Prevalence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} β-Lactamase Genes Among *Escherichia coli* Isolates in Foodborne Outbreak in Iran

Mohammad Mehdi Soltan-Dallal[1,2], Fereshteh Fani[3], Zahra Rajabi[2], Mohsen Karami-Talab[1], Hedroosha Molla Agha Mirzaei[2*]

[1]Devision Food Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[3]Professor Alborzi Clinical Microbiology Research Center, Faculty of Medicine, Shiraz University of Medical Sciences, Fars, Iran

*Corresponding Author:
Hedroosha Molla Agha Mirzaei,
Address: Food Microbiology
Research Center, Tehran
University of Medical Sciences,
Tehran, Iran.
Tel: +98-21-88992971,
Email: hedroosha_mirzaei@
yahoo.com

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Abstract
Background: FExtended spectrum beta-lactamases (ESBLs) are known as enzymes that are responsible for creating high level of antimicrobial resistance in gram negative microorganisms especially in *Escherichia coli*. Therefore, survey and identification of the genes generating these enzymes can be effective in controlling the resistance and helpful in using appropriate β-lactam drug against them.
Objective: In this study we focused on evaluating the prevalence of ESBL enzyme genes, including CTX-M, SHV, and TEM β-lactamase, among *E. coli* isolates in foodborne disease outbreak.
Materials and Methods: Four hundred stool samples from the individuals with diarrhea, nausea, vomiting, abdominal cramps, fever and headache were tested by IMVIC biochemical test for *E. coli*. ESBL genes were determined using antimicrobial susceptibility test based on the Clinical and Laboratory Standards Institute (CLSI), disk diffusion method, and confirmatory test (combined disk). Subsequently, polymerase chain reaction (PCR) was performed for following the relative genes.
Results: The highest resistance in *E. coli* isolates was reported against ampicillin (56%), and 36% of samples were ESBL-positive. As determined by PCR, the prevalence of SHV, TEM, and CTX-M1 genes was 0%, 18%, and 38%, respectively.
Conclusion: Due to the prevalence of β-lactamase genes, application of accurate and rapid identification methods such as PCR deems essential. Therefore, it can be considered as a routine method.

Background

Increasing challenge of antimicrobial resistance is focused on the worldwide increasing *Escherichia coli* that contain extended-spectrum beta-lactamases (ESBLs). These enzymes lead to resistance against the antibiotics such as cephalosporins and monobactams by hydrolyzing the β-lactam ring.[1] In *E. coli*, ESBL genes like bla_{CTX-M}, bla_{SHV} and bla_{TEM} are typically transported by movable genetic elements that have high facility in transition of resistance genes.[2] Pathogenic *E. coli* is one of the causes of intestinal and extraintestinal infections in humans.[3] *E. coli* is a major producer of β-lactamase enzymes[4] and one of the most important health problems worldwide, especially in developed countries where it is considered to be responsible for the death of 2.5 million children per year.[5,6] Different methods such as double disk synergy test

(DDST) have been suggested for the detection of ESBL in clinical isolates.[7,8]

In the past 15 years, many epidemics caused by ESBL-positive organisms have been reported worldwide. This prevalence is a serious threat to the treatment of infections. Experience has shown that there is no satisfactory outcome for treatment of infections caused by ESBL-positive bacteria.[9] The mortality rate of this group was significantly higher than the infections caused by drug-sensitive bacteria, varying from 42% to 100%. Nowadays, increasing use of antibiotics like cephalosporin has led to the emergence of a different group of β-lactamase genes that are compared with primary β-lactams (TEM-1, TEM-2, SHV-1). They have a higher activity spectrum, which can be described as general ESBLs which are wider than the first β-lactamases.[10] Therefore, the use of molecular

methods for the complete identification of these enzymes is important. The aim of this study was to identify ESBL-producing enzymes of *E. coli* using phenotypic methods and to evaluate TEM, SHV and CTX-M1 β-lactamase genes by polymerase chain reaction (PCRmethod in foodborne disease outbreaks.[11,12]

Materials and Methods

Within 9 months, 400 stool samples were collected from 100 foodborne outbreaks that had occurred across the country. Diarrheal samples of the patients with foodborne poisoning with symptoms of diarrhea, vomiting, nausea, abdominal cramps, fever and headache were taken.

Fifty *E. coli* isolates were identified via biochemical tests on colonies based on the following criteria: positive glucose fermentation, positive lactose fermentation, negative citrate, negative SH2, positive nitrate, methyl red positive (MR), Voges-Proskauer negative (VP), negative ureases, positive indole, and negative phenylalanine deaminase detected by using standard biochemical tests including the indole, methyl red, Voges-Proskauer, and citrate (IMViC) tests. All strains of *E. coli* were isolated from the samples that created a metallic mucilage colony on the Eosin Methylene Blue (EMB) agar (Merck, Germany) and stored in skim milk at -70°C until required for further tests.

Screening and Phenotypic Identification of ESBLs

According to the Clinical and Laboratory Standards Institute (CLSI), disk diffusion method was carried out using Muller-Hinton agar (pH 7.4 = 7.2) for the initial screening of *E. coli* isolates expressing ESBLs. Microbial suspensions with concentrations of 0.5 McFarland were consistently spread on the plates with Muller-Hinton agar medium. Antibiotic disks prepared by the company (Rosco Neo-Sensitabs, Denmark) included ampicilin (10 μg), azitromysin (15 μg), cotrimoxazole (25 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), meropenem (10 μg), gentamicin (10 μg), ceftriaxone (30 μg) and ceftazidime (30 μg) which were placed with distance of at least 2.5 cm from each other and incubated for 24 hours at 37°C. The inhibition zone was measured around each disc and compared to the CLSI standard. Resistant isolates of cephalosporin representatives were selected for confirmatory testing. Combined disk was

used for this purpose. Muller Hinton Agar medium was prepared. Microbial suspension with concentrations of 0.5 McFarland extended completely were diffused in the medium. Then, discs of ceftazidime (30 μg), ceftazidime-clavonic acid (10-30 μg), cefotaxime (30 μg), and cefotaxime-clavonic acid (10-30 μg) prepared from the company (Rosco Neo-Sensitabs Denmark) were placed with at least 2.5 cm from each other on the medium. After incubation for 24 hours at 37°C, an inhibition zone around the disk containing clavonic acid as an inhibitor of ESBL enzymes was measured relative to non-clavicic acid. If there was an increase in the zone of inhibition (ZOI) by ≥5 mm, strain was considered as an ESBL generator according to the CLSI standards.

Primer Design

Submitted sequences related to the *E. coli* TEM, CTX-M1, and SHV are present in GenBank, NCBI. All sequences were achieved and aligned using a MEGA 4 multiple-alignment program to identify analogs. The conserved sequences that had a high degree of homology were selected and used to design universal primers via the gene runner software (Table 1). Designed primers were evaluated using PCR as a final point.

PCR and Sequencing of the β-Lactamase Genes

For identification of the β-lactamase genes of SHV, CTX-M1, and TEM, genomic DNA of the strains were extracted according to the manufacturer's instructions (Bioneer Company). PCR process was followed on β-lactamase SHV (747 bp), CTX-M1 (593 bp) and TEM (445 bp) genes, using 3 pairs of universal primers under the following conditions: each PCR reaction contained 2.5 μL 10X buffer, 1μL 50 mM MgCl$_2$, 1 μL 10 mM dNTP, 1.5 μL 50 pmol primer each for the forward and reverse primers, 1 μL 5 U Taq DNA polymerase, 2 μL 50 pmol template DNA, and 14.5 μL H2O in a final volume of 25 μL. Markedly a mixture of template material was used as a negative control for SHV, CTX-M1 and TEM genes. In addition, *Klebsiella pneumoniae* ATCC 7881 , *E. coli* strain DF39TA and *E. coli* ATCC 25923 were used as positive controls for all genes. PCR was carried out under the following conditions: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and

Table 1. Primers used for amplification

Primer	Sequence (5′ to 3′ as Synthesized)	Expected Amplicon Size (bp)
Bla$_{SHV}$	F= 5'-ATGCGTTATATTCGCCTGTG-3' R = 5'-TGCTTTGTTATTCGGGCCAA-3'	747
Bla$_{TEM}$	F = 5'-TCGCCGCATACACTATTCTCAGAATGA-3' R = 5'-ACGCTCACCGGCTCCAGATTTAT-3	445
Bla$_{CTX-M}$	F = 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' R = 5'-TGGGTRAARTARGTSACCAGAAYCAGGG-3	593

elongation at 72°C for 1 minute. The final elongation step (72°C) occurred for 10 minutes. A multiplex PCR for the SHV and CTX-M1 genes was also performed, similar to the one abovementioned by adding both pairs of primers. Afterward, electrophoresis of PCR products was performed using a 0.8% agarose gel with 100 bp DNA ladder (Fermentas).

Results

Fifty *E. coli* were recognized from 400 stool samples that were collected from 100 foodborne outbreaks in the country. Achieved resistance pattern of isolates against 10 antibiotics is shown in Table 2. Their resistance pattern is shown in Figure 1.

The highest resistance was exposed to ampicillin (56%). Combined disk test identified 28 (56%) of isolates as ESBLs (Figure 2), from which 55.6% were from male stool and 44.4% from female stool. Provincial dispersion was as follows: Yazd had the highest rate with 7 (38.9%), Zanjan had 5 cases (27.8%), Sanandaj had 4 cases (22.2%), and Semnan had 2 cases (11.1%). Thirteen cases (72.2%) were urban and 5 cases (27.8%) were rural.

PCR data showed that among these isolates, 19(68%) were positive for bla_{CTX-M1}, 9(32%) for bla_{TEM} and 0(0%) for bla_{SHV} (Figure 3).

Discussion

Contaminated foods that possess *E. coli* with antimicrobial resistance genes can transiently inhabit the gastrointestinal tract for consequent infection.[13] *E. coli* is considered as an important foodborne pathogen that causes diarrhea both in children and adults.[14,15] The aim of this study was to investigate the prevalence of resistance genes which express ESBL enzymes. These genes are repeatedly associated with the plasmid-encoded mechanisms.[16] We focused on bla_{CTX-M}, bla_{SHV} and bla_{TEM} genes which are recognized to exhibit resistance to β-lactam antibiotics and are a major public health issue

Table 2. Pattern of Resistance Among 50 *Escherichia coli* Isolates Against 9 Antimicrobial Agents

Antibiotic	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
Ciprofloxacin	11 (22)	20 (40)	19 (38)
Cotrimoxazole	24 (48)	1 (2)	25 (50)
Nalidixic acid	25 (50)	6 (12)	19 (38)
Azithromycin	20 (40)	7 (14)	33 (66)
Meropenem	0 (0)	0 (0)	50 (100)
Ampicillin	28 (56)	1 (2)	21 (42)
Ceftazidime	2 (5)	7 (14)	41 (82)
Ceftriaxone	8 (20)	5 (10)	37 (70)
Gentamicin	0 (0)	0 (0)	50 (100)

worldwide.[17] The use of broad-spectrum beta-lactam antibiotics has increased rapidly along with resistance to these antibiotics.[18] Currently, there are several phenotypic tests recommended by the CLSI to detect ESBLs.[19] However, they are not sufficient for ultimate diagnosis of antibiotic resistant pathogens. In this study, recognizing ESBL-producing strains was performed according to the recommended tests by the CLSI. PCR was used to evaluate the prevalence of 13 lactamase enzymes and was successfully applied for the identification of ESBLs.[20] This method is useful for detection of foodborne outbreaks which require rapid detection to prevent broadening and spread of the disease and to control outbreak well timed. In this study we gathered all samples from foodborne outbreaks that had happened in the country and recognized the genes which played a significant role in creating the resistance to β-lactam antibiotics.

Tested samples showed high resistance to ampicillin. In the study conducted by Soltan Dallal et al, the frequency of broad-spectrum beta-lactamase genes of TEM, DHA, and MOX of 200 isolates of *E. coli* were tested and 74 (57%) of them were reported as having the TEM gene.[21]
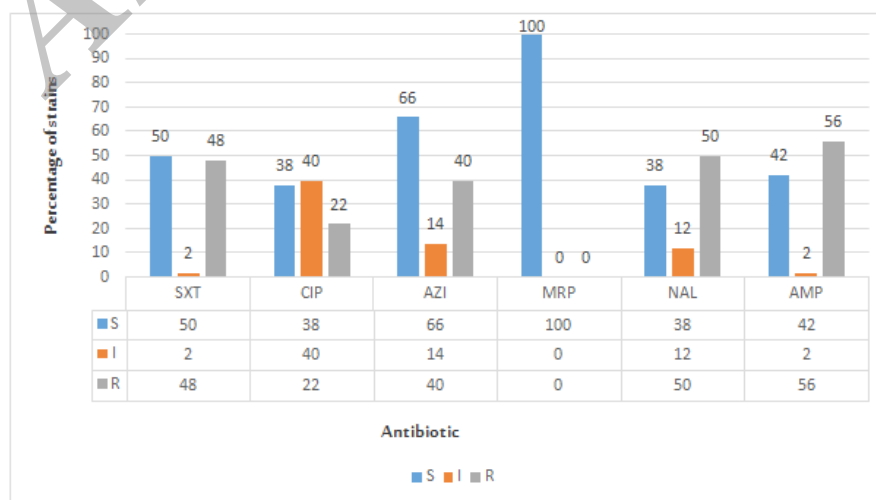

Figure 1. Pattern of Resistance Among *Escherichia coli* Isolates Against Antimicrobial Agents.
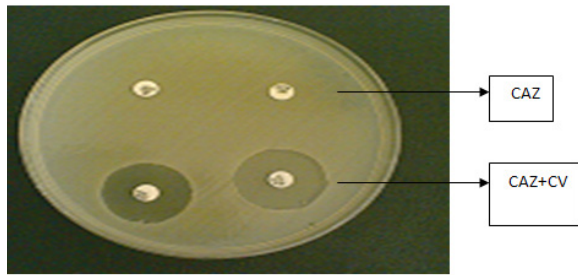
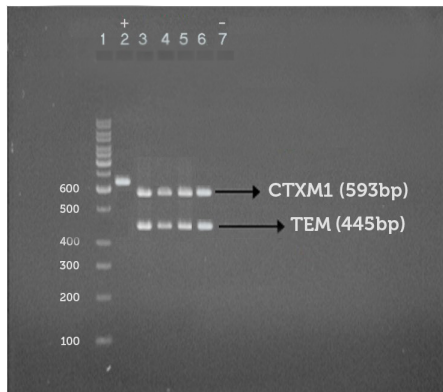**Figure 2.** Phenotypic Test for ESBL-Positive Isolates.

Figure 3. PCR Amplification. Lane 1: 100 bp DNA marker; lane 2: the positive control, lane: 3,4,5: bla_{TEM} and bla_{CTX-M} genes, clinical isolates that were negative for expression of the bla_{SHV} gene from clinical isolates, lane 7: negative control.

In another study, the molecular analysis of TEM broad-spectrum β-lactamase gene in *E. coli* isolated from clinical specimens indicated that out of 41 cases of isolated *E. coli* which were ESBL generators, 36 cases (87.8%) contained the TEM gene.[22-24] Based on these results, having accurate data about the resistance genes could be helpful in achieving a correct pattern for taking helpful antibiotics. Previous results on prevalence of gram-negative bacteria, generators of broad-spectrum beta-lactamases, have shown that ESBLs are rising rapidly.[25,26] In view of our survey, it was indicated that spread of ESBLs and their expression was associated with failures in treatment and increased mortality.

Conclusion

In order to control the proliferation of resistance genes in *E. coli* strains causing infection and to choose a rapid and appropriate treatment in emergency conditions like foodborne illnesses, it is suggested to gain more knowledge about the prevalence of different virulence genes in ESBL-producing *E. coli*. Therefore, molecular methods like PCR that has high sensitivity and specificity can be effective and helpful.

Authors' Contributions

MMSD and FF: concept and design of the study, critical revision, final approval of the study, and obtaining funding for the study. ZR and MKT: doing experiments. HAMA: writing of the manuscript.

References
1. Matsumura Y, Yamamoto M, Higuchi T, et al. Prevalence of plasmid-mediated AmpC beta-lactamase-producing *Escherichia coli* and spread of the ST131 clone among extended-spectrum beta-lactamase-producing *E. coli* in Japan. Int J Antimicrob Agents. 2012;40(2):158-162. doi:10.1016/j.ijantimicag.2012.04.013
2. Canton R, Gonzalez-Alba JM, Galan JC. CTX-M enzymes: origin and diffusion. Front Microbiol. 2012;3:110. doi:10.3389/fmicb.2012.00110
3. Dutta S, Guin S, Ghosh S, et al. Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. PLoS One. 2013;8(2):e56068. doi:10.1371/journal.pone.0056068
4. Khoshvaght H, Haghi F, Zeighami H. Extended spectrum betalactamase producing enteroaggregative *Escherichia coli* from young children in Iran. Gastroenterol Hepatol Bed Bench. 2014;7(2):131-136.
5. Soltan Dallal MM. Diarrhea caused by enteropathogenic bacteria in children. Arch Iran Med. 2001;4(4):201-203.
6. Azemi M, Ismaili-Jaha V, Kolgreci S, et al. Causes of infectious acute diarrhea in infants treated at pediatric clinic. Med Arch. 2013;67(1):17-21.
7. Bakhtiari R, Fallah Mehrabadi J, Molla Agamirzaei H, Sabbaghi A, Soltan Dallal MM. Evaluation of extended spectrum beta lactamase enzymes prevalence in clinical isolates of *Escherichia coli*. Microbiol Res. 2011;2(1):30-32. doi:10.4081/mr.2011.e8
8. Franiczek R, Sobieszczanska B, Turniak M, et al. ESBL-producing Escherichia coli isolated from children with acute diarrhea - antimicrobial susceptibility, adherence patterns and phylogenetic background. Adv Clin Exp Med. 2012;21(2):187-192.
9. Hamdy Mohammed el S, Elsadek Fakhr A, Mohammed El Sayed H, Al Johery SA, Abdel Ghani Hassanein W. Spread of TEM, VIM, SHV, and CTX-M beta-lactamases in imipenem-resistant gram-negative bacilli isolated from Egyptian hospitals. Int J Microbiol. 2016;2016:8382605. doi:10.1155/2016/8382605
10. Castanheira M, Farrell SE, Deshpande LM, Mendes RE, Jones RN. Prevalence of beta-lactamase-encoding genes among Enterobacteriaceae bacteremia isolates collected in 26 U.S. hospitals: report from the SENTRY Antimicrobial Surveillance Program (2010). Antimicrob Agents Chemother. 2013;57(7):3012-3020. doi:10.1128/aac.02252-12
11. Xia X, Meng J, Zhao S, et al. Identification and antimicrobial resistance of extraintestinal pathogenic *Escherichia coli* from retail meats. J Food Prot. 2011;74(1):38-44. doi:10.4315/0362-028x.jfp-10-251
12. Jakobsen L, Spangholm DJ, Pedersen K, et al. Broiler chickens, broiler chicken meat, pigs and pork as sources of ExPEC related virulence genes and resistance in *Escherichia coli* isolates from community-dwelling humans and UTI patients.

Int J Food Microbiol. 2010;142(1-2):264-272. doi:10.1016/j.ijfoodmicro.2010.06.025

13. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents. 2010;35(4):322-332. doi:10.1016/j.ijantimicag.2009.12.011

14. Turner PJ. Extended-spectrum beta-lactamases. Clin Infect Dis. 2005;41 Suppl 4:S273-275. doi:10.1086/430789

15. Wang L, Qu K, Li X, et al. Use of bacteriophages to control *Escherichia coli* O157:H7 in domestic ruminants, meat products, and fruits and vegetables. Foodborne Pathog Dis. 2017;14(9):483-493. doi:10.1089/fpd.2016.2266

16. Villa L, Garcia-Fernandez A, Fortini D, Carattoli A. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J Antimicrob Chemother. 2010;65(12):2518-2529. doi:10.1093/jac/dkq347

17. Ma J, Li N, Liu Y, et al. Antimicrobial resistance patterns, clinical features, and risk factors for septic shock and death of nosocomial *E coli* bacteremia in adult patients with hematological disease: a monocenter retrospective study in China. Medicine (Baltimore). 2017;96(21):e6959. doi:10.1097/md.0000000000006959

18. Zandi H, Tabatabaei SM, Ehsani F, Zarch MB, Doosthosseini S. Frequency of extended-spectrum beta-lactamases (ESBLs) in strains of *Klebsiella* and *E. coli* isolated from patients hospitalized in Yazd. Electron Physician. 2017;9(2):3810-3815. doi:10.19082/3810

19. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: Fifteenth Informational Supplement. 14th ed. Wayne, PA, USA: Clinical and Laboratory Standards Institute: 2005.

20. Zhu ML, Mai JY, Zhu JH, Lin ZL. [Clinical analysis of 31 cases of neonatal purulent meningitis caused by Escherichia coli]. Zhongguo Dang Dai Er Ke Za Zhi. 2012;14(12):910-912.

21. Soltan Dallal MM, Molla_Aghamirzaei H, Fallah Mehrabadi J, et al. Molecular detection of TEM and AmpC (Dha, mox) broad spectrum beta-lactamase in clinical isolates of Escherichia coli. Tehran Univ Med J. 2010;68(6):315-320.

22. Pornour M, Nahaei MR, Mobayen H, Mobasher A. Molecular study of TEM type extended-spectrum beta-lactamase genes in *Escherichia coli* and *Klebseilla pneumoniae* isolates. Med J Tabriz Univ Med Sci. 2010;32(2):4-30.

23. Li S, Qu Y, Hu D, Shi YX. Comparison of extended spectrum beta-lactamases-producing *Escherichia coli* with non-ESBLs-producing E. coli: drug-resistance and virulence. World J Emerg Med. 2012;3(3):208-212. doi:10.5847/wjem.j.1920-8642.2012.03.009

24. Tsui K, Wong SS, Lin LC, Tsai CR, Chen LC, Huang CH. Laboratory identification, risk factors, and clinical outcomes of patients with bacteremia due to *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum and AmpC type beta-lactamases. J Microbiol Immunol Infect. 2012;45(3):193-199. doi:10.1016/j.jmii.2011.11.003

25. Bayat Makoo Z, Binesh E, Hasani A, Naghili B, Alipour L. Study on prevalence of Extended-Spectrum Beta-Lactamase producing gram negative bacilli in clinical specimens isolated from hospitalized patients in Tabriz Sina hospital. Med J Tabriz Univ Med Sci. 2010;32(4):11-15.

26. Leylabadlo HE, Pourlak T, Bialvaei AZ, Aghazadeh M, Asgharzadeh M, Kafil HS. Extended-spectrum beta-lactamase producing gram negative bacteria in Iran: a review. Afr J Infect Dis. 2017;11(2):39-53. doi:10.21010/ajid.v11i2.6