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Short Communication

Detection of extended-spectrum β-lactamase (ESBL) and plasmid-borne *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes among clinical strains of *Escherichia coli* isolated from patients in the north of Iran

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ABSTRACT

Escherichia coli is an important cause of hospital-acquired infections worldwide. Antimicrobial resistance leads to treatment failure of hospital infections caused by *E. coli*. Production of extended-spectrum β-lactamases (ESBLs) is one of the major causes of antibiotic resistance in these bacteria. This study aimed to investigate the frequency of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes in ESBL-producing *E. coli* strains isolated from clinical specimens of patients admitted to six hospitals in the north of Iran. A total of 160 *E. coli* strains were isolated from various clinical samples of hospitalised patients. Antibiotic resistance patterns were determined by the Kirby–Bauer disk diffusion method. The double-disk phenotypic confirmatory test was carried out amongst β-lactam-resistant isolates to detect ESBL-producing strains. Plasmid DNA of ESBL-producing strains was extracted and subjected to PCR for detection of the *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes, and isolates were extensively verified by sequencing. The highest resistance rate was to amoxicillin; all *E. coli* isolates (100%) were susceptible to imipenem. Amongst the 160 clinical *E. coli* isolates, 83 (51.9%) were ESBL-positive, of which 27 (32.5%) and 72 (86.7%) were positive for *bla*$_{TEM}$ and *bla*$_{CTX-M}$, respectively. This study is the first report of an ESBL phenotype disseminated in hospitals in the north of Iran. These findings showed that there was a direct relationship between the development of resistance to β-lactam antibiotics and production of TEM and CTX-M enzymes.

1. Introduction

Hospital-acquired infections occur globally and affect both developed and developing countries. Increased mortality and morbidity amongst hospitalised patients are due to acquisition of infections in healthcare systems. *Escherichia coli*, a member of the Enterobacteriaceae, is one of the major causes of nosocomial infections, especially urinary tract infections (UTIs) [1,2]; >85% of UTIs are caused by this organism. Furthermore, some serotypes of *E. coli* are a major cause of diarrhoea in developing countries [1,3,4]. β-Lactam antibiotics, including penicillins, cephalosporins, carbapenems and monobactams, are one of the most common agents for the treatment of bacterial infections [5].

Nowadays, treatment of infections caused by *E. coli* has faced many troubles due to increased antibiotic resistance [4]. Production of extended-spectrum β-lactamases (ESBLs), which are normally plasmid-mediated enzymes, is one of the important resistance mechanisms in this bacterium [5]. Many studies in Iran, China, Italy and India have reported a high incidence of these enzymes in *E. coli* and *Klebsiella pneumoniae* [6]. ESBLs are encoded by TEM-type, SHV-type and CTX-M-type genes, which may hydrolyse penicillins, cephalosporins and monobactams [7]. The origin of such classic enzymes is the plasmid-encoded ESBLs of TEM (Temoniera), SHV (sulfhydryl variable) and OXA-type (oxacillinase) families [8].

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Escherichia coli is the major bacteria for ESBL production [9]. The first CTX-M (cefotaximase) enzyme was discovered in 1986, and this ESBL family now includes more than 60 diverse enzymes in five phylogenetic groups. Many strains that acquire CTX-M β-lactamases are multidrug-resistant [10].

In 1965, another ESBL enzyme, TEM β-lactamase, was first detected in an *E. coli* strain isolated from a patient called Temoniera in Greece [9]. A study by Ahmed et al. [11] revealed that the frequencies of bla_{TEM},bla_{CTX-M} and bla_{SHV} genes are fairly similar in *K. pneumoniae*, whilst in *E. coli* bla_{TEM} and bla_{CTX-M} are the most frequently found genes. The Clinical and Laboratory Standards Institute (CLSI) has suggested ESBL screening and confirmatory tests, and reporting of resistance to penicillins, cephalosporins and aztreonam if the ESBL confirmatory test is positive [6]. Since the frequency of ESBLs differs with geographical location and time, the aim of the present study was to determine the distribution of bla_{CTX-M} and bla_{TEM} amongst *E. coli* stains isolated from clinical samples in remedial hospitals in the north of Iran.

2. Materials and methods

2.1. Bacterial isolates

In a cross-sectional study, 160 consecutive non-duplicate *E. coli* were isolated from clinical specimens including urine, blood, wound, renal fluid and ascitic fluid of patients admitted to six major remedial hospitals in the north of Iran from June 2015 to November 2015 and with hospital-acquired infections due to *E. coli* during their hospitalisation. In other word, these patients were admitted to hospital not because of *E. coli* infection but they had acquired these infections within 3 days after hospitalisation. To isolate the *E. coli* strains, specimens were cultured on eosin methylene blue (EMB) agar (Merck KGaA, Darmstadt, Germany) and blood agar (Merck KGaA) media incubated at 37 °C for 24 h. Isolates were identified as *E. coli* if they had the following characteristics: gram-negative bacillus; triple sugar iron (TSI) agar test A/A; motile; LIA (lysine decarboxylase) negative; H_2S negative; citrate negative; urease negative; oxidase negative; Methyl Red/Voges–Proskauer broth (MR/VP) positive/negative.

2.2. Antimicrobial susceptibility testing of Escherichia coli isolates

The Kirby–Bauer disk diffusion technique was used to perform antibacterial susceptibility testing for all of the isolated organisms according to the recommendations of the CLSI (document M100-S23). Briefly, a suspension of 1.5×10^8 CFU/mL of each isolate, equivalent to a 0.5 McFarland turbidity standard, was transferred separately to Mueller–Hinton agar (Merck KGaA). Nineteen different antibiotic disks (MAST Chemical Co., Bootle, UK), including aztreonam (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), trimethoprim/sulfamethoxazole (25 μg), ofloxacin (5 μg), imipenem (10 μg), amoxicillin (25 μg), cefixime (5 μg), tetracycline (30 μg), cefepime (30 μg), cefalotin (30 μg), cefoxitin (30 μg), ampicillin (10 μg), nitrofurantoin (300 μg), nalidixic acid (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg) and amoxicillin/clavulanic acid (20/10 μg), were placed on cultured plates and were incubated at 37 °C for 24 h. The antibiotic potency of the disks was standardised against the reference strain *E. coli* ATCC 25922.

2.3. Detection of extended-spectrum β-lactamase-producing strains by the double disk method

Pairs of disks (ceftazidime + ceftazidime/clavulanic acid and cefotaxime + cefotaxime/clavulanic acid) were placed on cultured Muller–Hinton agar medium (Merck) with a 30-mm distance from the centre of each disk and were incubated for 24 h at 37 °C. The results were interpreted as recommended by the CLSI (document M100-S23).

2.4. DNA extraction and amplification of bla_{TEM} and bla_{CTX-M} genes by PCR

Plasmids of ESBL-producing isolates were extracted using a GeneJET Plasmid Miniprep Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's procedure. ESBL-producing isolates were subjected to PCR to detect the bla_{TEM} (1080 bp) and bla_{CTX-M} (544 bp).

PCR amplification was performed using the primers TEM-F (5′-ATAAAATTCTTGAAGACGAAA-3′), TEM-R (5′-GACAGTTAC-CAATGCTTAATCA-3′), CTX-M/F (5′-TTTGCGATGTGCAGTACCAG-TAA-3′) and CTX-M/R (5′-CGATATCGTTGGTGGTGCCATA-3′), which generated 1080-bp and 544-bp fragments, respectively [12].

PCR reactions were carried out in a final volume of 25 μL containing 4.25 μL of PCR Master Mix (SinaClon, Tehran, Iran), 17.75 μL of dH_2O, 1 μL of template plasmid DNA and 1 μL of each primer. Amplification was performed with pre-denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 1 min for bla_{TEM} and at 72 °C for 45 s for bla_{CTX-M}, and a final extension at 72 °C for 7 min.

The amplified PCR products were electrophoresed on 1.5% agarose gel containing SYBR® Safe (SinaClon) and were visualised using an ultraviolet transilluminator (Cambridge, UK) with a 100-bp ladder molecular weight marker. Furthermore, PCR products for sequencing and final approval of observed bands were sent to Bioneer Corp. (Daejeon, South Korea) in the determined volume and concentration along with their specific primers and the results were aligned with GenBank using the Basic Local Alignment Search Tool (BLAST).

2.5. Statistical analysis

Data were analysed using SPSS for Windows v.16.0 (SPSS Inc., Chicago, IL). Moreover, to evaluate the prevalence and qualitative variables, Fisher's exact test and χ^2 were used. The difference between the mean values was considered significant at $P < 0.05$.

3. Results

During the study period, a total of 160 *E. coli* strains were isolated from clinical specimens of patients, including 106 females (66.3%) and 54 males (33.8%), admitted to six different hospitals in the north of Iran. The mean patient age was 51.49 ± 26.67 years (range, 1 month to 90 years). Most samples were taken from urine, including 99 (70.7%) for females and 41 (29.3%) for males.

Amongst the tested isolates, 83 (51.9%) produced an ESBL. The frequency of ESBL-producing *E. coli* strains isolated from males and females was 35 (42.2%) and 48 (57.8%), respectively. The susceptibility of ESBL-producing and non-ESBL-producing isolates is shown in Table 1. All ESBL-producing *E. coli* strains were resistant to cefalotin, whereas resistance to amoxicillin, cefotaxime and ceftriaxone was seen in 98.8% of ESBL-producers. All ESBL-producing and non-ESBL-producing *E. coli* strains in this study were also sensitive to imipenem, as expected. Differences in resistance to the tested antibiotics between ESBL-producing and non-ESBL-producing isolates were significant, with the exception of imipenem ($P = 0.892$) and nitrofurantoin ($P = 0.362$).

The frequency of bla_{TEM} and bla_{CTX-M} genes in the 83 ESBL-producing *E. coli* isolates was 27 (32.5%) and 72 (86.7%) respectively. However, 24 isolates (28.9%) were indicated as having both genes. All *E. coli* strains harbouring bla_{TEM} were

Table 1
Antibiotic susceptibility of extended-spectrum β-lactamase (ESBL)-producing and non-ESBL-producing isolates.

Antibiotic	Non-ESBL-producing ($N = 77$)	ESBL-producing ($N = 83$)	
	Resistant [n (%)]	Resistant [n (%)]	
		TEM ($n = 27$)	CTX-M ($n = 72$)
Aztreonam	5 (6.5)	25 (92.6)	66 (91.7)
Gentamicin	5 (6.5)	13 (48.1)	40 (55.6)
Ciprofloxacin	20 (26.0)	16 (59.3)	59 (81.9)
Cefotaxime	13 (16.9)	26 (96.3)	72 (100)
SXT	42 (54.5)	19 (70.4)	7 (9.7)
Ofloxacin	18 (23.4)	16 (59.3)	58 (80.6)
Imipenem	0	0	0
Amoxicillin	47 (61.0)	26 (96.3)	72 (100)
Cefixime	7 (9.1)	22 (81.5)	59 (81.9)
Tetracycline	37 (48.1)	19 (70.4)	54 (75.0)
Cefepime	2 (2.6)	15 (55.6)	48 (66.7)
Cefalotin	28 (36.4)	27 (100)	72 (100)
Cefoxitin	4 (5.2)	6 (22.2)	18 (25.0)
Ampicillin	46 (59.7)	26 (96.3)	72 (100)
Nitrofurantoin	0	1 (3.7)	2 (2.8)
Nalidixic acid	36 (46.8)	19 (70.4)	56 (77.8)
Ceftriaxone	10 (13.0)	27 (100)	71 (98.6)
Ceftazidime	2 (2.6)	23 (85.2)	57 (79.2)
AMC	8 (10.4)	10 (37.0)	21 (29.2)

SXT, trimethoprim/sulfamethoxazole; AMC, amoxicillin/clavulanic acid.

resistant to cefalotin and ceftriaxone, whilst $bla_{\text{CTX-M}}$-positive strains were totally resistant to amoxicillin, ampicillin cefotaxime and cefalotin. There was only a significant relationship between the presence of bla_{TEM} and resistance to ciprofloxacin ($P = 0.002$), ofloxacin ($P = 0.003$) and nalidixic acid ($P = 0.003$). Finally, there was no significant relationship between the presence of $bla_{\text{CTX-M}}$ and resistance to antibiotics, with the exception of ampicillin ($P = 0.001$) and ciprofloxacin ($P = 0.004$).

4. Discussion

The result of this investigation revealed that ESBL-producing *E. coli* strains were prevalent in hospitals in the north of Iran. In the present study, the occurrence of ESBL in *E. coli* owing to the spread of $bla_{\text{CTX-M}}$ and bla_{TEM} enzymes was consistent with previous observations in Egypt, India, Iraq, Ireland, Italy, Portugal, South Korea, Sudan, Turkey, the UK and other countries. In the current study, the $bla_{\text{CTX-M}}$ gene was found more frequently than the bla_{TEM} gene in ESBL-producing *E. coli* isolates. The most predominant clinical samples were urinary samples, of which 99 (70.7%) were from women.

The present study described the susceptibility of isolates to 19 antibiotics using the Kirby–Bauer disk diffusion method. The majority of urinary and non-urinary isolates were resistant to amoxicillin (129; 80.6%) and ampicillin (127; 79.4%). Meanwhile, imipenem was the most effective antibiotic for all isolates. According to a report, the susceptibility of studied *E. coli* to imipenem was 100%, but the highest resistance rate was against carbenicillin (89%) and piperacillin (83%) [13].

In another study by AL-Temey and Al-Charrakh [14], 100 urine specimens were analysed, from which 60 uropathogenic *E. coli* strains were isolated. In that study, 22 *E. coli* isolates were ESBL-positive. All of the isolates (100%) were found to be resistant to ampicillin and amoxicillin, whereas the results of the current showed that the resistance rates to ampicillin and amoxicillin amongst ESBL-producing *E. coli* were 97.6% (81/83) and 98.8% (82/83), respectively. All 83 isolates (100%) were resistant to cefalotin. In a survey on 145 *E. coli* strains, 51 (35.2%) and 61 (42.1%) were resistant to ceftazidime and cefotaxime, respectively, and ESBL production was observed in 59 (40.7%) strains, comprising $bla_{\text{CTX-M-like}}$ genes in 22 (37.3%) and bla_{TEM} in 42 (71.2%) strains [15]. These results are contradictory to the current findings. In a study by Gholipour et al., the prevalence of bla_{TEM} in ESBL-producing *E. coli* was 12.1% (13/107) [16], which is less than the current finding (27/83; 32.5%). Also, all bacterial isolates were susceptible to imipenem, which is concordant with our study. As indicated by Riyahi Zaniani et al., the frequency of bla_{TEM} amongst ESBL-producing *E. coli* was 20.6% [9], which is lower than the current results. Another investigation revealed rates of 43.5% and 15.9% for bla_{TEM} and $bla_{\text{CTX-M}}$, respectively, amongst ESBL-producing *E. coli*, which is lower than the results of the current study [4].

In a previous study by Kaur and Aggarwal in India, amongst a total of 500 isolates from different clinical specimens, ESBL production was observed in 229 isolates (45.8%) by the CLSI confirmatory test [17]. Maximum ESBL production was seen amongst *E. coli* strains (46.43%) and the $bla_{\text{CTX-M}}$ gene was detected in 59.32% of cases. In their study, 315 (63.0%) of the 500 isolates were resistant to at least three cephalosporins (third- or fourth-generation). In another study by Bali et al. in Turkey, bla_{TEM} and $bla_{\text{CTX-M}}$ genes were observed in 72.72% and 22.72% of *E. coli* isolates, respectively [18], which is different from the current study.

Comparison of the results of the present study with the survey conducted by Abdi et al. in Iran [19] revealed that the frequency of $bla_{\text{CTX-M}}$ is similar but the frequency of bla_{TEM} (82%) was higher than the current results. Finally, the results of the study by Nimri and Azaizeh in Jordan [20] in a university hospital showed that 83 (50.3%) of 165 tested *E. coli* isolates were ESBL-producers. Also, of the 67 isolates with at least one ESBL gene, 47 (70.1%) had either $bla_{\text{CTX-M}}$ (28 isolates) or bla_{TEM} genes (19 isolates), whilst 20 (29.9%) isolates had both $bla_{\text{CTX-M}}$ and bla_{TEM} genes simultaneously; the rates of bla_{TEM} and $bla_{\text{CTX-M}}$ in the current study were 32.5% (27/83) and 72 (86.7%), respectively. Also, both $bla_{\text{CTX-M}}$ and bla_{TEM} genes were observed in 24 isolates (28.9%).

In conclusion, these findings showed a direct relationship between the development of resistance to β-lactam antibiotics and production of TEM and CTX-M enzymes. ESBL-producing bacteria have found their way out of the hospital, which could be considered as a risk factor for society.

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Competing interests

None declared.

Ethical approval

Not required.

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