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MOLECULAR CHARACTERIZATION OF TEM EXTENDED SPECTRUM BETA LACTAMASES IN URINE CULTURE IN ALZAHRA HOSPITAL IN ISFAHAN

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ABSTRACT

Extended-spectrum beta-lactamases (ESBL) enzymes are an important resistance mechanism in gram-negative bacteria. Diagnosis of such resistance would seem necessary in order to adopt new therapeutic measures.

In this study, 150 bacteria isolated from urine specimens of hospitalized patients and 100 bacteria isolated from outpatients were sent to the laboratory of Isfahan Al-Zahra Hospital. The specimens were studied individually. Kirby - Bauer Method was used to determine sensitivity of bacteria to antibiotics. All resistant isolates to cefotaxime were evaluated by DoubleDisk Synergy test in order to confirm ESBL production. Colony PCR was used to detect TEM gene.

8 cases produced ESBL among 43 E.coli isolates (18.6%) resistant to cefotaxime (18%), 6casesproduced ESBL among 10Acetobacterisolates (60%),19 cases produces ESBL among 23

klebsiella isolates (82.6%), 2 cases produced ESBL among 32 Pseudomonas aeroginosa isolates (6.25%) and 5 cases produced ESBL among 7 Enterobacter isolates (71.42%) based on double disk synergy tests. The ESBL-producing bacteria (based on double disk synergy tests) were studied in molecular terms with PCR. PCR products were sequenced. The results of all sequences confirmed presence of TEM and authenticity of PCR.

It is essential to diagnose these infections using molecular and phenotypic methods and control antibiotics doses.

Keywords: TEM, Extended-spectrum beta-lactamases (ESBL), urinary tract infection, Kirby-Bauer, double disk synergy

INTRODUCTION

Urinary tract infection is one of the most common nosocomial and society-acquired infections. Resistance to antibiotic treatment in patients with urinary tract infection is an example of development of antimicrobial resistance problem. Recent data has shown that urinary tract infection is caused by ESBL-producing bacteria, which has infected many individuals around the world. ESBL enzymes are encoded by different genes located on either chromosome or plasmid (1).Among various types of antibiotic resistance produced by bacteria, those bacteria with beta lactamase capabilityare clinically important because these bacteria are effective against third generation cephalosporins, especially ceftazidime,cefotaxime and cefpodoxime. This group of bacteria are called expanded spectrum of β-ESBLs (2). For the first time in 1983, Knotle *et al.* described transferable resistance tobroad-spectrum cephalosporins in clinical samples of *Serratia marcescens* and *Klebsiella pneumoniae* (3). Paterson *et al.* examined the prevalence of ESBL from 1983 to 2004. The relevant results indicated an increase in ESBL-producing strains during these years (4). The prevalence of hospital-acquired infections caused by ESBL-producing bacteria was first reported in France (5) and Germany (6) in 1985followed by other cases in America in early 1990, which was reported in the late 1980s. (7) The prevalence of ESBL-producing *klebsiella pneumoniae* was studied and compared in various hospitals in a study in France from 1988 and 1990(8). Many TEM and SHV-type ESBL enzymes in Enterobacteriaceae strains such as Klebsiella were detected by 1990s. Then, CTX-M-producing E.coli was detected (9).

Isolation of *Klebsiella pneumoniae* strains from urinary tract infections and respiratory diseases were reported in numerous studies in Iran. High frequency ESBL producing *Klebsiella pneumoniae*(22%) were isolated from the patients with urinary and respiratory infections in Tehran from 2003 to 2004.

TEM and SHV plasmids were tested and isolated from *E.coli* and klebsiella samples in a study in Iran University of Medical Sciences from 2005 to 2007. CTX-M ESBL-producing gram-negative bacteria specimens(with high frequency of ESBL production)were isolated from urinary tract infections among which E.coli produced ESBL more than other bacteria in a study in Kurdistan (9).

ESBL enzymes emerged as an important mechanism of resistance in Gram-negative bacteria. Detecting resistance to ESBL seems necessary in order to adopt new therapeutic methods. In this study, two groups of outpatients and the patients diagnosed with urinary tract infection in Isfahan Al-Zahra hospital were examined individually in terms of production of ESBL enzymes.

MATERIALS AND METHODS

Time of implementation

In this study, 250 urinary tract pathogens were taken from Al-Zahra Hospital in Isfahan from October 2012 to May 2014 and were studied individually.

Location of sample collection

In this study, 250 urine samples (150 samples from hospitalized patients and 100 samples from outpatients) were sent to Al-Zahra hospital laboratory and were studied individually. The samples were cultured in Al-Zahra hospital laboratory. They strains of urinary infection pathogens were detected and transferred to the Research Laboratory in Islamic Azad University in Falavarjan.

Determining sensitivity of bacteria to antibiotics

The antibiotics used in this study were prepared from PadtanTeb Corporation and included streptomycin (10µg), nalidixic acid (30µg), gentamicin (10µg), ciprofloxacin (5µg), chloramphenicol (10µg), cefotaxime (30µg), amoxicillin (30µg), sulfamethoxazole (10µg) and ceftazidime (30µg).(10)

Kirby-Bauer Method was used to determine sensitivity of bacteria. The specimens were cultured in Mueller Hinton broth with 5.0 McFarland turbidity. Then, agar was densely inoculated using a sterile swab over the surface of Mueller Hinton broth. Antibiotic discs were placed on surface of the medium

with an appropriate distance between the disks after 15 minutes. Culture medium was kept at 37°C for 24 h. Then,growth inhibition zone was measured in millimeters. The results were reported as sensitive, semi-sensitive and resistant based on standard tables (11,12).

Determination of ESBL enzymes

All isolates resistant to cefotaxime were evaluated to confirm ESBL production. Double disk synergy test was used to detect ESBL. The presence of ESBL was examined using combined disks of cefotaxime and cefotaxime with clavulanic acid antibiotics (10µg/30µg). The specimens were cultured in Mueller Hinton broth with 5.0 McFarland turbidity. Then, agar was densely inoculated using a sterile swab over the surface of Mueller Hinton broth. Antibiotic discs were placed on surface of the medium with an appropriate distance between the disks after 15 minutes. The cultured plates were incubated at 37°C from 18h to 24h. Then, diameter of growth inhibition zone around each disk was measured. The results were compared with each other. Diameter of growth inhibition zone less than or equal to 3 mm to 5 mm indicated ESBL isolates (13).

Primer Synthesis

Homology of genes were examined with BLAST server and Gene runner software was used to design following protected primers with Ftem 5'-TAAAgTTCTgCTATgTggCg-3'and Rtm5'-ATCgTTgTCAgAAgTAAgTTg-3' sequences. The primers were synthesized.

Colony PCR

In Colony PCR Technique, a small part of colony is added to 5.19-microliter di-ionized water and placed in thermocycler for 5 minutes at 95°C. Then, Master Mix is prepared, which included1-macro liter dNTp, 1.5 macro liter $MgCl_2$,5 macro liter PCR buffer, 2 macro liter dose for each primer, 0.4 macro liter Taq polymerase enzyme and chromatography water. The Master Mix was divided equally into the tubes. Then, DNA specimens were added to the tubes. The tubes were placed in thermocycler. PCR program was regulated for 35 cycles of DNA production including 95°C in 5 minutes for the first denaturation, 94°C in 30 seconds for denaturation, 55°C in 3 minutes for annealing,72°C in 1 minute for extension and 72°C in 10 minutes for the last extension. PCR products were electrophoresed on 1% agarose with 100bp markers. Then, PCR products were sequenced.

Statistical analysis method

Data analysis was performed using SPSS version 20. Data normality was assessed

with Q-Q plots at first. Then, statistical test of equality of variances (Levene)was used to check equality of means. Then, the t-test for two independent samples was used.

RESULTS

Among 150 urine samples of the patients hospitalized in Al-Zahra Hospital in Isfahan, 26 *E.coli* bacteria, 15 klebsiella, 28 *Pseudomonas aeruginosa*, 51 Staphylococcus, 7 Enterobacter and 23 Acetobacter cases were detected.

Among 100 urine specimens of the outpatients who visited Al-Zahra Hospital in Isfahan, 23 *E.coli* bacteria, 18 klebsiella, 23 *Pseudomonas aeruginosa*, 2 Staphylococcus, 13Acetobacter and 12 Enterobacter cases were detected.

Only gram-negative bacteria were isolated from the specimens sent to Flavarjan Research Laboratory. The remaining tests were performed on these bacteria. Antimicrobial resistance pattern of gram-negative bacteria against different antibiotics from hospitalized patients in table 1 and from outpatients in table 2 has been shown.

Bacteria were resistant to cefotaxime among gram-negative bacteria isolates in outpations and hospatalezed pations were shown in **Figure 1**.

Figure 2 Shows resistant to ceftazidime among gram-negative bacteria isolates in outpations and hospatalezed pations**.**

Table 1: Antimicrobial resistance pattern of gram-negative bacteria against different antibiotics from hospitalized patients

	streptomycin	**nalidixic acid**	**gentamicin**	**ciprofloxacin**	**chloramphenicol**	**cefotaxime**	**amoxicillin**	**sulfamethoxazole**	**ceftazidime**
E.coli	26	26	24	21	13	22	22	19	21
Acentobacter	23	23	17	10	7	8	14	23	5
Klebsiella	15	15	13	-	11	10	15	15	10
P.aeroginosa	28	28	27	27	23	21	28	28	22
Enterobacter	7	7	1	1	3	4	2	-	-

Table 2: Antimicrobial resistance pattern of gram-negative bacteria against different antibiotics from outpatients

	streptomycin	**nalidixic acid**	**gentamicin**	**ciprofloxacin**	**chloramphenicol**	**cefotaxime**	**amoxicillin**	**sulfamethoxazole**	**ceftazidime**
E.coli	32	26	28	23	10	21	27	24	18
Acentobacter	13	13	11	5	3	2	**8**	13	4
Klebsiella	14	15	15	12	9	13	18	18	10
P.aeroginosa	18	18	16	15	10	11	18	18	15
Enterobacter	12	12	3	3	2	3	3	-	-

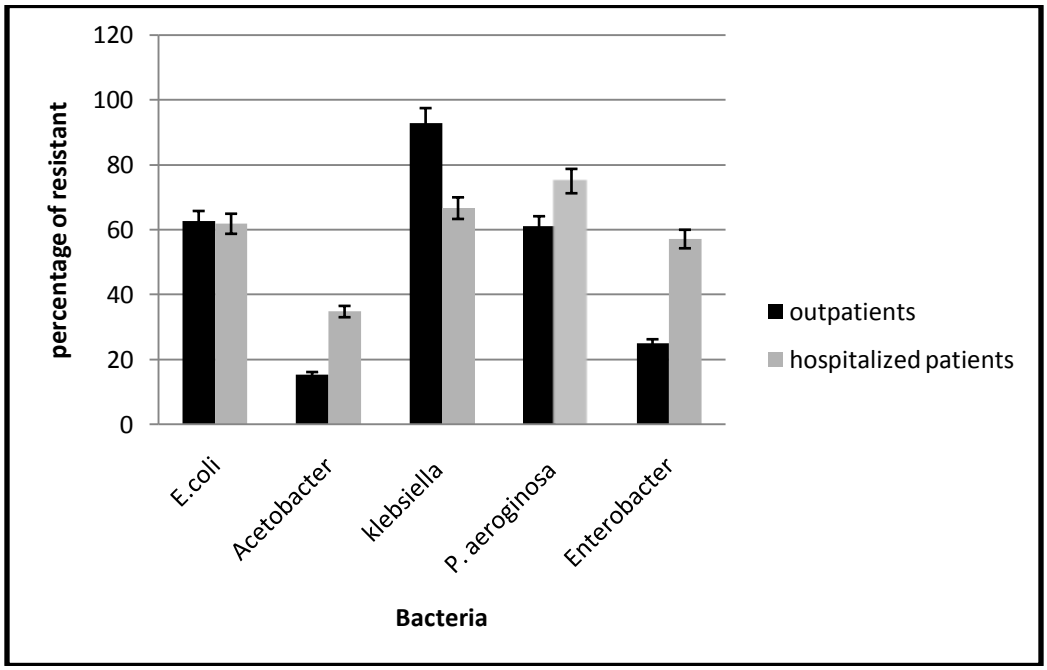

Figure1: Bacteria were resistant to cefotaxime

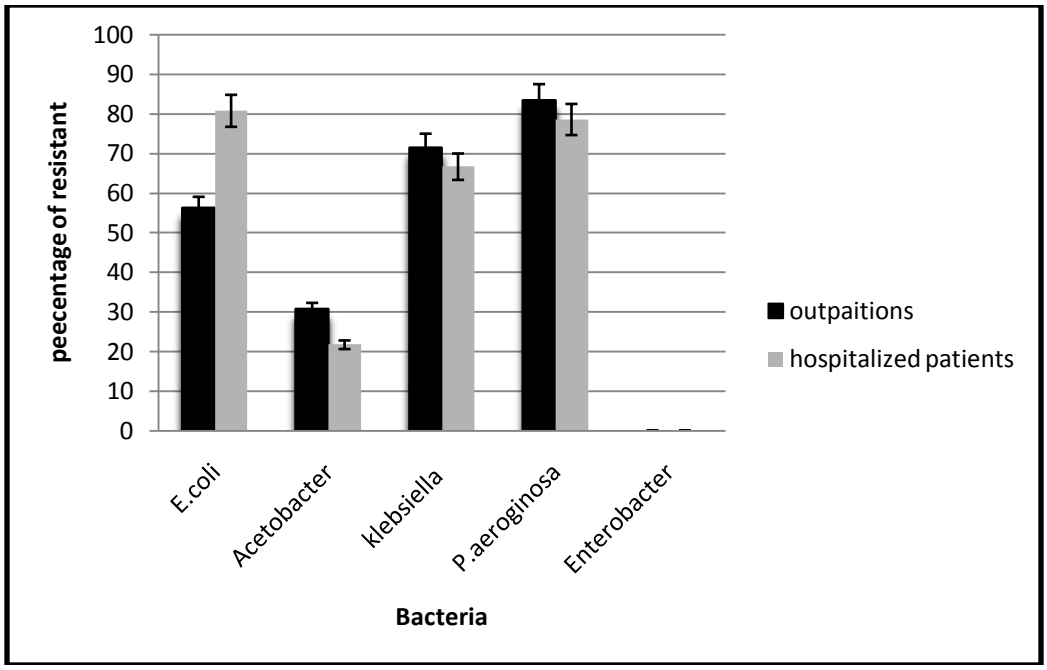

Figure 2: Bacteria were resistant to ceftazidime

No resistant case to ceftazidime was detected among Enterobacteria isolated from urine samples of both outpatients and hospitalized patients.

Following cases were resistant to cefotaxime among isolates from both

Antibiotic resistance patterns were examined in gram-negative bacteria (causes of urinary tract infections) isolated from outpatients and hospitalized patients in Isfahan Al-Zahra Hospital. Those bacteria resistant to cefotaxime were isolated and individually examined by double disk synergy tests. The bacteria with a difference between diameters of growth inhibition zone less and equal to 5mm were detected and isolated for molecular test.

18.6% *E.coli* isolates, 60% Acetobacter isolates, 82.6% klebsiella isolates, 6.256% *Pseudomonas aeroginosa* isolates, 71.42% Enterobacter isolates produced ESBL among isolates based on double disk synergy test

The ESBL-producing bacteria were studied in molecular terms. PCR was performed on these bacteria. PCR products were sequenced. The results confirmed presence of TEM and authenticity of PCR.

DISCUSSION

Unfortunately, indiscriminate application of antibiotics in recent decades has increased emergence of multi-drug resistant strains in infectious bacteria (14). These bacteria are resistant to such antibiotics as penicillin and cephalosporin by producing ESBL enzymes.(15) This type of resistance is more

frequent in Iran than other countries due to indiscriminate application of beta-lactam antibiotics. Thus,it is essential to identify and detect the organisms that produce beta-lactamase enzymes (16).

In this study, the *Escherichia coli* strains isolated from hospitalized patients and outpatients were resistant to sulfamethoxazole by 74.13%. In another study conducted in Russia, bacterial resistance to these antibiotics was 27% (17). In this study, *E. coli* resistance to ciprofloxacin was 75.86%. In an other study conducted in Taiwan in 2005, *Escherichia coli* strains isolated from nosocomial infections were resistant to ciprofloxacin by 37.3% (18). In the present study, *E. coli* resistance to cefotaxime and ceftazidime were respectively as 74.13% and 67.24%. However, *E.coli* resistance to these antibiotics was respectively obtained as 5% and 19% in a study conducted in Russia (16).Nevertheless, *E.coli* resistance to these antibiotics was reported as 14.6% in a study conducted in Brazil (19).

In this study, 18.6% *E. coli* cases, 60% Acinetobacter cases, 82.6% Klebsiella cases,6.25% *P. aeruginosa* cases and 71.42% Enterobacter cases were tested positive for ESBL.

Soltan Dala *et al.* conducted a study on 200 *E.coli* isolates in Tehran in 1999 in which 115 (89.8%) cases produced ESBL (20). Shah cheraghi *et al.* also conducted a study in Pasteur Institute in Iran in 2007. They showed that 105 clinical cases (52.5%) were test positive for ESBL among 200 E. coli isolates. Mirsalehian *et al.* conducted another study on enterobacter isolates from clinical samples at three hospitals and examined 33 E. coli isolates in which 20 cases (61%) were ESBL positive (21). Shah *et al.* also showed that ESBL producing isolates in different countries varies from 0 to 40% (22). BehzadianNejad *et al.* conducted another study on 280 clinical samples in Tehran and showed that 40 isolates were ESBL positive with phenotypic methods (23).Another study was conducted on Enterobacteriaceae in various hospitals in three cities in South Koreain 2005. In this study, 41 isolates of five strains of *Klebsiella pneumoniae*, *Escherichia coli*, c*itrobacter freundii*, enterobacter and *Serratia marcescens* were ESBL positive (24).Behrouzi *et al.* conducted an other study in Milad Hospital in 2009 and reported that *Klebsiella pneumoniae* produced ESBL with 12%frequency (25). Ling *et al.* conducted a study in China and reported that *E.coli* produced ESBL with 16% frequency (26).

The difference between these results is due to differences in patterns of antibiotic use, region, differences in resistance patterns in

different regions and indiscriminate use of antibiotics in our country. High resistance of the strains resistant to some antibiotics represents undue consumption of antibiotics in our country.

Significance test of mean of all bacteria isolated from urine samples in hospitalized patients and outpatients (t-test) showed no difference between means of bacteria isolated from hospitalized patients and outpatients. There was also no statistically significant difference between bacterial resistance to all antibiotics among Gram-negative bacteria isolated from urine samples of hospitalized and outpatients.

CONCLUSION

According to the study, it can be stated that the ESBL-producing genes are frequent among Gram-negative bacteria. It is essential to detect ESBL production in laboratory carefully given high level of bacterial resistance to beta-lactam antibiotics. Understanding mechanisms of resistance to antibiotics by physicians and using beta-lactam inhibitor antibiotics effectively reduce frequency of resistance to antibiotics.

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