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Determination of the Pattern of Antibiotic Resistance and Investigation of Extended-Spectrum Beta-Lactamase Production of Enterobacteriaceae Isolates of Clinical Specimens

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Abstract

***Background*:** Extended-spectrum beta-lactamase (ESBLs) producing bacteria are issued as a serious problem considering their ability to hydrolyze most of beta-lactam antibiotics. The outbreak of infections derived by ESBL-producing enterobacteriaceae is increasing throughout the world. Therefore, this study aims to determine a pattern of antibiotic resistance and investigate the extended-spectrum beta-lactamases production of enterobacteriaceae isolates separated from clinical specimens.

***Materials and Methods*:** In this study, 170 various strains of enterobacteriaceae isolated from clinical specimens in teaching hospitals of Arak cultured and identified applying standard methods during one year (2010-2011). The antibiotic resistance of isolates was investigated through disk Agar diffusion according to CLSI criteria. The resistant isolates against ceftazidime and cefotaxime antibiotics were studied through the combined disk test for the final confirmation of ESBL-production. The minimum inhibitory concentration was determined through micro broth dilution.

***Results*:** In this study, the resistance rate of various strains of enterobacteriaceae against amoxiclav, cefotaxime, ceftriaxone, ceftazidime, cefoxitin, cefotetan, meropenem and imipenem were respectively, 91.1%, 70%, 68.8%, 62.9%, 28.2%, 11.1%, 11.1% and 1.7%. Among 125 resistant enterobacteriaceae isolates against ceftazidime or cefotaxime, 108 isolates (86.4%) had ESBL-positive phenotype and 17 isolates (13.6%) had ESBL-negative phenotype. The MICs of the resistant isolates were indicated within a range of 16 to 512 µg/ml for ceftazidime and 64 to 512 µg/ml for cefotaxime.

***Conclusion*:** According to the results of this study, imipenem is the most effective antimicrobial antibiotic. On the other hand, the present study indicates that the bacteria within the family of ESBL producing enterobacteriaceae are highly prevalent among the patients. The increase in rate of such cases is often resulted by irrational antibiotic prescription. Application of new antimicrobials, limitation of the use of antimicrobial factors and increasing the utilization of infection control tools are all required in order to solve this problem.

Introduction

Antibiotic resistance is considered as a basic problem in treatment and infection control. During recent years extended-spectrum beta-lactamases have been extremely prevalent throughout the world. The *Enterobacteriaceae* family is the dominant pathogen for urinary tract infection. 30 to 40% of septicaemias and over 70% of urinary infections and many intestinal infections are caused by the bacteria of this family. The wide use of antimicrobial drugs during the recent decades has created resistant strains against them. Beta-lactamases are the most prevalent factors which create resistant bacteria against beta-lactam antibiotics [1, 2].

Extended-spectrum beta-lactamase-producing bacteria are issued as a basic problem in medical societies due to their ability to hydrolyze the majority of beta-lactam antibiotics [3]. Since identification of these enzymes in 1983, we have observed their extreme prevalence throughout the world caused by their fast propagation. So far, more than 150 types of ESBL are reported from all over the world, most of which are isolated from bacteria of *Enterobacteriaceae* family [4].

Today, as a consequence of the ongoing increase in ESBL-producing organisms, we see many reports indicating its vast prevalence among clinical specimens. This is frequently caused by irregular consumption of extended-spectrum beta-lactam drugs. TEM-1 is the first plasmid related to the beta-lactamase in gram-negative bacteria which was obtained from the Escherichia coli isolates isolated from the patients' blood culture in Greece in 1960 [5]. Bush, Jacoby and Medeiros classified ESBLs in four main groups (A, B, C and D) [6], which are mainly restrained by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. These

enzymes are usually coded by large plasmids (100 kb) which are transferable between one bacterial strain and another one or other bacterial types. ESBLs are produced through a mutation or after it during the substitution of amino acids specially in the active position of SHV and TEM beta-lactamases and this major structural changes promote the beta-lactamase activities of these mutants into a third generation of cephalosporin [3].

The preliminary screening for the reduced sensitivity toward the suggested antibiotic through disk agar diffusion test is the best method for the identification of extended-spectrum beta-lactamases (CLSI: Clinical and Laboratory Standards Institution) and performance of combined disk confirmatory tests in order to prove the effect of synergism between a cephalosporin marker and a beta-lactamase inhibitor comes next [3].

Therefore, this research investigates and evaluates the antibiotic resistance pattern and the production of extended-spectrum beta-lactamases by the enterobacteriaceae isolates isolated from clinical specimens.

Materials and Methods

In this descriptive-analytical research, 170 strains of *Enterobacteriaceae* were isolated from clinical specimens (urine, blood, trauma, etc) in teaching hospitals of Arak during one year (May, 2010- May, 2011). The separated isolates included *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter cloacae* and *Citrobacter freundii*. After the culturing and identification based on differential tests, they were identified applying biochemical and diagnostic standard methods related to *Enterobacteriaceae* family [7]. The *Enterobacteriaceae* isolates were then lyophilized and kept in a -20ºC holding environment (glycerol-containing culturing environment) for further tests. The preliminary screening of the ESBL-producing organisms was performed through disk agar diffusion method according to CLSI criteria. In this method, the microbial suspension was provided in 0.5 McFarland turbidity after providence of a Mueller-Hinton agar medium.

This suspension was spread in the Mueller-Hinton agar environment by a sterile swab, then regarding the standard space, amoxiclav (AC: 30 µg), cefotaxime (CTX: 30 µg), ceftriaxone (CTR: 10 µg), ceftazidime (CAZ 30 µg), cefoxitin (CX: 30 µg), cefotetan (CTN: 30 µg), meropenem (MPR: 10 µg) and imipenem (IMP: 10 µg) (provided by Mast group. Ltd; UK) antibiotic disks were cultured in the environment. After 24 hour incubation in 35ºC, the halos of non-growth surrounding each disk was measured by a ruler and the resistance and sensitivity of isolates was investigated by a standard table. In order to investigate the presence of extended-spectrum beta-lactamases in isolated strains from clinical specimens the combined disk method was applied using cefazidime & cefazidime-clavulanic acid and cefotaxime & cefotaxime- clavulanic acid disks (provided by Mast group. Ltd; UK) [8]. Firstly, the Mueller-Hilton agar medium was provided, then a 0.5 McFarland turbidity bacterial suspension was utterly spread in the surface mentioned environment, finally, cefazidime & cefazidime-clavulanic acid and cefotaxime & cefotaxime-clavulanic acid disks were placed there with a minimum distance of 20 mm. After 24 hours incubation in 35ºC, the diameter of the obtained halos of non-growth was measured by a millimetre ruler and the diameters equal or bigger than 5 mm were determined as ESBL-producing bacteria for each single cefalosporine or cefalosporines with clavulanic acid according to CLSI criteria. For the quality control in this test, *Klebsiella pneumonia* (ATCC700603) and *Escherichia coli* (ATCC25922) were applied as positive and negative controls respectively [9].

MIC (Minimum Inhibitory Concentration) test was performed on the resistant ESBL positive specimens against cefazidime or cefotaxime applying Micro-broth dilution method. 512, 4, 8, 16, 32, 64, 128 and 256 µg/ml delusions of pure antibiotic powder were provided for cefazidime and cefotaxime (provided by Mast group. LTD; UK). 2X Mueller-Hilton Broth medium (German, Merck) was applied as the culturing environment and 1.25 ml sodium chloride solution ($CaCl_2.2H_2O$) and 2.5 ml magnesium chloride solution ($MgCl_2.6H_2O$) was added per each 1000 ml.

A bacterial suspension with a concentration of 1×10^6 CFU/ml was provided as well an equal amount of which was added to each unit. It was incubated in 37ºC for 24 hours. The minimum concentration in which no turbidity is observed and it prevents the bacteria from growing was reported as MIC [10]. Then the statistical analysis of data was performed through SPSS-18 software. The level of significance determined for these findings was $p\leq0.05$.

Results

In this study, 99 (58.2%) strains of *Escherichia coli*, 63 (37%) strains of *Klebsiella pneumonia*, 6 (3.5%) strains of *Enterobacter cloacae* and 2 (1.1%) strains of *Citrobacter freundii* were isolated from clinical specimens in order to determine the antibiotic resistance pattern. χ^2 test indicated that the studied specimens of urine culture had the dominant percentage (p=0.05) (Table 1).

Figure 1 illustrates a general view of resistance against these antibiotics. The least resistance was observed in imipenem.

The results of screen test: According to the results achieved through disk Agar diffusion screen test, 125 specimens entered to the ESBL-producing confirmatory stage, which means that they were at least resistant against one of cephalosporin markers (Fig. 2).

The results of confirmatory test: According to results achieved through the combined disk confirmatory test, 108 (86.4%) isolates had ESBL-positive phenotype and 17 (13.6%) ones had ESBL-negative phenotype (Fig. 3 & Table 2).

According to the results of MIC test achieved through micro broth dilution method, 92 (85.1%) specimens showed MIC≥16 µg/ml toward cefazidiem and 105 (97.2%) specimens showed MIC≥64 µg/ml toward cefotaxime (Table 3).

Table 1. The frequency of the bacteria of enterobacteriaceae family in collected clinical specimens

Specimen Bacteria	Urine N (%)	Blood N(%)	Trauma N(%)	Total N(%)
Escherichia coli	89(58.9)	7(58.3)	3(42.8)	99(58.2)
Klebsiella pneumonia	54(35.7)	5(41.6)	4(57.1)	63(37)
Enterobacter cloacae	6(3.9)	-	-	6(3.5)
Citrobacter freundii	2(1.3)	-	-	2(1.1)
Total	151(88.8)	12(7)	7(4.1)	170

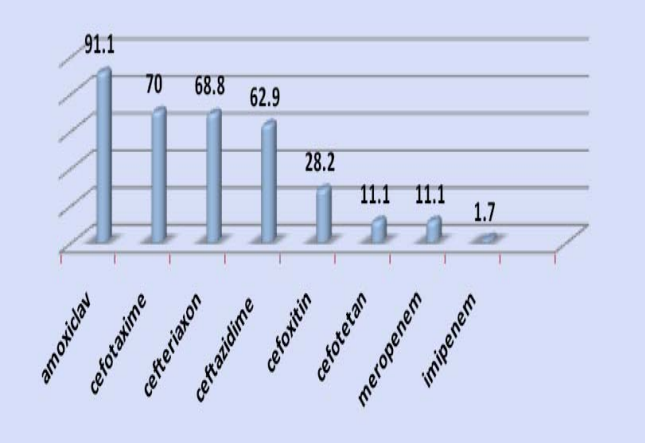

Figure 1. Illustrates a general view of resistance against these antibiotics. The least resistance was observed in imipenem

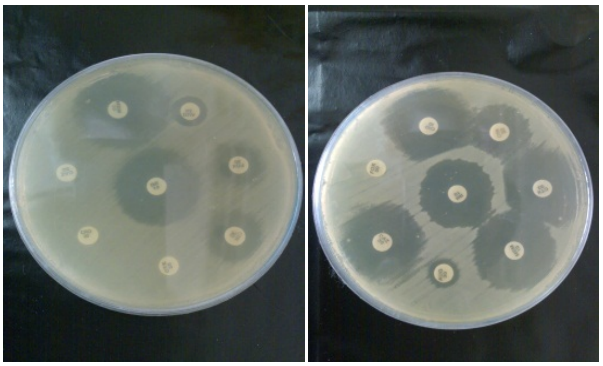

Figure 2. Preliminary screening with disk Agar diffusion (DAD)

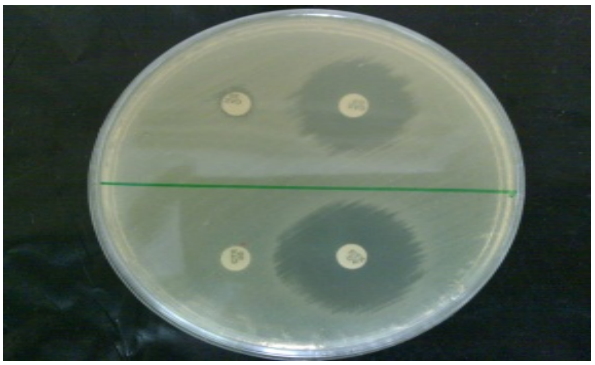

Figure 3. Confirmatory phenotypic test through combined disk method

Table 2. Frequency distribution of isolated ESBL-producing enterobacteriaceae specimens

Percentage	Number	Enterobacter cloacae	Klebsiella pneumonia	Escherichia coli	ESBL frequency
86.4	108	3	47	58	ESBL-positive phenotype
13.6	17	1	7	9	ESBL-negative phenotype
Negative	125	4	54	67	total

Table 3. The results of MIC test

Concentration µg/ml	2≤	8	16	32	64	128	256	512≥
Ceftazidime (number of specimen)	7	9	27	24	16	13	9	3
Cefotaxime	2	1	0	0	8	22	29	46

Discussion

The present study has investigated a total number of 170 bacteria strain including 99 (58.2%) strains of *Escherichia coli*, 63 (37%) of *Klebsiella* pneumonia, 6 (3.5%) strains of *Enterobacter cloacae* and 2 (1.1%) strains of *Citrobacter freundii*. Among these strains, 108 (86.4%) isolates had ESBL-positive phenotype and 17 (13.6%) isolates had ESBL-negative phenotype. Extended-spectrum beta-lactamases have increased considerably during the last two decades. These bacteria have caused a lot of problems for treatment due to disabling a broad range of beta-lactam drugs specially the third generation of cephalosporins and monobactams. The emersion and propagation of these bacteria seems to be mainly caused by the vast use of extended-spectrum beta-lactam drugs among the patients. Therefore, today, we see an ongoing increase in rate of ESBL-producing bacteria in the hospitals. Due to the fact that the members of *Enterobacteriaceae* family are the main factors which cause infection in hospitals and society and some of them create opportunistic infections as the members of normal flora, it seems necessary to adopt a modern strategy to diagnose and treat these strains [11].

In a study performed by Hakim and Zaoud, the highest rate of ESBL-production was reported to be 34.8 for *Klebsiella* pneumonia and 28.1% for *Escherichia coli* [12], also in another similar research performed by Mendes et al., among a total number of 76 specimens, *Klebsiella pneumonia* (27.84%), *Escherichia coli* (27.27%) and *Enterobacter cloacae* (23.9%) were the most frequent isolates collected from different divisions of hospitals. Performing disk agar diffusion screening test, 89 (88.66%) specimens entered the final stage of ESBL-production. All of the investigated specimens were sensitive toward imipenem [13].

In a similar investigation by Tsu-Lanwn et al., performed in ICU divisions in hospitals of Taiwan, after perfuming screening and confirmatory tests on 188 ESBL-producing isolates, *Klebsiella pneumonia* (25.56%), *Enterobacter cloacae* (29.54%), and *Escherichia coli* (18,18%) were the most frequent ones [14]. In another similar study in state of Berkeley in US the reported rate of ESBL prevalence was 44% [15]. Although the reported rate of ESBL prevalence is 20% in Southeast Asia, it has reached a rate higher than 60% in some areas [16].

The ESBL prevalence rate in Europe was indicated to be 18.4% in a study which reports a rate of 40% for Netherland and a rate of 3% for Sweden [17] and in an investigation performed by Mirsalehian et al., the highest rate of ESBL prevalence was reported 76.74% for *Klebsiella pneumonia* and 60.6% for *Escherichia coli* [18]. In a study by Binesh et al., it was indicated that 62.9% of *Klebsiella pneumonia* specimens include ESBL Enzyme which shows the high pathogenic potential of these strains in various hospital divisions [19].

Investigation of the patients' files indicated that cefazidime, cefotaxime and cefteriaxon have been (merely or simultaneously) used in most cases. This condition truly reveals the role of selective pressure caused by wide and unlimited drug consumption, in creation of such resistance.

It should be mentioned that the irregular and long-term use of extended-spectrum cephalosporins is one of the reasons of the emersion of resistant ESBL-producing types and according to many reports; the prevalence of these organisms would decrease applying infection control methods and limiting the consumption of oxyimino-ephalosporins. With the help of laboratory specialists and after the determination of an accurate sensitivity pattern, the bacterial infection would be treated using beta-lactam in a combination with a beta-lactamase inhibitor. In this way, the prevalence of extended-spectrum beta-lactamases would reduce among the various bacterial strains, which prevents the expansion of resistant infections and the ongoing increase in death in medical centers.

According to the comparison between the results of this study and those achieved through other mentioned researches; ESBL-producing organism is more prevalent in various divisions of the selected centers in our country. Although *Escherichia coli* and *Klebsiella pneumonia* are the main ESBL-producing organisms based on the results of this study, it seems that the variety of the bacteria of *Enterobacteriaceae* family isolated from studied centers and fast propagation of the extended-spectrum beta-lactamase-coding plasmid genes are among the important factors.

Finally, we hope that a right pattern of antibiotic use, limitation of taking beta-lactam drugs specially extended-spectrum cephalosporins, and application of an antibiotic rotation program would reduce the prevalence of extended-spectrum beta-lactamase-producing strains and other drug resistance patterns among the patients.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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