Molecular Characterization of Antibiotic Resistance Associated with TEM and CTX-M ESBL in Uropathogenic E. coli Strains Isolated from Outpatients

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**KEYWORDS**

E. coli; urinary tract infection; ESBL; antibiotic resistance

**ABSTRACT**

**Background & Objective:** Escherichia coli (E. coli) is a leading cause of urinary tract infections becoming resistant against beta-lactams and cephalosporins through different mechanisms, including ESBL production due to the presence of ESBL specific genes, including blaCTX-M and blaTEM. The purpose of the present study was to detect the uropathogenic E. coli strains producing the ESBL.

**Methods:** A total of 100 isolates of uropathogenic E. coli were randomly selected in a period of 6 months and their resistances to a number of antibiotics including amoxicillin, amikacin, gentamicin, ciprofloxacin, ceftazidime, cefotaxime, ceftriaxone, cefotizoxime, nalidixic acid, and nitrofurantoin were determined. Then, DDT test was used to detect the presence of ESBL. Finally, the presence of blaCTX-M and blaTEM resistance genes was analyzed by PCR method.

**Results:** The resistance profile of bacterial isolates to the antibiotics was as follows: amoxicillin: 16.7%, amikacin: 7.8%, gentamicin: 20.3%, ciprofloxacin: 35.5%, ceftazidime: 35.0%, cefotaxime: 40.0%, ceftriaxone: 41.3%, nalidixic acid: 64.0%, nitrofurantoin: 9.7%, and cefotizoxime: 100%. Of these, 28 isolates (28%) were selected in a period of 6 months and their resistances to a number of antibiotics including amoxicillin, amikacin, gentamicin, ciprofloxacin, ceftazidime, cefotaxime, ceftriaxone, cefotizoxime, nalidixic acid, and nitrofurantoin were determined. Then, DDT test was used to detect the presence of ESBL. Finally, the presence of blaCTX-M and blaTEM resistance genes was analyzed by PCR method.

**Conclusion:** Regarding the production of ESBL by some E. coli isolates, phenotypic detection of ESBL-producing isolates is routinely suggested in the laboratories. Likewise, the treatment regimen should be selected regarding the ESBL production to avoid treatment failure.

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resistance against ceftazidime and cefotaxime (12, 13).

The TEM-1 enzyme was originally found in E. coli isolated from blood culture of a Greece patient called Temoniera and hence was named TEM (13). This enzyme caused resistance to penicillin and first generation cephalosporins such as cephalothin and cephaloridine (14). Various types of TEM beta-lactamases have been obtained through substituting amino acids in the activation site of TEM so that more than 130 types of TEM have been identified (15). The prevalence of some of these enzymes is different in various parts of the world (16). Today, the number of organisms producing TEM enzymes has been increased, while this has been considered as a crisis in the treatment of infections caused by these bacteria (17).

Since the prevalence of drug resistance is increasing and it has delayed the treatment of patients, especially those admitted to hospitals, the present research was performed to assess the detection and identification of ESBL-producing E. coli strains being resistant to beta-lactam antibiotics in Tehran's Baghiyatallah Hospital.

Material and Methods

Isolation of Bacteria from Urine Specimens
This was a cross-sectional descriptive study. In a period of 6 months, 100 urine samples were collected from patients referred to Baghiyatallah Hospital of Tehran, and cultured on selective media of Eosin Methylene Blue (EMB) agar. Then, the plates were incubated at 37°C for 24 hours. The colonies grown underwent biochemical tests using MRVP broth, Simon citrate agar, TSI agar, SIM agar, MacConkey agar as well as Urea broth media. Finally, the E. coli isolates were identified and the corresponding colonies were kept at -70°C in the Skim Milk medium to be used in the later stages.

Identification of ESBL-producing E. coli Strains
The pattern of antibiotic susceptibility was studied by disk diffusion (Kriby-Bauer method) according to the instructions of Clinical and Laboratory Standards Institute (CLSI) using antibiotic disks manufactured by MAST Company (UK) including cefotaxime (30 mg), ceftriaxone (30 mg), ceftazidime (30 mg), cefpod-oxime (30 mg), nalidixic acid (10 mg), cefitzoxime (30 mg), cefixime (30 mg), nitrofurantoin (10 mg), ami-kacin (10 mg), and gentamicin (10 mg) (18). In order to perform disk diffusion, at first microbial suspensions were prepared as 0.5 McFarland from 18-hour colonies and then cultured on the surface of Mueller-Hinton agar (Merck, Germany), using a sterile swab. After 15 minutes, antibiotic disks were placed on the surface of the media every 20 mm from each other. After 15 minutes, plates were incubated at 35 to 37°C for 16 to 18 hours. The diameter of inhibition zone was measured and interpreted according to the criteria of CLSI. Isolates with inhibition zone diameters of ≤22, ≤25, and ≤27 for ceftazidime, ceftriaxone, and cefotaxime, respectively, were evaluated for the presence of ESBL (18).

Phenotypic Confirmatory Test
To confirm ESBL production in candidate organisms, phenotypic confirmatory Double Disk Test (DDT) was used according to the CLSI command. The hybrid disks containing ceftazidime (30 mg) + clavulanic acid (10 mg) and cefotaxime (30 mg) + clavulanic acid (10 mg) were prepared from MAST Company. Afterward, the plates were incubated at 37°C for 24 hours. Then the diameter of inhibition zone was measured using a millimeter ruler and the results were interpreted according to the CLSI standards.

If the inhibition zone diameter of a colony around the combination disk was at least 5 mm bigger than the inhibition zone diameter of the single disk of the same antibiotic, it was considered as ESBL-producing isolate (18). In this test, the bacteria E. coli with the code of ATCC25922 and Klebsiella pneumonia with the code of ATCC700603 were used as ESBL positive and negative controls, respectively.

DNA Extraction and PCR
After performing the initial phenotypic confirmatory test, the samples containing resistant isolates were selected for DNA extraction. Boiling method was used for DNA extraction (19) and PCR reaction was done to detect beta-lactamase genes including bla-CTX-M and bla-TEM under the conditions presented in Table 1. The forward and revs primers used in the present study were designed using the Primer3 software and their specificity was determined by Primer-blast online software (http://www.ncbi.gov/tools/primer-blast). Moreover, to assure the quality of the primers, the Oligo analyzer program was used. The reaction mixture in a volume of 20 ml contained: 10 ml of master mix, 2 microliters of forward and revs primers (20, 21) (Table 2), 1 mL of extracted genome, and 7 mL of sterile distilled water.

Finally, electrophoresis of PCR products was performed in order to identify specific fragments with the sizes of 593bp (for blaCTX-M gene) and 867bp (for bla-TEM gene) on a 1.5% agarose gel with a size marker of 100 bp.

Results
The percentage of the identified isolates being resistant to the antibiotics are listed in Table 3.

Based on the results of disk diffusion screening test, 28 samples (28%) were resistant to cefotaxime, ceftazidime, and ceftriaxone, simultaneously. In DDT test, 21 cases were confirmed to be ESBL producing isolates.

According to the PCR results, the presence of blaCTX-M and blaTEM genes in 21 ESBL positive
isolates were 21% and 20% for mentioned genes, respectively (Figures 1 and 2). Additionally, 19 samples (19%) contained isolates harboring both of the genes.

Table 1. PCR condition for amplification of ESBL gens

| Step            | Temperature (˚C) | Time (min) | Cycle repeats |
|-----------------|------------------|------------|--------------|
|                 | **blaTEM**       | **blaCTX-M** |              |
| 1. Initial denaturation | 95               | 96         | 5            | 1             |
| 2. Amplification  | Denaturation      | 94         | 96           | 1             |
|                 | Annealing         | 52.2       | 60           | 1             |
|                 | Extension         | 72         | 72           | 1             |
| 3. Final Extension | 72               | 72         | 7            | 1             |

Table 2. The primers used for PCR

| Primer | 5’-sequence-3´ | Expected-size (bp) | Reference |
|--------|----------------|--------------------|-----------|
| TEM-F  | ATGAGTATCAACATTTCCG | 867                | 18        |
| TEM-R  | CTGACAGTTACCAATGCTTA |                |         |
| CTX-M-F | ATGTGCAGYACCAGTAARGT   | 593                | 19        |
| CTX-M-R | TGGGTRAARTARGTSACCAGA |                |         |

Table 3. Antibiotic resistance pattern for the examined antibiotics

| Antibiotic | Number of resistant isolates (%) |
|------------|----------------------------------|
| Amoxicillin| 2 (16.7)                         |
| Amikacin   | 4 (7.8)                          |
| Gentamicin | 12 (20.3)                        |
| Cephalexin | 5 (29.4)                         |
| Ceftazidime| 21 (35.0)                        |
| Ceftriaxone| 26 (41.3)                        |
| Cefotaxime | 4 (40.0)                         |
| Cefizoxime | 2 (100)                          |
| Nitrofurantoin | 6 (9.7)                        |
| Nalidixic acid | 32 (64.0)                     |
| Ciprofloxacin | 22 (35.5)                      |

**Fig. 1.** Electrophoresis of PCR products for **blaCTX-M** gene. Lane 1: size marker (100 bp DNA ladder); lane 2: negative control, lane 3: positive control for **blaCTX-M** gene; lanes 4-7: PCR positive samples (593 bp) for **blaCTX-M** gene; lane 8: negative sample with no band.

**Fig. 2.** Electrophoresis of PCR products for **blaTEM** gene. Lane 1: size marker (100 bp DNA ladder); lane 2: positive control for **TEM** gene; lanes 3, 4, 6, and 7: PCR positive samples for **bla-TEM** gene (867bp); lane 5: negative sample with no band; lane 8: negative control.
Discussion

Bacterial beta-lactamase genes, especially $ESBL$s genes are among the effective factors that increase their resistance to beta-lactam antibiotics such as broad-spectrum cephalosporins. $ESBL$ producing bacteria have created many health problems in recent years and novel methods are required to detect these bacteria in the clinical microbiology laboratories (21, 22). $ESBL$ phenotypic detection is an appropriate method for differentiation between $ESBL$-producing isolates and isolates that use other mechanisms of beta-lactam antibiotics resistance (23). In the present study, among the analyzed samples, 21 samples (21%) were $ESBL$ producers which indicate a high rate of $ESBL$-producing $E. coli$ isolates in patients with urinary tract infection. These isolates were susceptible to the clavulanic acid (a broad spectrum beta-lactamase) and one case was not susceptible to this antibiotic which could be due to the production of enzymes such as AmpC (24). The prevalence of $ESBL$ production in $E. coli$- and Klebsiella pneumoniae-positive samples is different in various countries; for example, in Korea, the prevalence of these organisms is in the range of 4.8%-7.5% and 22.5%-22.8% for $E. coli$ and Klebsiella pneumoniae, respectively. In India, the frequency of $ESBL$ production is 34.2% and 27.3% for $E. coli$ and Klebsiella pneumoniae, respectively (25). These results had no statistically significant difference compared to our results.

Several studies have indicated that $ESBL$ production by nosocomial and non-nosocomial $E. coli$ strains, has been quickly spread around the world, due to the emergence of $ESBL$ type $CTX$-$M$. Lewis et al., in 2007 using phenotypic tests and molecular techniques, examined $ESBL$ production on 94 urine samples of patients infected with uropathogenic $E. coli$ and reported that among the samples, the most common $ESBL$ type was $CTX$-$M$ (26).

In the present research, in line with the other studies, susceptibility to gentamicin in the isolates producing $CTX$-$M$ was more than that in the isolates producing $TEM$. Recent studies in Canada, Italy, Spain, Greece, and the United Kingdom revealed that $ESBL$ production in $E. coli$, especially those that produce $CTX$-$M$ have multiple resistances to trimethoprim, Sulfamethoxazole, tetracycline, gentamicin and ciprofloxacin (27).

Pitout et al. (2005) found that Enterobacteriaceae (like $E. coli$) isolated from urinary tract infections that produce $ESBL$ type $CTX$-$M$ are resistant to quinolones; in other words, resistance to quinolones is often associated with $CTX$-$M$ (27). This relationship is confirmed in our study as in the isolates having $CTX$-$M$, 11 cases with resistance to ciprofloxacin were observed while was not observed in any other type of $ESBL$s. Moreover, the isolates in our study had the characteristics of $ESBL$-mediated resistance because a high percentage of isolates were resistant to the third generation cephalosporins, including: 35%, 41.3%, 40%, 100%, 50%, and 66.7% for ceftazidime, ceftriaxone, cefotaxime, cefixime and cefpodoxime, respectively. In a number of countries such as Iran, cephalosporins are the antibiotics of choice using to treat UTIs and practitioners use these antibiotics in abundance.

In a study conducted by Pour Akbari et al. in 2012, 100 $E. coli$ strains isolated from UTI in patients aged 2 to 12 years underwent antibiotic susceptibility testing using disk diffusion method. Their results indicated a high percentage of susceptibility of $E. coli$ to antibiotics including amikacin and nitrofurantoin (95% and 91%, respectively) (28). Meanwhile, in another research in the United States, the sensitivity to antibiotics obtained as 90% (29). We found similar results in our study regarding the susceptibility to the two antibiotics, amikacin (72.6%), and nitrofurantoin (83.9%).

Conclusion

The use of amikacin as empiric therapy is a good choice for patients with urinary tract infection as well as the use of nitrofurantoin as an antibiotic to prevent urinary tract infections. Moreover, concerning the production of $ESBL$ by some isolates, phenotypic detection of $ESBL$-producing isolates is routinely suggested. The treatment should be selected regarding the $ESBL$ production to avoid treatment failure.

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None.

Conflict of Interest

The authors declared no conflicts of interest.

References

1. Zaniani FR, Meshkat Z, Nasab MN, Khaje-Karamadini M, Ghazvini K, Rezaee A, et al. The prevalence of TEM and SHV genes among extended-spectrum beta-lactamases producing Escherichia coli and Klebsiella pneumoniae. Iran J Basic Med Sci. 2012;15(1):654-60. [PMID] [PMCID]

2. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization,
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epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 2001;14(4):933-51. [DOI:10.1128/CMR.14.4.933-951.2001] [PMID] [PMCID]

3. Kim YK, Pai H, Lee HJ, Park SE, Choi EH, Kim J, et al. Bloodstream infections by extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in children: epidemiology and clinical outcome. Antimicrob Agents Chemother. 2002;46(5):1481-91. [DOI:10.1128/AAC.46.5.1481-1491.2002] [PMID] [PMCID]

4. Haj Ebrahim Tehrani F, Moradi M, Ghorbani N. Bacterial Etiology and Antibiotic Resistance Patterns in Neonatal Sepsis in Tehran during 2006-2014. Iran J Pathol. 2017;12(4):356-61. [DOI:10.30699/ijp.2017.27992] [PMID] [PMCID]

5. Livermore DM. beta-Lactamas in laboratory and clinical resistance. Clin Microbiol Rev. 1995;8(4):557-84. [DOI:10.1128/CMR.8.4.557] [PMID] [PMCID]

6. Tenover FC, Raney PM, Williams PP, Rasheed JK, Biddle JW, Oliver A, et al. Evaluation of the NCCLS extended-spectrum beta-lactamase confirmation methods for Escherichia coli with isolates collected during Project ICARE. J Clin Microbiol. 2003;41(7):3142-6. [DOI:10.1128/JCM.41.7.3142-3146.2003] [PMID] [PMCID]

7. Darvishi M, Forootan M, Nazer MR, Karimi E, Noori M. Nosocomial Infections, Challenges and Threats: A Review Article. Iran J Med Microbiol. 2020;14(2):162-81. [DOI:10.30699/ijmm.14.2.162]

8. Eliopoulos GM, Bush K. New β-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin Infect Dis. 2001;32(7):1085-9. [DOI:10.1086/319610] [PMID]

9. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamas: a clinical update. Clin Microbiol Rev. 2005;18(4):657-86. [DOI:10.1128/CMR.18.4.657-686.2005] [PMID] [PMCID]

10. Mirzae M, Pourmand M, Chitsaz M, Mansouri S. Antibiotic resistance to third generation cephalosporins due to CTX-M-Type extended-spectrum β-lactamases in clinical isolates of Escherichia coli. Iran J Public Health. 2009;38(1):10-7. [Article]

11. Pallecchi L, Bartoloni A, Fiorelli C, Mantella A, Di Maggio T, Gamboa H, et al. Rapid dissemination and diversity of CTX-M extended-spectrum beta-lactamase genes in commensal Escherichia coli isolates from healthy children from low-resource settings in Latin America. Antimicrob Agents Chemother. 2007;51(8):2720-5. [DOI:10.1128/AAC.00026-07] [PMID] [PMCID]

12. Chen Y, Delmas J, Sirot J, Schoiehet B, Bonnet R. Atomic resolution structures of CTX-M beta-lactamas: extended spectrum activities from increased mobility and decreased stability. J Mol Biol. 2005;348(2):349-62. [DOI:10.1016/j.jmb.2005.02.010] [PMID]

13. Gupta V. An update on newer beta-lactamas. Indian J Med Res. 2007;126(5):417-27. [PMID]

14. Mlynarczyk G, Mlynarczyk A, Bilewska A, Dukaczewska A, Golawski C, Kicman A, et al. [High effectiveness of the method with cefpirome in detection of extended-spectrum beta-lactamas in different species of gram-negative bacilli]. Med Dosw Mikrobiol. 2006;58(1):59-65. [PMID]

15. Zaman zad B, Deyham B, Nafisi M, Karimi A, Farokhi E. The Frequency of TEM-1 Gene in Extended Spectrum Beta Lactamas Producing Escherichia coli, Klebsiella pneumoniae and Enterobacter Strains Isolated from Hospital Clinical Samples Using PCR. Sci J Hamadan Univ Med Sci. 2008;14(4):19-25.

16. Perilli M, Dell’Amico E, Segatore B, de Massis MR, Bianchi C, Luzzaro F, et al. Molecular characterization of extended-spectrum beta-lactamas produced by nosocomial isolates of Enterobacteriaceae from an Italian nationwide survey. J Clin Microbiol. 2002;40(2):611-4. [DOI:10.1128/JCM.40.2.611-614.2002] [PMID] [PMCID]

17. Haghi F, Zeighami H, Keramati N, Hemmati F, Hajiahmad F. Frequency of TEM extended spectrum beta lactamase producing Escherichia coli in clinical specimens by phenotypic and molecular methods in Zanjan. Zanjan Univ Med Sci. 2013;21(85):55-63.

18. CLSI. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 21st Informational Supplement. M100-S21. CLSI Wayne, PA; 2011. [Link]

19. Queipo-Ortuño MI, Colmenero JDD, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. Clin Vaccine Immunol. 2008;15(2):293-6. [DOI:10.1128/CVI.00270-07] [PMID] [PMCID]

20. Oliver A, Weigel LM, Rasheed JK, McGowan JE, Jr., Raney P, Tenover FC. Mechanisms of
decreased susceptibility to cefpodoxime in Escherichia coli. Antimicrob Agents Chemother. 2002;46(12):3829-36. [DOI:10.1128/AAC.46.12.3829-3836.2002] [PMID] [PMCID]

21. Cavallo JD, Leblanc F, Fabre R, Fourticq-Esqueoute A, Group d'Etude le la Resistance de PaaB. [Survey of the antibiotic sensitivity of Pseudomonas aeruginosa in France and the distribution of beta-lactam resistance mechanisms: the GERPB 1999 study]. Pathol Biol (Paris). 2001;49(7):534-9. [DOI:10.1016/S0369-8114(01)00213-9]

22. Rupp ME, Fey PD. Extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae: considerations for diagnosis, prevention and drug treatment. Drugs. 2003;63(4):353-65. [DOI:10.2165/00003495-200363040-00002] [PMID]

23. Pitout JD, Hossain A, Hanson ND. Phenotypic and molecular detection of CTX-M-beta-lactamases produced by Escherichia coli and Klebsiella spp. J Clin Microbiol. 2004;42(12):5715-21. [DOI:10.1128/JCM.42.12.5715-5721.2004] [PMID] [PMCID]

24. Bell JM, Chitsaz M, Turnidge JD, Barton M, Walters LJ, Jones RN. Prevalence and significance of a negative extended-spectrum beta-lactamase (ESBL) confirmation test result after a positive ESBL screening test result for isolates of Escherichia coli and Klebsiella pneumoniae: results from the SENTRY Asia-Pacific Surveillance Program. J Clin Microbiol. 2007;45(5):1478-82. [DOI:10.1128/JCM.02470-06] [PMID] [PMCID]

25. Akram M, Shahid M, Khan AU. Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in JNMC Hospital Aligarh, India. Ann Clin Microbiol Antimicrob. 2007;6(1):4. [DOI:10.1186/1476-0711-6-4] [PMID] [PMCID]

26. Lewis JS, Herrera M, Wickes B, Patterson JE, Jorgensen JH. First report of the emergence of CTX-M-type extended-spectrum beta-lactamases (ESBLs) as the predominant ESBL isolated in a US health care system. Antimicrob Agents Chemother. 2007;51(11):4015-21. [DOI:10.1128/AAC.00576-07] [PMID] [PMCID]

27. Pitout JD, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. J Antimicrob Chemother. 2005;56(1):52-9. [DOI:10.1093/jac/dki166] [PMID] [PMCID]

28. Pourakbari B, Ferdosian F, Mahmoudi S, Teymuri M, Sabouni F, Heydari H, et al. Increase resistant rates and ESBL production between E. coli isolates causing urinary tract infection in young patients from Iran. Braz J Microbiol. 2012;43(2):766-9. [DOI:10.1590/S1517-83822012000200041] [PMID] [PMCID]

29. Mohammadi-Mehr M, Feizabadi M. Antimicrobial resistance pattern of Gram-negative bacilli isolated from patients at ICUs of Army hospitals in Iran. Iran J Microbiol. 2011;3(1):26-30. [Article]