

Molecular identification of types TEM, SHV and CTX extended-spectrum-β-lactamase of escherichia coli, edwardsiell and erwinia spp. Isolated from feces of carriers

Molecular Identification

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Abstract

Aim: Healthy ESBL carrier patients are the major challenge in control of infections produced by members of Enterobacteriaceae. The aims of the present study were to investigate the isolation of TEM, SHV, and CTX type ESβLs producing E. coli, Edwardsiella, and Erwinia spp. from feces of carriers.Material and Method: Two hundred fresh stool samples collected from non-hospitalized and hospitalized patients were cultured on MacConkey agar supplemented with 2 mg/L cefotaxime. After 24 hr. Incubation at 37°C the E. coli, Erwinia and Edwardsiella spp were identified by routine biochemical tests. Combined tests were carried out to select ESBLs producing bacteria and susceptibility of isolates was determined by disc diffusion method. Multiplex-PCR was used to identify TEM, SHV and CTX type ESβLs producing isolates. Results: Of the 34.5% bacteria resistant to cefotaxime, 81.63% and 55% ESBL producing organisms were recovered from inpatients and outpatients respectively. E. coli was the predominant ESBL-producing organism; One Erwinia and three Edwardsiella producing ESBL were detected. Overall, carbapenems including imipenem and meropenem and amikacin were the antibiotics most active against the ESBL-producing organisms. The overall prevalence of these ESBL genes was 73.92%, including the blaTEM and blaSHV genes alone in 27.45% and 5.88% respectively; blaCTX-M were not distinguished alone in any of the isolates. Discussion: More than one ESBL was produced by most isolates carried by patients and Carbapenems, imipenem and meropenem continue to show good in vitro activity against the isolates. Patients can act as a source of ESBL producing bacteria in hospitals.

Keywords

Healthy Carrier; ESBL; E. Coli; Erwinia; Edwardsiella

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Aim

Some genera among *Enterobacteriaceae* family carrying extended-spectrum β-lactamases (ESBLs) have emerged as significant pathogens. Infections due to such strains of these genera are associated with extended hospital stays, increased healthcare costs and, in the setting of bloodstream involvement, development of mortality if proper therapy is delayed. [1]

The most important species of genus *Edwardsiella*, that 2is, *E. tarda, E. ictaluri* and *E. hoshinae* have a wide environmental distribution. Of these, *E. tarda* has been shown to be pathogenic in humans.[2]Most members of this genus characteristically produce diseases of plants, vegetables, and fruits.[3]Reports of *Erwinia* isolates as human pathogens are limited, but the phytopathogen *E. persicina[4]* has been isolated from human urinary tract infections.[5] *Escherichia coli* are the common opportunistic pathogen, isolated from different infections of humans and have demonstrated an increasing antimicrobial resistance to most antibiotics.[6] In the past 2 decades, antibiotic-resistant strains have emerged among the *Enterobacteriaceae* members by a production of extended-spectrum β-lactamases (ESBLs). [7] Recently, a group of ESBLs that hydrolyze cefotaxime (CTX), the CTX-M β-lactamase, has been detected and reported with increasing frequency.[8] Several papers have recently reported the distribution of ESBLs in the community, mainly in patients with chronic conditions.[9-11] The increase of drug resistance among these organisms has made therapy of different infections difficult and has led to greater use of expensive broad-spectrum antibiotics such as the third generation of cephalosporin. Hence, regular monitoring of such resistance at local, national and international levels is necessary as control plan by most national and international organizations.[12,13] ESBLs are frequently plasmid-mediated and can hydrolyze penicillins, third-generation cephalosporins, and monobactams.[14] Distribution of human clinical isolates and capacity to produce ESBLs has been found out among inpatients, as well as among those in the community.[15] we know carriers of ESBL producing organisms exist, but in general practice, this condition has rarely been reported. Until now, more than 600 ESBL variants have been reported. Among them, CTX-M, SHV, and TEM enzymes so far considered to be important in *Enterobacteriaceae.[16]* This is nearly known that resistance caused by ESBLs is often associated with resistance to some other antibiotics such as fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole.[17,18] In the recent few years, there has been an increase in the finding of ESBL-producing strains in the general community[19] but few studies have been published about ESBL dissemination in healthy humans, explaining prevalence between 6 and 7%.[20,21] Percentage of the carriage in the stool is changeable and is found to be higher in rural than the urban population.[2] Recently, ESBL producers have also been reported increasingly among infection-associated members of *Enterobacteriaceae* family in France[22,23], Italy[24], the Czech Republic[25], and Austria.[24] These studies were the reason to look for ESBL producers among healthy carriers.[27]This study was carried out to determine rates of ESBL mediated resistance in fecal isolates of *E. coli*, *Erwinia and Edwardsiella spp.* in hospitalized and outpatients without suffering from diarrhea.

Material and Method

Specimen collection and Screening for and confirming the presence of ESBLs

Two hundred fresh stool samples were collected from non-hospitalized (n=100) and hospitalized patients after 48 hours admission (n=100) in Shahid Madani training and treatment center from November 2014 to February 2015. Those with gastrointestinal illness and diarrhea were excluded from the study. Stool samples were cultured on MacConkey agar supplemented with 2 mg/L cefotaxime ('CTX-MacConkey') and incubated at 37C for 24 h. The isolated bacteria were identified by routine biochemical tests and *E. coli*, *Erwinia and Edwardsiella spp.* stored at -20°C in trypticase soy broth containing 12% glycerol. This study was approved by the ethical committee of regional Medical Research of the Tabriz University of Medical Science, and all patients provided written informed consent for this research (TBZMED.REC.1394.352). ESBL expression was confirmed by the disc diffusion method on Mueller–Hinton agar using cefotaxime (30μg) or ceftazidime (30 μg) with and without clavulanic acid (10 μg), as recommended by the CLSI, and each set of samples was tested with CLSI quality control strains *E. coli* ATCC 25922.[28]

Antimicrobial susceptibility testing

All ESBL-producing *E. coli*, *Erwinia and Edwardsiella* isolates were tested for susceptibility to imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, and tetracycline by the disc diffusion method using Mast Discs (Mast Ltd, UK) according to the CLSI and manufacturer's instructions.[28]

blaTEM, SHV, CTX genes identification

The *bla*TEM, SHV, CTX genes were identified by Multiplex- PCR using DNA extracted by boiling suspensions of isolates. DNA samples at a concentration of 1 μL were used as PCR templates, and the genes were amplified using the primers (Cinnagen Co, Tehran, Iran) as described previously.[29,30]

SHV	ATGCGTTATATTCGCCTGTG	(747 bp)
SHV	TGCTTTGTTATTCGGGCCAA	
TEM	TCGCCGCATACACTATTCTCAGAATGA	(445 bp)
TEM	ACGCTCCCGGCTCCAGATTTAT	
CTX	ATGTGCAGYACCAGTAARGTKATGGC	(593 bp)
CTX	TGGGTRAARTARGTSACCAGAAYCAGCGG	

A loopful of bacteria colonies harvested from a blood agar plate was suspended in 0.5 ml of sterile water and heated at 95°C for 10 min. After centrifugation at 5,000 rpm for 5 min at 4°C, the DNA-containing supernatant was used as the source of template for further amplification.[31] PCR reactions were performed with an automated thermal cycler (Eppendorf mastercycler gradient, Germany) with the PCR cycling conditions of initial cycle at 94ºC for 10 min, 35 cycles of denaturation at 94ºC for 30 s, annealing at 60 ºC for 30 s, extension at 72 ºC for 1 min, and final cycle extension at 72 ºC for 10 min. Gel electrophoresis was performed for 60-120 min in a 1.2% agarose gel at 75 V. DNA profiles were visualized by ultraviolet (UV) light after ethidium bromide staining on a UV transilluminator. The gels were photographed using a gel documentation system

(UVP, USA) for the analysis of bands. Molecular marker (Fermentase; 100 bp DNA ladder) was used to assess PCR product size. Multiplex PCR reactions were carried out in a final 25 µL volume containing 2.5 µL of 10X PCR reaction buffer, 1 µL DNA solution, 0.5 µL MgCl2 (50 mM), 0.5 µL of each gene-specific primer (10 pmoL), 0.5 µL (3 U/mL) Hot Star Taq Mastermix DNA polymerase (Qiagen) and 0.5 µL deoxynucleoside triphosphates mix (dNTPs, 10 mM).

Results

Bacterial isolates other than *E. coli, Erwinia and Edwardsiella spp.* That grew on the MacConkey agar were disregarded. Of the 200 stool samples tested, 69 (34.5%) bacteria resistant to cefotaxime and ceftazidime, were isolated. Out of the 49% and 20% resistant isolates to cefotaxime and ceftazidime, 40 (81.63%) and 11 (55%) ESBL producing organisms were recovered from inpatients and outpatients respectively (Table 1, Fig 1). *E. coli* was the predominant ESBL-producing organism; it was recovered from 38 (95%) of inpatients and 9 (81.81%) of outpatients. One *Erwinia* and three *Edwardsiella* producing ESBL were detected. Overall, carbapenems including imipenem and meropenem and amikacin were the antibiotics most active against the ESBL producing organisms. The susceptibility data for the isolates are shown in Table 2. The overall prevalence of these ESBL genes among inpatients and outpatients was 73.92% (51/69). The PCR assay detected the results for each of the resistance genes as follows: the *bla*TEM and *bla*SHV genes alone in 27.45% (14/69) and 5.88% (3/69) respectively; *bla*TEM and *bla*SHV identified in 13.72% (7/69); *bla*TEM and *bla*CTX-M recognized in 13.72% (7/69); *bla*SHV and *bla*CTX-M detected in %7.84 (4/69) and *bla*TEM, *bla*SHV and *bla*CTX-M were distinguished in 31.37% (16/69) of the isolates (Fig. 2).

Table 1. Distribution of Extended-Spectrum β-lactamase (ESBL)- Producing Fecal Isolates of Escherichia coli, Erwinia and Edwardsiella spp.

		No. (%) of isolates		
Study group	No. of individuals	Caz-Ctx* resistant	ESBL producing carrier	Non-ESBL Producing carrier
Inpatients	100	49 (49)	40(40)	60(60)
Outpatients	100	20(20)	11(11)	89(89)
All	200	69(34.5)	51(25.5)	149(74.5)

*Note. Caz, ceftazidime; Ctx, cefotaxime.



Fig1. A positively combined disc (CD) using ceftazidime (CAZ 30 µg), ceftazidime /clavulanic acid (30µg/10µg) discs. A representative of E. coli isolates showing a ≥ 5 mm zone size enhancement in the CD test indicating inhibition of ESBL production.

Table 2. Antimicrobial Susceptibility Profiles of Extended-Spectrum β-lactamase–Producing Fecal Isolates of Escherichia coli, Erwinia and Edwardsiella spp.

Study group	No. of isolates	No. (%) of isolates susceptible, by agents					
		Cfep	Cip	Gm	Pip-Taz	Ak	Car*
Inpatients	40	3 (7.5)	9 (22.5)	19 (47.5)	23 (57.5)	32 (80)	40 (100)
Outpatients	11	0 (0)	5 (45.45)	7 (63.63)	8(72.72)	10 (90.90)	11 (100)
All	51	3 (5.88)	14 (27.45)	26 (50.98)	31(60.78)	42 (82.35)	51(100)

Note. Ak, amikacin; Car, carbapenem; Cfep, cefepime; Cip, ciprofloxacin; Gm, gentamicin; Pip-Taz, piperacillin- tazobactam.
* Imipenem and/or meropenem.

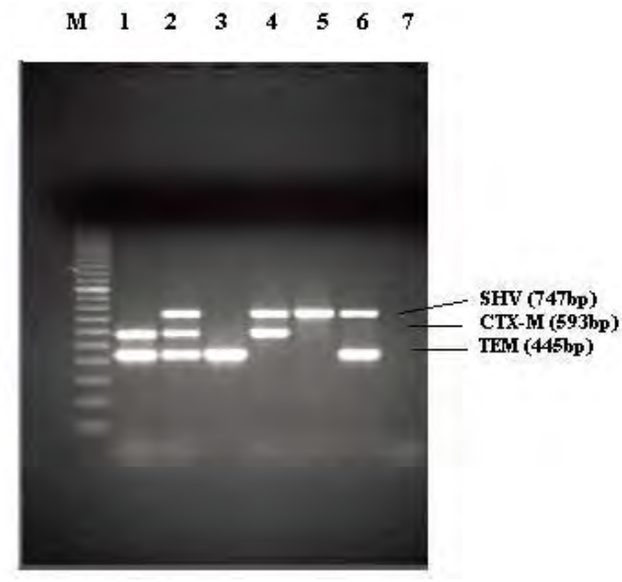


Fig 2. Multiplex PCR detection of SHV, CTX-M and TEM β-lactamase genes in E. coli (EC), Erwinia and Edwardsiella isolates. Lanes M, 100bp ladder molecular size marker; Lanes 1, 2, 4, 6, EC isolates; Lane 3, Edwardsiella, Lane 5, Erwinia; and Lane 7, No template (water)

Discussion

Although *E. coli, Erwinia and Edwardsiella spp,* normally live harmlessly in the gut, they can cause various types of infection, most commonly urinary tract infection.2,5-6 Study of susceptibility patterns for 2,302 gram-negative pathogens obtained from urine samples in Saudi Arabia found that 10.2% of the isolates were ESBL-producing *E. coli*.[32] In another study, the rate of fecal carriage of ESBL-producing organisms among inpatients (26.1%) was higher than that among outpatients (15.4%).[33] In a study done in Lebanon, the rate of fecal carriage for ESBL-producing *E. coli* in inpatients was reported to be 80.5%.[34] These correspond nearly with our result obtained for inpatients (40%) that is much higher than the results of outpatients (11%). In our hospital, most of the inpatients before surgery and also in surgery process receive prior therapy with third-generation cephalosporins and/or fluoroquinolones

as prophylactic. The routine consumption of these antimicrobial agents could clarify the higher spread of fecal carriage of ESBL-producing bacteria in the hospital, a contrast to the rate in the community. The same reason could cause the greater resistance of amikacin, piperacillin-tazobactam, and ciprofloxacin in the ESBL-producing isolates obtained from inpatients, compared with those obtained from outpatients.

The spread of ESBL-producing organisms to the community could be the result of previous hospital infection, as some inpatients maintain to carry ESBL-producing bacteria over prolonged periods, and such carriage may contribute to their propagation outside the hospital.[35] In the present study, none of the outpatients or inpatients had any acute gastroenteritis problem, and there was no evidence of any recent hospitalization. In our study, the fluoroquinolone resistance rate was high both in isolates recovered from inpatients (31 of 40, 77.5%) and in those recovered from outpatients (5 of 11, 54.54%). Although we could not obtain enough information about recent exposure to antibiotics among the outpatients, the unlimited sale of antibiotics in developing countries is probably the most important reason for the creation of resistant organisms in the population. Some antibiotics such as amoxicillin-clavulanate, and fluoroquinolones are often purchased and used without prescriptions. Therefore, it can be deduced that even if clinical laboratories to attempt to detect ESBL-producing bacteria in individuals with community-onset infection, the exact prevalence rate of ESBL producing organisms will not be determined in the community, and asymptomatic carriers may remain unnoticed for long periods. The presence of ESBL producing bacteria in the gut will cause problems because the intestinal colonization for the majority of infections caused by ESBL-producing bacteria is essential.[36] Monstein et al in Sweden by multiplex-PCR method detected the presence of genes in K. pneumoniae as follows: blaSHV in 8% (3/37), blaSHV and blaTEM in 2.7% (1/37), and blaTEM, blaSHV and blaCTX-M in 8% (3/37) of isolates.29 The ESBL prevalence in our study showed a higher prevalence compared with the Sweden results. The variation in the prevalence of ESBL producing isolates in our results could be due to poor infection control, distribution of hospital-acquired ESBLs into the community and vice versa. Therefore, epidemiologists and clinical microbiologists to control the prevalence of ESBL-producing organisms need sufficient knowledge about the status of these bacteria not only in the hospital environment but also in the community. The restricted availability of treatment choices for infections caused by ESBL-producing organisms requires prevention of these infections by limiting the use of antibiotics, along with the performance of prompt infection control actions. To control or reduce the high rate of carriage for these organisms, helpful measures should be taken to forbid the sale of antibiotics without a prescription and to increase knowledge of the hazards of taking antibiotics without medical consultation among the population.

Conclusion

Most commonly one or more than one ESBL was produced by many strains, and this was correlated with increased resistance levels. Carbapenems, imipenem, and meropenem continue to show good *in vitro* activity against isolates. Patients as healthy carrier can act as a source of ESBL producing bacteria to influx resistant bacteria to hospitals and vice versa. The necessity of antimicrobial resistance surveillance is warranted in light of these findings.

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