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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

Molecular study of extended-spectrum beta-lactamase *(TEM-1)* gene in *Escherichia Coli* isolates collected from Ostad Alinasab Hospital in Tabriz Iran

Iran Tebriz Ostad Alinasab Hastanesi'nin örneklerinden elde edilen *Escherichia coli* izolatlarında genişletilmiş-spektrum beta-laktamoz *(TEM1)* geninin araştırılması

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ABSTRACT

Objectives: Emergence of antibiotic resistance is an important problem in microbial infection control. Certain enzymes can lead to resistance against the third generation cephalosporins by the hydrolysis of monobactams. This study was conducted to evaluate antibiotic sensitivity profiles and the presence of bla_{TEM} gene in *E. coli* isolates collected from clinical specimens of Ostad Alinasab Hospital in Tabriz.

Materials and Methods: *E. coli* (100 isolates) were detected by using conventional bacteriologic tests and then antibiotic sensitivity tests were performed according to Kirby-Bauer method. Confirmatory test was also performed by combined disc test method. Finally bla_{TEM} gene was detected by using polymerase chain reaction (PCR) technique.

Results: Out of 100 *E. coli,* 23% of isolates contained bla_{TEM} gene. 18% of isolates were resistant to ceftazidime, while 30% of isolates were resistant to cefotaxime and the remaining was sensitive. 46% of isolates were extended-spectrum beta lactamase (ESBL) producers from which 23% of them contained bla_{TEM} gene.

Conclusion: High resistance of *E. coli* isolates to the third generation cephalosporins underlines need for accurate sensitivity tests as well as avoidance of inappropriate use of antibiotics.

Keywords: Extended-spectrum beta-lactamases (ESBLs), bla_{TEM} gene, *Escherichia coli,* Polymerase chain reaction

ÖZ

Amaç: Antibiyotik direncinin gelişmesi bakteriyel enfeksiyonların kontrolünde önemli bir sorundur. Bu çalışma, antibiyotik duyarlılık profillerini değerlendirmek ve Tebriz'de Ostad Alinasab Hastane'sinin klinik örnekleriden elde edilen *E.coli* izolatlarında bla_{TEM} geni varlığını değerlendirmek amacıyla planlanmıştır.

Gereç ve Yöntemler: *E. coli* (100 izolatları) geleneksel bakteriyolojik testler ile elde edilmiş ve daha sonra antibiyotik duyarlılık testleri Kirby-Bauer yöntemine göre değerlendirilmiştir. Doğrulayıcı testler ise "kombine disk" testleri ile gerçekleştirilmiştir. Son olarak bla_{TEM} geni varlığı polimeraz zincir reaksiyon (PCR) tekniği kullanılarak değerlendirilmiştir.

Bulgular: Yüz *E. coli* izolatından %23'nün bla_{TEM} genini içerdiği belirlenmiştir. İzolatların %18'inde seftazidim, % 30'unda sefotaksim direnci saptanmıştır. Izolatların % 46'sında geniş spekturumlu beta-lakamaz (GSBL) sentezi belirlenmiş ve bu izolatların % 23'de de bla_{TEM} geninin varlığı saptanmıştır.

Sonuç: *E. coli* izolatlarında saptanan üçüncü kuşak sefalosporinlere karşı yüksek direnç, uygunsuz antibiyotik kullanımından kaçınılması gerektiğinin ve tedavi öncesi doğru duyarlılık testlerinin uygulanması gerektiğinin önemini göstermektedir.

Anahtar kelimeler: Geniş spektrumlu beta-laktamaz (GSBL), bla_{TEM} geni, *Escherichia coli,* Polimeraz zincir reaksiyonu

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Introduction

Since sulfonamides and penicillin first appeared, a new opportunity was created in the treatment of diseases. In the early days of application of these medicines, they could suppress numerous pernicious epidemics. However, diseases resulting from infectious organisms still remain as a serious challenge [1,2]. There are two important mechanisms through which resistance to antibiotics and other medicines emerge. The first one occurs as a result of a

spontaneous mutation at approximately 10^{-5} Hz, which alters sensitivity to medicine, changes it into a merely selective agent and enables tolerant organisms to survive [1-3]. The second mechanism is to create resistance in genetic exchange wherein genetic information can control bacterial resistance transferred from a (resistant) cell to another (sensitive) by transformation, conjugation and transduction [2-4]. Hospitalized patients are exposed to hospital infections particularly organisms resistant to some medicines (gram negative bacillus species) as a major infection agent and its associated fatality [1,4]. Since antibiotics are frequently prescribed in intensive care units (ICU) to accelerate the natural treatment process, the problem become more serious, thus, having hydrolysis potential, a wide variety of extended-spectrum beta-lactamases (ESBLs), ESBL-generating strains (Beta-lactamase) are of critical importance. ESBL-generating bacteria together with AmpC-encoded class C cephalosporinase are the most common resistance mechanism of gram positive bacillus to this antibiotic [5-7]. Since the second half of 1980s, the wide geographic spread of ESBL variants has been a known epidemiological phenomenon [8]. This paper focuses on clinical isolates of *E. coli* collected from Ostad Alinasab Hospital in Tabriz in order to propose a sensitivity pattern to the analyzed antibiotics and to study their molecular structure to check presence of bla_{TEM} from ESBLs.

Materials and Methods

Various clinical specimens including mucus, urine, blood, tracheal tube secretions, wound secretions, throat secretions, catheter, cerebrospinal fluid, ascites, and peritoneal fluid were collected from hospitalized patients and outpatients of Ostad Alinasab Hospital in Tabriz by Simple Random Sampling method.

The bacteria extracted from patients' specimen were purified using MacConkey Agar, Blood Agar. Then, they were identified routinely by using citrate, urea, Methly red (MR), Voges-Proskauer (VP) Triple Sugar Iron Agar (TSI) and Sulfide Indole Motility (SIM) culture (all culture media were made by Merck, Germany) [9].

Combined Disc Test

This test was conducted by using ceftazidime disc (30µg), cefotaxime (30µg), ceftazidime/clavulanic acid (30µg/10µg) and cefotaxime/clavulanic acid (30µg/10µg) made in Mast Company, UK. To conduct this test, suspension isolates equal to 0.5 McFarland standard turbidity were prepared. They were, then cultured by cotton swabs on Muller-Hinton Agar in three orientations, and the aforementioned discs were superimposed. Growth inhibition zone around the discs were recorded after 24 hour incubation at 37 ºC. Over 5mm increase in the size of growth inhibition zone around ceftazidime/clavulanic acid (30µg/10µg) and cefotaxime/clavulanic acid (30µg/10µg) in comparison to that of ceftazidime discs (30µg) and cefotaxime (30µg) indicated ESBL samples were positive and the result proved positive (Fig. 1). In this test, negative control strain was *E. coli* ATCC 25922 and negative control strain was *E. coli* ATCC 35218 [9].

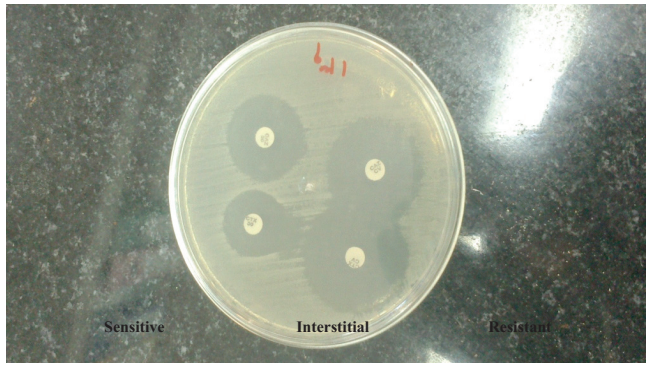

Fig. 1. Schematic representation of increase in the size of growth inhibition zone in *E. coli* isolates using combined disc test for isolate no.139.

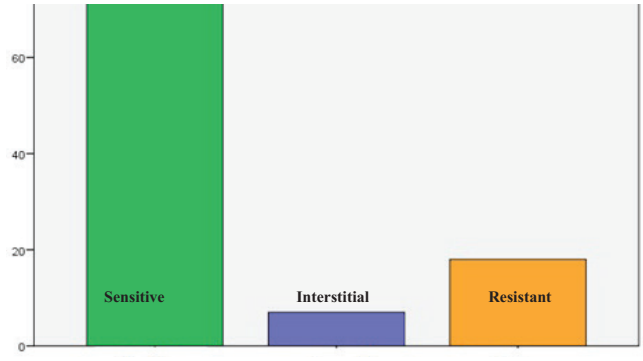

Fig. 2.1. Sensitivity test of *E. coli* isolates to ceftazidime using Disc Agar Diffusion

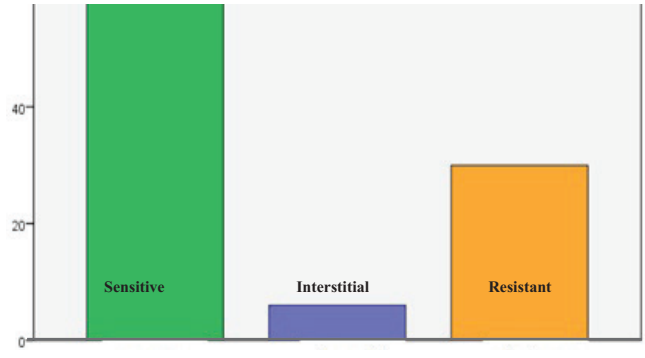

Fig. 2.2. Sensitivity test of *E. coli* isolates to cefotaxime using Disc Agar Diffusion

DNA Isolation and PCR Cloning

First, DNA plasmids were isolated by using Bioneer Kit based on the guidelines of the manufacturing company (RTP® Bacteria DNA Mini Kit). Then, polymerase chain reaction (PCR) test was conducted to identify beta lactamase bla_{TEM} under the following circumstances (Table I). Table II shows the primers used in PCR analysis. Also, negative control strain was *E. coli* ATCC 25922 and negative control strain was *E. coli* ATCC 35218. %12 poly acryl amid gel and SMO323 Ladder (Fermantase as Size Marker) were used for electrophorese PCR products [10-12].

Table I. Terms used in PCR

Test procedure	Temperature (° C)	Time
Initial denaturation step	94	4 min
Denaturation step	94	1 min
Annealing step	53	45 min
Extension/elongation step	72	1 min
Final extension/ elongation step	72	10 min
Number of cycles	35	

Table II. Primers used in PCR reactions for beta lactamase gene type *TEM* (Pornour et al. [20])

Gene sequence	Nucleotide sequence
Forward -	ACA TGG GGG ATC ATG TACT-3 - 5
Reverse -	5- GAC AGT TAC AAT GCT TACT-3

Results

A hundred isolates of *E. coli* consisted of the following numbers of samples: 80 urine (80%), 10 tracheal tube secretions (10%), 7 blood culture (7%), 1 catheter (1%), 1 pleural fluid (1%), and 1 mucus (1%). 18 isolates (18%) were ceftazidime-resistant, 30 were cefotaxime-resistant and the rest were sensitive. Also, 46 isolates (46%) were ESBL-positive. Of 100 isolates tested for PCR, 23 (23%) contained bla_{TEM}. In this paper, an isolate containing bla_{TEM} was identified, which was ESBL negative (Fig. 3.1-4).

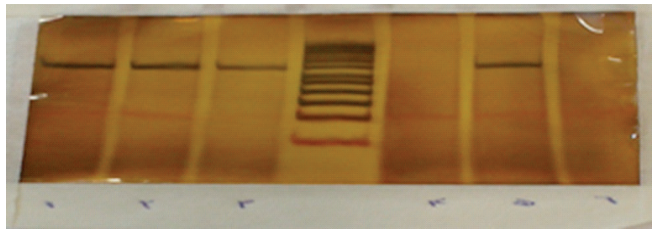

Fig. 3.1. Results of PCR reaction in search for positive control (no.3) and negative control (no.4) bla_{TEM} gene
Nos.1, 2 and 5 bla_{TEM} gene
No.6 lacks bla_{TEM} gene

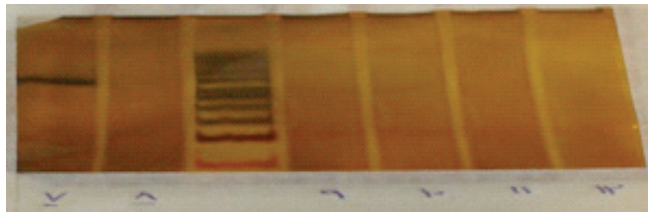

Fig. 3.2. Schematic representation of bla_{TEM} containing isolates No.7 contain bla_{TEM} gene and Nos. 12, 11, 10, 9, 8 lack it

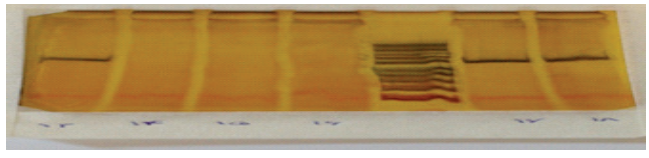

Fig. 3.3. Schematic representation of bla_{TEM} containing isolates Nos.18, 17, 13 contain bla_{TEM} gene and Nos. 16, 15, 14 lack it

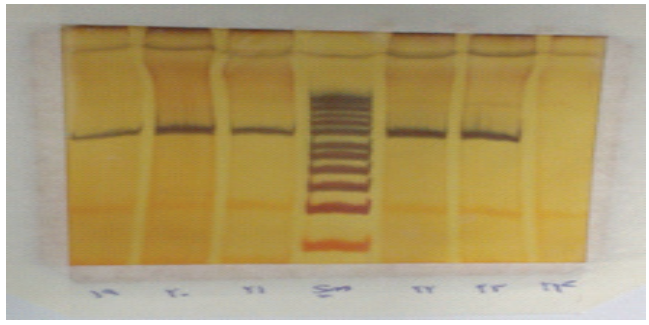

Fig. 3.4. Schematic representation of bla_{TEM} containing isolates Nos.23, 22, 21, 20, 19 contain bla_{TEM} gene and No.24 lacks it

Thorough examinations of *E.coli* isolates suggested that for ceftazidime: of 23 bla_{TEM}-containing isolates, 20 (86.9%) were resistant and 3 were sensitive (13.1%). For

cefotaxime: of of 23 bla_{TEM}-containing isolates, 21 (88.89%) resistant and 6 sensitive (13.1%). Of 100 tested *E. coli*, one isolate was bla_{TEM}-lacking and cefotaxime-resistant (2.44%) and 2 lacked bla_{TEM} with intermediate resistance (4.88%). For ceftazidime / clavulanic acid, of 23 tested isolates containing bla_{TEM}, 21 showed increase in the size of growth inhibition zone (91.3%). For cefotaxime / clavulanic acid, of 23 tested isolates containing bla_{TEM}, 20 showed increased growth inhibition zone (91.3%). Of 100 *E.coli* isolates tested, one bla_{TEM}-containing isolate showed no increase when exposed to either ceftazidime / clavulanic acid or cefotaxime / clavulanic acid (Fig. 2.1, 2.2)

Discussion

Extended-spectrum beta-lactamases (ESBLs) are able to hydrolyze most commonly used beta-lactam antibiotics. Therefore, they are referred to as ESBLs. Seemingly, ESBLs are inherently produced by class C chromosomal gene cephalosporinase and participate in creating resistance mechanisms in gram negative bacteria. Given these descriptions, ESBLs are class A or D beta lactamase molecules that can hydrolyze oxyimino-cephalosporins 10% or more than that of benzylpenicillin. The organisms carrying these genes increase pathogenicity [1,9].

In a similar study conducted by Mazinani and colleagues in 2008 in Vali Asr Hospital, Tehran, 47 isolates of 76 clinical samples (60%) contained *TEM* gene [13]. Masjedian showed that, 84.6% of 148 *E.coli* strains contained *TEM* isolates [14]. Mirsalehian et al. suggested that 39.45% of *E.coli* strains contained beta lactamase *TEM* isolates [15]. A comparison made between the present study and above studies indicates high prevalence of this enzyme type in *E.coli* strains in Iran. A study performed by Bradford in the United States showed that *TEM* beta lactamase has the highest abundance of ESBLs enzymes [15,16].

Between 2001 and 2006, Hong Fang showed that among 87 *E.coli* isolates phenotypically known as ESBL producers in Sweden, 63% had beta lactamase genotype [17], while in the present paper 46% of the isolates were ESBL positive and 50% of ESBL producers had a *TEM* type genotype. In Spain, Emilio David Valverde revealed that 30% of *E.coli* samples taken from 11272 patients of Salamanca Hospital produced ESBLs, which *TEM* family was 22.1% [18]. High levels of ESBLs throughout the world were reported in the recent literature. For example, distribution of ESBLs was as follows; Northern America: *K. pneumonia* (4.2%-4.4%), *E. coli* (3.3%-4.7%), *P. mirabilis* (3.1%-4.7%), Far-East-Western region: *K. pneumoniae* (11.3%-51%), *E. coli* (9.9%-23.6%), *Salmonella* (3.4%) and *P. mirabilis* (1.8%). In Europe expansion and prevalence of ESBL in *Enterobacteriaceae* in 1610 *E.coli* and 785 *K. pneumoniae* specimen collected from 31 stations of 10 European countries varied from 1%-5% in Northern Europe (Germany) to almost 39%-47% in East Europe (Russia, Poland, Turkey). In a joint study compiled by Paul-Ehrlich-Gesellschaft in 2001, protein extracts from 8.2% of *K. pneumonia*, 0.8% of 612 *E.coli*, and 1.3% of 152 *K. oxytoca* specimen contained ESBL [8,9,19], while ESBL was 46% in the present paper. Overconsumption of antibiotics particularly ceftazidime, hospitalization in ICU wards, and excessive uses of urinary catheter are all factors of breaking out ESBLs. The results of research in recent years on ESBL producing *E.coli* various strains in different countries are: Pakistan 53.3% (2006), India 68% (2002), Spain 51.8% (2005), South Korea 9.2% (2004), Lebanon 13.3% (2005), Israel 22% (2005), Germany 10.3% (2005), France 4% (2006), Turkey 17% (2004), Bangladesh 43.2% (2004), United States 2%-10% (2003), China 13%-35% (2002) [20] .

Eslamy et al. studied breakout of ESBLs on 200 isolates of *E. coli* collected from hospitals in Arak City by combined disc phonotypical method, which suggested that 47% ESBL were positive [9], similar to the present paper. Pornour et al. classified 41 *E.coli* isolates in terms of resistance to medications: 87.8% resistant to aztreonam, 80.49% to piperacillin/tazobactam and ceftazidime, 78.05% to cefotaxime, cefuroxime and ceftriaxone, 65.85% to cefepime, and 4.88% to amikacin. But none of these isolates resisted Imipenem. Also, 97.56% of ESBL was positive and 36 out of 41 isolates under PCR reaction (87.8%) contained *TEM* beta lactamase gene [20]. In our research, the resistance to ceftazidime and cefotaxime were 86.9% and 88.89%, respectively. Soltan Dallal, et al. found that 57.8% of 200 *E.coli* isolates contained *TEM* genes [21]. Zaman zad and colleagues (2008) reported that abundance of *TEM-1* genes of *E.coli* is 48.7% among 83 isolates of intestinal bacteria producing ESBL including *E.coli* strains, *K. pneumonia* and entrobacter from clinic specimen taken from educational hospitals in Shar e Kord [22,23]. This was 50% in our research. In Taiwan, Chin-fulin et al. (2010) reported the prevalence of *TEM* gene in 69 *E.coli* isolates [24]. Shakil et al. reported that *TEM* is the most common resistance gene in *E.coli* of Neonatal Intensive Care Units of Indian hospitals [25].

Conclusion

We found out that 86.9% and %88.89 bla_{TEM} containing *E.coli* isolates resisted ceftazidime and cefotaxime, respectively. This represents the role of bla_{TEM} gene in making resistance to cephalosporins. With regard to growing increase of various types of ESBL enzymes and their unbalanced effects on various antibiotics, precise determination of different types of *TEM* enzyme and other ESBL enzymes using other molecular methods including PCR-RFLP, REP-PCR and sequencing these genes is inevitable. Beta lactam-based antibiotics specially, third generation of cephalosporins are the main antibiotic therapies in clinical centers. It should be noted that increased resistance of pathogenic bacteria to these medications particularly ceftazidime and cefotaxime (for *E.coli*) questions the clinical effect of such medications. Thus, prescription of antibiotics suited to precise sensitivity test are recommended to avoid expansion of this medicinal resistance, ESBL secretion, and infections with likely ESBL-producing organisms.

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