



# Characterization of Antimicrobial Resistance Pattern and Molecular Analysis among Extended Spectrum $\beta$ -Lactamase-Producing *Escherichia coli*

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## ABSTRACT

**Background:** Infection is a serious problem in medicine and appropriate antibiotic therapy is very important. Because of broad spectrum activity and low toxicity of  $\beta$ -lactam antibiotics, they are the most commonly used drugs. But, bacterial resistance to  $\beta$ -lactam antibiotics, has been considered as the global healthcare concern. The aim of study was to evaluate the antimicrobial resistance pattern and molecular characterization among ESBL-producing *Escherichia coli* isolated from patients with diarrhea admitted to a hospital in Ilam, Iran.

**Methods:** Totally, fifty *E. coli* isolates were investigated. Confirmatory tests for phenotypic detection of ESBLs were performed. Molecular identification of the *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes was carried out by PCR method. To identify genetic relatedness among isolates, Randomly Amplified Polymorphic DNA (RAPD) analysis was performed.

**Results:** The antibiotic susceptibility results showed that the most effective antibiotic was imipenem and minimum effect was related to gentamicin. Thirty-one isolates (62%) were ESBL-producing organisms according to phenotypic method. The distribution of *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes among ESBL-producing isolates were 20 (64.5%) and 6 (19.3%), respectively. RAPD-PCR typing among isolates gave us eight different types. Twelve isolates were clustered in genotype A and all of them were ESBL-producer.

**Conclusion:** The present study showed noticeable incidence of ESBL-producing *E. coli* isolated from outpatients and hospitalized patients with diarrhea. Therefore, it seems that constant supervision is crucial to monitor the ESBL-producing microorganisms in hospitals and community.

## Introduction

Infection is a serious problem in several areas of medicine and appropriate antibiotic therapy is very crucial. Because of broad spectrum activity and low toxicity of  $\beta$ -lactam antibiotics, they are the most commonly used drugs against infections.<sup>1</sup>

But over the past decade, bacterial resistance to antibiotics, especially  $\beta$ -lactam type, has been considered as the global healthcare concern.  $\beta$ -lactam resistance occurs by the mobilization of genes that encode efficient drug modifying enzymes such as  $\beta$ -lactamase.<sup>2-5</sup>

Extended-spectrum  $\beta$ -lactamases (ESBLs) are

considered as one of the most prevalent mechanisms of resistance to  $\beta$ -lactam agents especially in enteric gram-negative bacilli.<sup>6,7</sup> The enzymes, which are usually plasmid-encoded, have the ability to hydrolyze numerous  $\beta$ -lactam agents including penicillins, third-generation cephalosporins and aztreonam. They are inhibited by  $\beta$ -lactamase inhibitor including tazobactam and clavulanic acid.<sup>8,9</sup>

These phenotypic features can assist to detection of ESBL-producing bacteria by routine laboratory methods such as double disk diffusion test.<sup>10</sup> In addition, detection of specific genes is generally

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applied for final verification. According to the literature, ESBLs production is related to presence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>PER-1</sub>, *bla*<sub>GES</sub> and *bla*<sub>CTX</sub> genes.<sup>11,12</sup> Some researchers reported that most ESBLs are originated from *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes.<sup>13</sup>

ESBLs are frequently produced by *Escherichia coli* and *Klebsiella pneumoniae* isolates which are responsible for nosocomial and community-acquired infections.<sup>14</sup> *E. coli* is the most common causative microorganism of gastroenteritis, urinary tract infections and other hospital-acquired infections. Increasing the multidrug resistance strains is due to the widespread prescription of antibiotics against bacterial isolates.<sup>15,16</sup>

Therefore, determination of antimicrobial susceptibility and ESBL production patterns are essential for treatment of related infections. Additionally, identification of the genetic relatedness among isolates can be helpful for infection source control and making preventive procedures. The aim of study was to determine the frequency of ESBL-producing *E. coli* and their genetic diversity, isolated from gastroenteritis patients admitted to clinical laboratory of Imam Khomeini Hospitals in Ilam, west of Iran.

## Materials and Methods

### Bacterial strains

In this cross sectional study, 50 isolates of *E. coli* were collected from patients with diarrhea admitted to clinical laboratory of Imam Khomeini hospital in Ilam during July 2014 to January 2015. In order to genus confirmation, biochemical tests (oxidase, citrate, fermentation of glucose, lactose, motility, gas production, and SH<sub>2</sub> production) were performed.<sup>17</sup>

### Antimicrobial susceptibility

Antibiotic sensitivity tests were done by disc diffusion method on Mueller Hinton agar (Merck Co, Germany) according to the Clinical Laboratory Standards Institute (CLSI) guideline 2015.<sup>18</sup> The tested antibiotics (Mast Co., UK) were: imipenem (10 µg), gentamicin (10 µg), levofloxacin (5 µg), amikacin (30 µg), ciprofloxacin (5 µg) and trimethoprim/ sulfamethoxazole (1.25/23.75 µg).

### Phenotypic confirmatory test for determination of ESBL production

Combined disc method was utilize for identification of ESBL-producing organisms

according to CLSI guidelines.<sup>18</sup> Briefly, sensitivity tests to cefotaxime (30 µg), cefotaxime/clavulanate (10 µg), ceftazidime (30 µg) and ceftazidime/clavulanate (10 µg) disks (Mast Co., UK) were determined on Muller-Hinton agar (Merck Co, Germany). The plates were incubated for 18-24 h at 37 °C. ESBL-producing strains were recognized by at least 5 mm increase in zone diameter around cefotaxim/clavulanate and ceftazidime/clavulanate disks compared to disks without clavulanic acid.<sup>19</sup> *Escherichia coli* ATCC 25922 was used as control strain.

### Molecular detection of ESBLs related genes

DNA of the ESBL-producing isolates was extracted by boiling method. The PCR method for detection of *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes was carried out as described previously.<sup>20</sup> Briefly, the PCR protocol was 30 cycles of 60 s at 95 °C, 45 s at 48 °C (for *bla*<sub>SHV</sub>) and 50 °C (for *bla*<sub>TEM</sub>), and 45 s at 72 °C. A final extension step was done at 72 °C for 5 min. PCR products were separated by electrophoresis in 1% agarose gels. Oligonucleotide primers were mentioned in Table 1.

### Molecular genotyping

Randomly Amplified Polymorphic DNA (RAPD) analysis was also performed to identify genetic relatedness among isolates as described previously with a little modification. RAPD-PCR oligonucleotide primer was 5'-AAGAGCCCG-3'.<sup>21</sup> Briefly, the PCR protocol was a predenaturation step at 95 °C for 5 min, followed by 35 cycles of 60 s at 95 °C, 45 s at 42 °C, and 50 s at 72 °C. A final extension step was performed at 72 °C for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gels. After agarose gel electrophoresis, banding patterns were analyzed by visual examination. Classification as a distinct RAPD type was based on difference between two or more band of each banding pattern. In other words, isolates were classified as identical RAPD types when their banding patterns were similar or different in one band only.<sup>22</sup>

### Statistical analysis

Resistance to antimicrobial agents between ESBL-producing and non ESBL-producing isolates was investigated by Chi-square test. A p-value of <0.05 was considered as statistically significant.

**Table 1.** Oligonucleotide primers used for ESBL detection.

Target gene	Primer sequence	Reference
<i>bla</i> <sub>TEM</sub>	F: 5'-ATGAGTATTCAACATTTCCG-3'	20
	R: 5'-CCAATGCTTAATCAGTGAGC-3'	
<i>bla</i> <sub>SHV</sub>	F: 5'-CTTTACTCGCTTTATCG-3'	20
	R: 5'-TCCCGCAGATAAATCACCA-3'	

**Table 2.** Antibiotic resistance pattern of ESBL and non ESBL-producing isolates.

Antibiotics	Resistance rate (n=50)	ESBL-producing isolates (n=31)	Non ESBL-producing isolates (n=19)
Imipenem	0 (0%)	0 (0%)	0 (0%)
Ciprofloxacin	13 (26%)	9 (29.3%)	4 (21%)
Levofloxacin	1 (2%)	1 (3.2%)	0 (0%)
Gentamicin	19 (38%)	13 (41.9%)	6 (31.5%)
Amikacin	7 (14%)	3 (9.6%)	4 (21%)
Trimethoprim/Sulfamethoxazole	16 (32%)	11 (35.4%)	5 (26.3%)

**Results**

The antibiotics susceptibility results showed that the most effective antibiotics were imipenem followed by levofloxacin and amikacin. Resistance rates to gentamicin, ciprofloxacin and trimethoprim/ sulfamethoxazole were 38%, 26% and 32% respectively. Resistance to almost all tested antibiotics among ESBL-producing isolates were more than non ESBL-producing isolates without significant correlation (Table 2).

In this study, of fifty collected *E. coli* isolates, 31 isolates (62%) were ESBL-producing based on phenotypic method (Table 3).

**Table 3.** The prevalence of ESBL-producing *E. coli* by disk diffusion method.

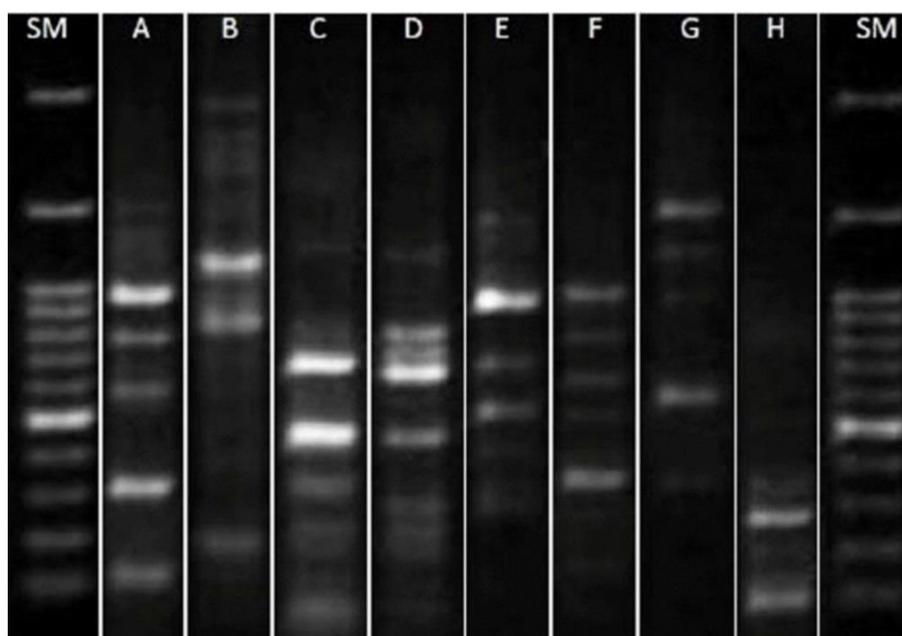
ESBL-positive N (%)	ESBL-negative N (%)
31 (62)	19 (38)

Also, the distribution of *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes among ESBL-producing isolates was 20 (64.5%) and 6 (19.3%), respectively (Table 4).

RAPD-PCR genotyping gave us 8 (A-H) different types (figure 1). The most prevalent type was A, containing 12 isolates. All of them were ESBL-producing stains. Of these 12 strains, 9 isolates carried *bla*<sub>TEM</sub> gene and only one of them had *bla*<sub>SHV</sub> gene. Subsequent prevalent type was B, consisting of 8 isolates which 5 strains were ESBL producer. In B genotype *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes were found in 3 and 2 strains respectively. In our analysis, the H genotype was unique and containing only on isolate. This strain was ESBL producer but had not *bla*<sub>SHV</sub> or *bla*<sub>TEM</sub> gene.

**Table 4.** The frequency of *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes among ESBL-producing *E.coli* isolates.

<i>bla</i> <sub>TEM</sub> positive N(%)	<i>bla</i> <sub>SHV</sub> positive N(%)	Total N(%)	<i>bla</i> <sub>TEM</sub> and <i>bla</i> <sub>SHV</sub> negative N(%)
20 (64.5)	6 (19.3)	26 (83.8)	5 (16.2)



**Figure 1.** RAPD-PCR patterns of *E. coli* isolates, Lanes 1 and 10; DNA size marker (100bp), Lanes A-H; eight RAPD genotypes. Patterns A and B were related to 12 and 8 isolates respectively. Pattern H was seen in 1 isolate exclusively.

## Discussion

The emergence of ESBL producer gram negative bacteria in the clinical setting has been reported from around the world.<sup>8,23,24</sup> The present study was designed to evaluate the frequency of ESBL-producing *E. coli* and the presence of *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes among ESBL-producing isolates. Our results indicated that, the prevalence of ESBL-producing *E. coli* was 62%. The rate of prevalence in our study was higher than other studies in different regions of Iran. In two reports from north of Iran, ESBL-producing rate among *E. coli* isolates were about 26% and 30%.<sup>25,26</sup> In a research in northeast of Iran, prevalence ESBL-producing of *E. coli* and *K. pneumoniae* were 43.9% and 56.1% respectively.<sup>27</sup> These variations may be related to geographical difference.

Phenotypic method showed that, 62% of *E. coli* isolates were ESBL producer, but *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes were found among 83.8% of them. ESBL-producing among isolates which were negative for *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes (for example, type H), may be related to other ESBL responsible genes. The genes such as, *bla*<sub>CTX</sub>, *bla*<sub>VEB</sub>, and *bla*<sub>PER-1</sub> have been reported among clinical isolates in various studies in Iran.<sup>26,28,29</sup> Our findings indicated that frequency of *bla*<sub>TEM</sub> gene was high among isolates (64.5%) which is accordance with the several studies.<sup>29,30</sup>

ESBL positive strains show resistance to other antimicrobial classes such as aminoglycosides, trimethoprim/ sulfamethoxazole and tetracyclines.<sup>31</sup> In the present study, resistance to almost all tested antibiotics among ESBL-producing isolates were more than non ESBL-producing isolates.

In present study RAPD-PCR used for typifying isolates. Strains, which had similar RAPD type, showed same antimicrobial resistance patterns approximately. Molecular investigation indicated that most prevalent genotype was A. Most of genotype A isolates harbored *bla*<sub>TEM</sub> gene. It seems that *bla*<sub>TEM</sub> mediated resistance is most common in drug resistant infections.

Most of the *bla*<sub>TEM</sub> positive isolates were clustered in an identical genotype (type A). These findings were in contrast to a previous study that the *bla*<sub>TEM</sub> positive strains were heterogeneous completely according to RAPD-PCR genotyping.<sup>32</sup> This difference may be related to type of samples. In Pokhrel RH, *et al.*'s study the isolates were collected from various specimen, whereas the isolated of present study were obtained from diarrheal patients only. Similarly, in a RAPD analysis study among ESBL-producing *E. coli* isolates at a tertiary care centre in Lebanon, genomic variability was demonstrated.<sup>33</sup> Regardless of geographic region, it seems that closely related isolates, which had *bla*<sub>TEM</sub> gene had been colonized in evaluated gastroenteritis patients

in Ilam.

In order to control the wide spread of ESBL-producing bacteria,  $\beta$ -lactam prescription should be managed carefully. Furthermore, according to recent studies, utilizing the new drugs or approaches can be effective against infectious diseases.<sup>34-36</sup>

## Conclusion

We found most susceptibility and resistance to imipenem and gentamicin among *E. coli* isolates respectively. The phenotypic method demonstrated 31 ESBL producer isolates whereas only 26 isolates carried *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes. Antimicrobial resistance among ESBL-producing isolates was more than non ESBL-producing isolates.

In molecular analysis, eight different RAPD types were determined. The most prevalent type was A. All of type A isolates were ESBL-producer and most of them had *bla*<sub>TEM</sub> resistance gene. Therefore, it seems that a *bla*<sub>TEM</sub> positive ESBL-producing *E. coli* was common among investigated patients.

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## Conflict of interests

The authors claim that there is no conflict of interest.

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