freundii* was the predominant ESBL-producing organism 4(1%). No *Salmonella typhi* was detected but two (0.5%) *Salmonella typhimurium* from in patients were isolated. Overall, imipenem and amikacin were the antibiotics most active against the ESBL producing organisms.  $bla_{CTX-M}$ ,  $bla_{TEM}$ ,  $bla_{SHV}$  were noticed in 75%, 50%, and 25%, of *citrobacter* isolates, respectively. Patients are one of the most important carriers of ESBLs to hospitals. In addition, our findings suggest the concurrent society spread of different ESBL genotypes, not a development of special ESBL genes.

**Key words:** *Salmonella*, *Citrobacter*, *Enterobacter*, ESBLs, Carriers

### INTRODUCTION

In the last 10 years, extended-spectrum  $\beta$ -lactamase-producing enterobacteria (ESBL-E) have become one of the main challenges for antibiotic treatment of enterobacterial infections. Salmonellosis is a common disease among humans as well as animals(1, 2).Typhoid fever was a major cause of morbidity and mortality in the United States and Europe in the 19th century (3). The disease remains a serious public health problem in developing countries

(4). Studies have demonstrated that *Salmonella enterica* subsp. *enterica* serovar Typhi colonizes the gall bladder and remains there long after symptoms subside, serving as a reservoir for the further spread of the disease (5).

*Citrobacter spp.* and *Enterobacter cloacae* complex are an important cause of healthcare-associated infections, and serious nosocomial outbreaks due to *Citrobacter* and *Enterobacter spp.* have been reported (6, 7). Species of both genera can cause a wide variety of infections, including urinary tract, respiratory, intra-abdominal, CNS and bloodstream infections (8, 9).

These organisms are transmitted via the faecal-oral route. Therefore, the global distribution of the disease is limited to areas with poor standards of hygiene and sanitation, which facilitate transmission (10).

Mary Mallon, an asymptomatic carrier who infected 51 people while working as a cook and laundress in New York in 1906–15, is not the only one, scientists have calculated between 1% and 6% of individuals infected with *Salmonella typhi* become chronic, asymptomatic carriers (11). Strikingly, over 1.1 billion ESBL-E carriers appear to be present in the community populations of Southeast Asia. The Western Pacific and Eastern Mediterranean regions rank second and third, with 280 and 180 million carriers, respectively, ahead of Africa, where 110 million carriers are estimated to be present. America and Europe appear to be far behind, with 48 and 35 million carriers, respectively (12).

Surprisingly, the rates of colonization in Switzerland in 2012 were as high as 15% in pigs and 63% in chickens (13), despite the rather strict antibiotic policy in that country (14). Over three decades have passed since the identification of the first extended-spectrum  $\beta$ -lactamase (ESBL)-producing bacteria. Since then, the prevalence of ESBL-producing strains has increased, and new types and variants have been described. The first ESBLs were derived from TEM (temoniera) and SHV (sulfhydryl-variable)  $\beta$ -lactamases, which are mainly found in healthcare-associated infections. Cefotaximase (CTX-M) ESBLs have increased in importance due to the increased frequency of community-acquired infections caused by strains carrying this enzyme (15, 16). The frequency and predominant types of ESBL vary from region to region and even between institutions within the same region (17).

Plasmid-borne *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> wild-type penicillinase genes were early observed almost exclusively in hospitals, first in Europe and subsequently in other parts of the world (18) especially in intensive care units (ICU), where they sometimes generated large-scale outbreaks (19).

Despite the global extent of the pandemic, there are currently no precise guidelines about how to screen for and deal with ESBL-E carriers in hospitals (20). This is partially due to the paucity of hospital-acquired infections in non-outbreak situations.

We badly lack adequate recommendations to prevent the emergence and spread of ESBL-E through fecal carriage in our community and yet not much is known about ESBL producing bacteria in commensal flora carriers amongst patients in Tabriz.

Knowing the proportion of Tem, SHV, and CTX producing *Salmonella*, *Citrobacter* and *Enterobacter spp.* among healthy carriers is really significant for intervention and may necessitate differing control measures. Therefore, we examined the prevalence of ESBL carriage in outpatients and inpatients, in order to estimate influx of these bacteria into hospitals.

## MATERIALS AND METHODS

One government hospital, Sina teaching and treatment center in Tabriz was selected for this study. The Medical Ethics Committees of the hospitals approved of the study protocol (5/4/8965- 2015).

During the period from April 2015 to July 2016 stool samples were obtained from 200 out patients at beginning of admission and 200 inpatients after 48 hours hospitalization. None of these patients suffered diarrhea or other medical problems in gastrointestinal tract. Only a single sample was taken from each member of the study group. Specimens were cultured in CTX-MacConkey agar (2mg/L cefotaxime) and selenite F broth for salmonella enrichment. Following 24 hours incubation at 37°C, subcultures were made to CTX-MacConkey (2mg/L cefotaxime) from selenite F broth and after overnight incubation at 37°C, all cefotaxim resistant bacteria were identified by biochemical tests such as IMVIC test (Indole production, Methyl Red test, Vogues-Proskauer test,

Citrate test), Urease test, and Triple Sugar Iron agar test(21)and ESBL-producing *Salmonella*, *Citrobacter* and *Enterobacter spp.* were detected using double-disk synergy test (DDST)in which an amoxicillin–clavulanate (AMC) (20 µg/10 µg) disk was placed in the centre with ceftazidime (CAZ) (30µg) and cefotaxime (CTX) (30µg) disks at a 15 mm distance from AMC (24). Strains producing ESBL were defined as those showing synergism between AMC and any one of CTX and CAZ. Susceptibility of ESBL-producing isolates was also determined by disk diffusion method using ampicillin (10µg) chloramphenicol (30µg), TMP—SMX (1.25-23/75µg), ciprofloxacin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), amoxicillin-clavulanic acid (30 µg), gentamicin (10 µg), ceftiofloxacin (30 µg), amikacin (30 µg), according to Clinical Laboratory Standards Institute instructions(22).

**PCR detection of genes coding for ESBLs:** Detection of type SHV, TEM, and CTX-M ESBL genes was performed by PCR using following primers (Table 1) with DNA extracts from isolates with a positive DDST result. A single colony of each test isolate was re-suspended in 500 µl of water and boiled for 15 min, and 2 µl of the supernatant was used as template DNA in a 25 µl PCR mixture (23).

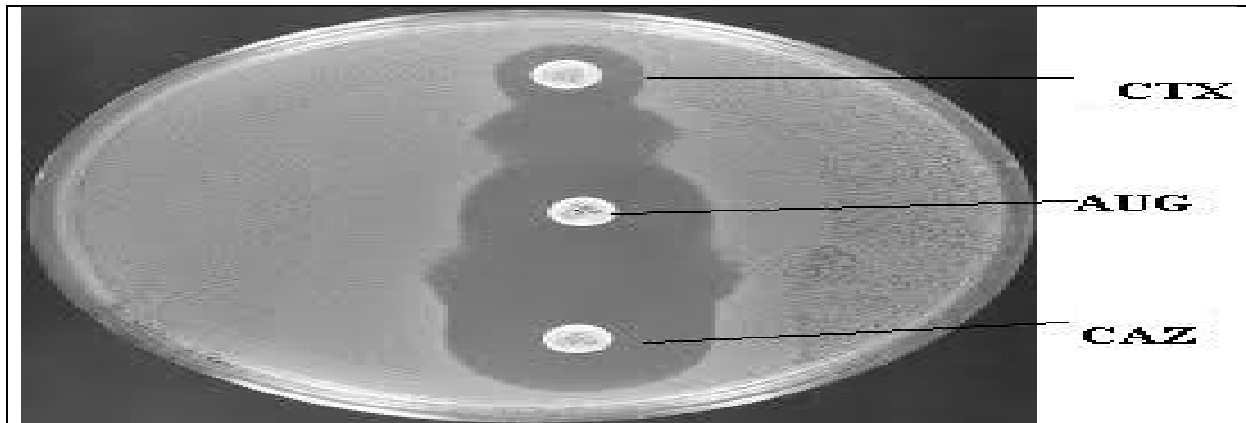
Primers	Nucleotide Sequences (5'-3')	References (GenBank no)	Expected amplicon size (bp)
SHV-F	CGCCTGTGTATTATCTCCCT	EF125011	293
SHV-R	CGAGTAGTCCACCAGATCCT		
TEM-F	TTCGTGTGCGCCCTTATTCC	AB282997	403
TEM-R	ATCGTTGTCAGAAGTAAGTTGG		
CTX-M-F	CGCTGTTGTTAGGAAGTGTG	DQ303459	569
CTX-M-R	GGCTGGGTGAAGTAAGTGAC		

Reaction mixtures of 25 µl containing 1.5 U Taq DNA polymerase, 1.5mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.2 µM selected primer and 2 µl DNA template were prepared. The amplification was done in a DNA thermal cycler(Eppendorf master cycler gradient, Germany), and programmed for a cycle of 5 min at 96 C°, followed by 35 cycles of 1 min at 96 C°, 1 min at 58 C° for *bla*<sub>TEM</sub> or at 60 C° for *bla*<sub>SHV</sub> and 1 min at 72 C°, with a final extension of 10 min at 72 C°. The thermal cycling conditions for the CTX-M gene were one cycle of 7 min at 94 C°, followed by 35 cycles of 50 s at 94 C°, 40 s at 55C° and 1 min at 72 C°, with a final extension of 5 min at 72 C°. Aliquots (10 µl) of each PCR product were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide (0.5 µg mL<sup>-1</sup>) and Photographed on a gel documentation system(UVP, USA)for the analysis of the bands (all the PCR materials including primers were provided by CinnaGen; NedayehFan Co., Iran)(24).

## RESULTS

Bacterial isolates other than *Salmonella*, *Citrobacter* and *Enterobacter spp.* that grew on the MacConkey agar were disregarded. Of the 400 stool samples tested, 9 (2.25%) ESBL producing bacteria and resistant to cefotaxime were isolated. Out of the 9 (2.25%) resistant isolates to cefotaxime, 2 (1%) and 7 (3.5%) ESBL producing organisms were recovered from outpatients and inpatients respectively. Inpatients were not further classified among ward patients.(Table 1, Fig 1). *Citrobacter freundii* was the predominant ESBL-producing organism 4(1%); it was recovered from 3 (0.75%) of inpatients and 1 (0.25%) of outpatients. Three (0.75%) *Enterobacter cloacae*, one from out patients and 2 from inpatients were isolated. No *Salmonella typhi* was detected but two (0.5%) *Salmonella typhimurium* from in patients were isolated. The sex ratio (male/female) was 1: 03 and the median age was 28 years (9 to 40 years).

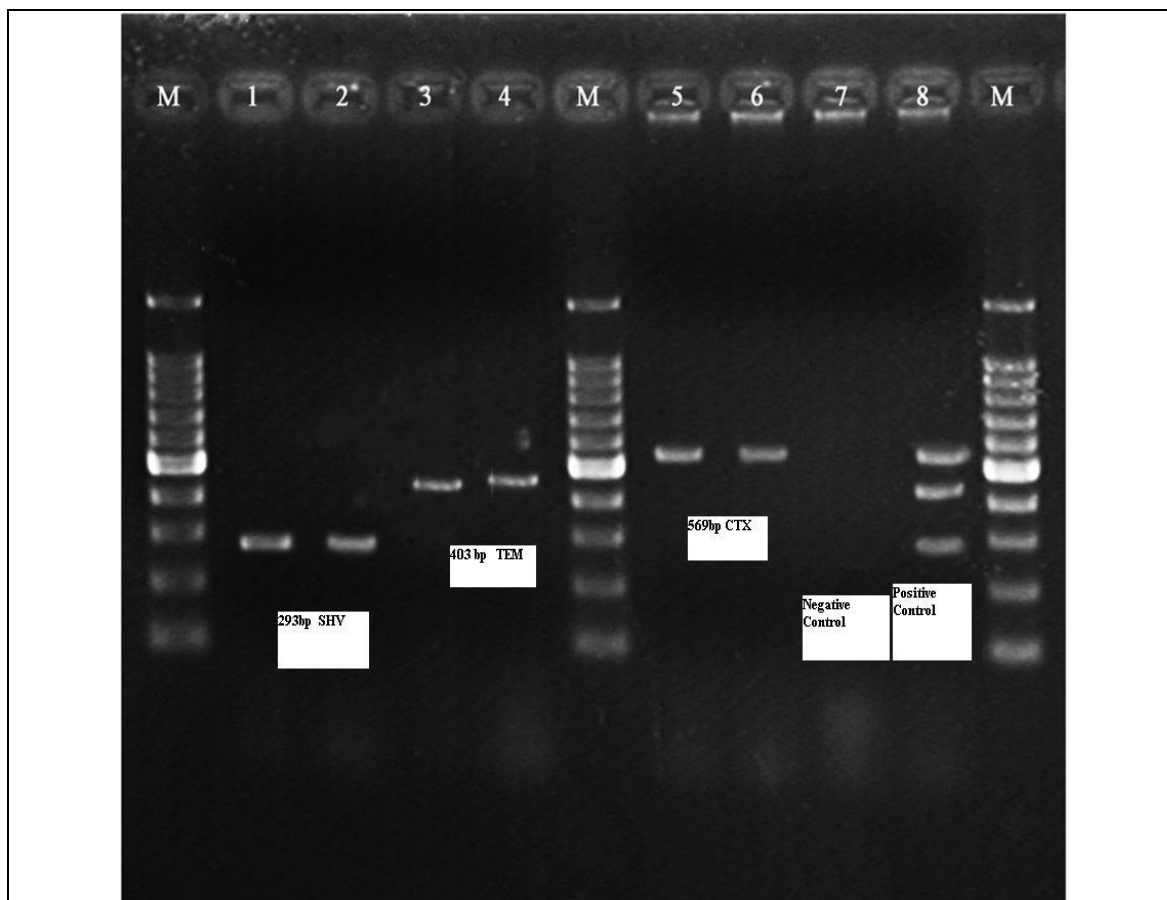
Overall, imipenem and amikacin were the antibiotics most active against the ESBL producing organisms. The susceptibility data for the isolates are shown in Table 2. All isolates from inpatients and outpatients contained one or more genes producing ESBL. The majority of ESBLs in *Salmonella* were *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> type b-lactamase. *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, were noticed in 75%, 50%, and 25%, of citrobacter isolates, respectively. The results for each of the resistance genes was as follows: the *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes alone in 11.11% (1/9) and 11.11% (1/9) respectively; *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> identified in 22.22% (2/9); *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> recognized in 11.11% (1/9); *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> detected in 22.22% (2/9) and *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> were distinguished in 22.22% (2/9) of the isolates (Fig. 2).



**Fig-1: A positive Double-disc synergy test (DDST) using ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), and Augmentine (AUG = Amoxicillin 20µg plus Clavulanic acid 10 µg) discs. A representative of *Citrobacter* spp isolates showing distinct extension of the zone of inhibition towards Augmentine disc indicating ESBL production**

**Table 1. Distribution of Extended-Spectrum β-lactamase (ESBL)- Producing Fecal Isolates of *Salmonella typhimurium*, *Citrobacter freundii* and *Enterobacter cloacae***

Study group	No. of individuals	Cefotaxime resistant and ESBL producing carrier No. (%)
Inpatients	200	7(3.5)
Outpatients	200	2(1)
All	400	9(2.25)



**Fig 2: PCR detection of SHV (293bp), CTX-M (569bp) and TEM (403bp) β-lactamase genes in *Salmonella*, *Citrobacter* and *Enterobacter* SPP. Lanes M, 100bp ladder molecular size marker; Lanes 1, 2, SHV, 3, 4, TEM; Lane 5, 6, CTX isolates; Lane 7, Negative control No template (water); and Lane 8, Positive control**

**TABLE 2. Resistance rates to various antimicrobial agents for ESBL producing isolates (%)**

Antibiotic tested	<i>E.cloacae</i> (n=3)	<i>C. freundii</i> (4)	<i>S. typhimurium</i> (2)
Ceftriaxone	32	57.5	15
Ceftazidime (30 µg),	46	70	10
Cefotaxime (30 µg),	100	100	100
Cefepime	88	77.5	85
Cefoxitin (30 µg)	100	87.5	89
Ciprofloxacin (5 µg),	0	67.5	25
Gentamicin (10 µg)	66	70	100
Piperacillin	86	80	100
Piperacillin-tazobactam)	40	30	0
Imipenem (10 µg),	0	7.5	0
ampicillin (10µg)	100	100	57
chloramphenicol (30µg),	47	52	52
TMP-SMX (1.25- 23/75µg)	100	100	70
amoxicillin-clavulanic acid (30 µg	100	100	43
Amikacin	0	0	0

## DISCUSSION

Human carriers are the only infection source for *S. typhi*. Prevalence of this bacterium differs from one country to another according to number of carriers and health policy. Typhoid is endemic in some countries, which could justify the findings. Nath and colleagues reported a carrier rate of 13.1% in India using nested-PCR (25). Birhaneselassie and colleagues reported positive results in one out of 107 stool specimens taken from food handlers; they used bacteriological characteristics, biochemical and serologic methods to spot the organism (26).

In another study, Pratap and colleagues designed a new nested-PCR primer methodology to target the *staA* gene, which is a member of the fimbrial gene family specific to *S. typhi* only (27). The prevalence of carriers in some countries like India was reportedly as high as 17.4% to 79% (28, 29). In the report of Senthil Kumar and colleagues the authors collected stool samples from the suspected food handlers and used culture characteristics, biochemical tests, antibiotic sensitivity test (disc diffusion), agarose gel electrophoresis, and conjugation protocols to spot the intended organism(29). In another study, Francis and colleagues collected smear swabs and nail cuts of hotel workers to screen for *S. typhi* carriers. They used bacteriological characteristics as well (28). In another report from typhoid endemic area, the Ethiopia, a rate of 1.6%, carriers were found (30). No *S. typhi* was cultured among 400 samples in our research. Other reports from Iran demonstrated typhoid carrier prevalence of 0.94% in Hamedan and 0.6% in Sanandaj respectively(31). They used bacteriological and serological methods for the detection of asymptomatic carriers of *S. typhi* among food handlers.

The reason for such findings could be due to the improvement in the health status of the country and the low typhoid carriers because of massive vaccination in the country for decades.

*S. typhimurium* was isolated only from two hospitalized patients carrying TEM and SHV type ESBL producing genes. Demographic information showed previous enteritis of these patients following food poisoning. In our research *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, were noticed in 75%, 50%, and 25%, of citrobacter isolates, respectively. These findings corresponded the results of Shahid, M. (7) that reported *bla*<sub>CTX-M</sub>(67.5%), *bla*<sub>TEM</sub>(40%), *bla*<sub>SHV</sub>(25%), in isolates of *Citrobacter spp.*

Colonization with ESBL-producing isolates is a prerequisite for infection. The importance of detection of carriers harboring ESBL-producing bacteria has been emphasized not only in patient populations but also in healthy people (32). ESBL-producing bacteria may be transmitted from human-to human or through the environment resulting in an increase in the proportion of carriers in the community (33). The admission of carriers harboring ESBL-producing bacteria to hospitals increases the risk of infection in other hospitalized patients (34, 35). Antibiotic selective pressure in hospitals may amplify the number of carriers harboring ESBL-producing bacteria and enhance the opportunity for these bacteria to cause infections (36). Several studies have focused on beta-lactam resistance in Enterobacteriaceae isolated from stools in healthy people, although specific detection of ESBLs was neither performed nor reported (36, 37). Mirelis *et al.*, (38) reported that 2.1% of outpatients were fecal carriers of ESBL-producing bacteria in 2001 and this percentage increased to 3.8% one year later. Valverde *et al.*,(39)also reported that rates of fecal carriage of ESBL producing isolates increased significantly ( $p < 0.001$ ) in both hospitalized

patients and outpatients, from 0.3% and 0.7%, respectively, in 1991 to 11.8% and 5.5%, respectively, in 2003. In the present study, Out of the 9 (2.25%) resistant isolates to cefotaxime, 2 (1%) and 7 (3.5%) ESBL producing organisms were recovered from outpatients and inpatients respectively. Although the numbers of isolates were relatively low but they harbored one or more kind of ESBL producing genes such as SHV, TEM and CTX indicating potential of isolates to make multidrug resistant infections.

#### CONCLUSION

Influx of ESBL producing bacteria into hospitals and distribution of such organisms to community by already hospitalized patients are predictable. In addition, our findings suggested the simultaneous community spread of diverse ESBL genotypes, not an expansion of particular ESBL genes. The low rate of typhoid carrier state detected in this study requires further studies with more sensitive diagnostic methods on a larger sample size to have better evaluation of their presence.

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