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Extended-spectrum beta-lactamases detection and prevalence of *bla$_{TEM}$* gene in clinical isolates of *Klebsiella pneumoniae* from hospitals in North of Iran

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ABSTRACT

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family which plays an important role in creating various infections in the community. These strains are resistant to multiple β-lactam antibiotics due to the production of β-lactamases enzyme (ESBL). The purpose of this study was to assess the phenotypic and genotypic characteristics of ESBL-producing *K. pneumoniae* strains from clinical specimens in three hospitals in Mazandaran, Iran. In this cross-sectional study, *K. pneumoniae* samples (N = 100) were identified from different clinical specimens after standard biochemical and microbiological tests. Disc agar diffusion test was applied for antibiotic-resistant examinations. Phenotypic detection of ESBL-producing isolates was performed using mixed disk method. The presence of *bla$_{TEM}$* gene was investigated in ESBL-producing isolates using PCR method. The ESBL test analysis was positive for 40 isolates (40%) of *K. pneumoniae*. The prevalence of *bla$_{TEM}$* gene in ESBL-producing *K. pneumoniae* isolates was 55%. Tetracycline, tobramycin, and ampicillin were the most active antibiotics against *K. pneumoniae* isolates, showing 85%, 81% and 73% sensitivity, respectively. The highest antibiotic resistance in isolated *K. pneumoniae* was found for ceftriaxone (48%) and cefotaxime (46%) antibiotics. There was a significant correlation between ESBL production and *K. pneumoniae* isolates resistance to cefotaxime ($p = 0.000$), ceftazidime ($p = 0.001$), ciprofloxacin ($p = 0.001$), tobramycin ($p = 0.044$), and ceftriaxone ($p = 0.000$). The prevalence of ESBL-producing *K. pneumoniae* was high and increasing. The high prevalence of *bla$_{TEM}$* gene in these isolates may be a reason for their pathogenesis and multiple-antibiotic resistance. Therefore, there is a need to develop the strategies to manage antibiotic resistance in these isolates.

Keywords: Beta-lactamase; Bla$_{TEM}$ gene; *Klebsiella pneumoniae*; PCR; Phenotypic and genotypic studies.
Article type: Research Article.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen and a member of the *Enterobacteriaceae* family which causes severe infections and serious public health problems such as urinary tract infection and pneumonia in hospitalized patients (Sarojamma & Ramakrishna 2011; Taneja *et al*. 2010). These complications can be associated with mortality and morbidity in patients, especially in immunocompromised patients (Moini *et al*. 2015). *K. pneumoniae* isolates are resistant to multiple β-lactam antibiotics due to the production of extended-spectrum β-lactamases (ESBL) enzymes. ESBL are plasmid-mediated enzymes that mediate resistance to a wide range of antibiotics such as penicillins, cephalosporins, and clavulanic acid (Keynan & Rubinstein 2007). Therefore, increase in the spread of antimicrobial resistance in *K. pneumoniae* isolates, which has been reported by epidemiological studies, is due to the acquisition of plasmid-containing ESBL genes (Giamarellou 2010). So that, these isolates have become an important threat to human health worldwide and, therefore, treatment of these multiple antibiotic-resistant strains is now considered as a great challenge in the community (Ahmed *et al*. 2012).

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The *bla$_{TEM}$* genes, which are located on a family of related β-lactamase plasmids, are a main reason for antibiotic resistance and pathogenesis of *K. pneumonia*e clinical isolates (Lagha *et al*. 2014). The presence of ESBL-producing *K. pneumonia*e and their clinical resistance to multiple antibiotics has also been verified in many countries. However, the prevalence of ESBL-producing isolates varies from a geographical place to another (Read *et al*. 2013, Deguchi *et al*. 2003). Therefore, molecular characterization of ESBL-producing isolates in each place is necessary in allowing hospitals and also the clinical and laboratory standard institutes to identify the sources of these pathogenic bacteria as well as to prevent the spread of resistant strains.

Currently, there is a relatively little information about the ESBL-producing *K. pneumoniae* isolates from Mazandaran Province, north of Iran. Thus, a molecular characterization study was performed on urine specimens infected by ESBL-producing *K. pneumoniae,* collected from three hospitals in the province. This study constituted a primer report on high prevalence of blaTEM gene in ESBL-producing isolates of *K. pneumoniae* and denotes the need of more extensive studies on these antibiotic genes to determine the magnitude of the problem of antibiotic resistance exiting in this locality.

MATERIALS AND METHODS

Samples collection

In this cross-sectional study, a total number of 100 urine samples, positive for *k. pneumoniae* upon initial culture, collected from the laboratories of three hospitals (Imam Ali, Imam Reza and Dr. Shafaei) in Amol, Iran during February to June 2017. The strains were collected from patients with urinary infection. Isolates were initially cultured in MacConkey agar media for 24 h at 37 ºC. Standard biochemical and microbiological tests such as gram staining, eosin methylene blue, catalase, oxidase, lactose, sucrose, glucose and D-sorbitol fermentation, indole, sulfide, motility (ISM), spore and gas production, triple sugar iron agar (TSI), Voges-Proskaure (VP), citrate and urease tests were performed for the identification of *K. pneumoniae* isolates.

Antibacterial activity test

Disc agar diffusion (DAD) test using Bauer method according to CLSI procedure was applied for the assessment of antibacterial effects of several antibiotics against *K. pneumoniae* isolates. The clinical *K. pneumoniae* strains (1.5×10^8 CFU mL^{-1}) were spread onto the surface of the Muller Hinton Agar (MHA) with a sterile swab. Ampicillin (10 µg), amoxicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), tetracycline (30 µg), ciprofloxacin (10 µg), tobramycin (10 µg), co-trimoxazole (25 µg), and ceftriaxone (30 µg) disks were used as antibiotics. All antibiotics were purchased from the PADTAN TEB Company (Tehran, Iran). The agar plates were incubated for 24 h at 37 ºC and the diameter of the zone of inhibition for each microorganism was measured. All tests were performed as triplicate.

Phenotypic detection of ESBL-producing isolates

K. pneumoniae isolates were initially cultured in MHA and then extended-spectrum beta-lactamase (ESBL) production was tested with the CLSI confirmatory test using both ceftazidime (30 mg) and cefotaxime (30 mg) disks alone and in combination with clavulanic acid (10 mg). Plates were incubated at 37 ºC for 24 h and the diameter of the zone of inhibition was evaluated. When an increased growth-inhibitory zone was found around either the ceftazidime or the cefotaxime disk with clavulanic acid (5 mm), or a greater diameter was observed around the disk containing cefotaxime or ceftazidime alone, the isolates were considered positive for ESBL production.

Detection of *bla$_{TEM}$* gene by PCR

The *bla$_{TEM}$* gene was investigated in ESBL-producing isolates by PCR method using specific primers designed in NCBI (Table 1). *K. pneumoniae* isolates were initially cultured in 5 mL nutrient broth medium at 37 ºC for 24 h. Cultured isolates were then transferred into a 1.5 mL microtube and centrifuged at 4000 rpm for 5 min. Supernatants were removed and pellets were applied for DNA extraction. DNA was extracted using a specific commercial kit provided from CinnaGen Company (Iran, Tehran). Briefly, 100 µL prelysis and 20 µL ribotinase solutions were added to the pellets and incubated at 55 °C for 30 min. Then, 400 µL lysis buffer was added to tubes and vortexed for 5 second, followed by adding 300 µL precipitation solutions to these tubes and transferring the solution to a spin column using a collection tube and centrifuging at 13.000 rpm for 1 min. Afterwards, spin column was placed in a new collection tube and 400 µL wash buffer I was added to it and centrifuged at 13000

rpm for 1 min, followed by placing the spin column in a new collection tube, adding wash buffer II and centrifuging at 13000 rpm for 1 min. Column was transferred to a new 1.5 mL-tube, then 50 µL elution buffer was added and incubated for 3-5 min at 65 °C. The solution was centrifuged at 13000 rpm for 1 min and DNA was separated from the supernatants. The quantity and quality of extracted DNA were evaluated using Nanodrop (Thermo 2000) and agarose gel electrophoresis methods, respectively (Ramazanzadeh *et al*. 2015). The supernatants containing the DNA were stored at -20 °C for further analyses.

Table 1. Primers used for *bla*$_{TEM}$ amplification

Primer	Sequences	Product size
Forward	5'- AGTGCTGCCATAACCATGAGTG -3'	432 bp
Reverse	5'- CTGACTCCCCGTCGTGTAGATA -3'	

PCR amplification was carried out in a 20 µL reaction mixture with each primer (contained 2.5 µL buffer, 0.75 µL $MgCl_2$, 0.5 µL dNTP, 0.2 µL Taq DNA polymerase, and 1 µL of each primer) as the following steps: an initial denaturation step at 94 °C for 5 min; followed by 33 cycles, including denaturation at 94 °C for 60 sec, annealing at 61 °C for 60 sec, elongation at 72 °C for 60 sec and a final extension at 72 °C for 5 min. The PCR products were electrophoresed in a 1% agarose gel for 20 min at 70-120 V. The gels were then stained with ethidium bromide and visualized using UV transilluminator.

Statistical analyses

Descriptive statistics was applied for the analysis of frequencies among patients group. Data were analyzed using SPSS software (version 19).

RESULTS

In this study, *Klebsiella pneumoniae* was isolated from clinical samples of 100 patients with urinary infections. The mean age of patients was 36.6 ± 17.22 years (ranged from 7 days to 75 years) and 68% out of them were female. The number of isolated *K. pneumoniae* from each ward is summarized in Table 2. Most of the isolates were from outpatients unit (80%), while only a few numbers were from NICU (2%) and surgery (2%).

Table 2. Distribution of *K. pneumoniae* isolates in each ward

Clinical specimens (N = 100)	Frequency (%)
Outpatients unit	80 (80%)
Emergency ward	7 (7%)
Internal medicine	6 (6%)
PICU	3 (3%)
NICU	2 (2%)
Surgery	2 (2%)
Total	100

NICU: Neonatal Intensive Care Unit; PICU: pediatric intensive care unit.

The biochemical and microbiological test results for *K. pneumoniae* isolates are shown in Table 3. The results for oxidase, SIH, methyl red tests, and spore production were negative, while the catalase test, lactose, glucose, sucrose and D-sorbitol fermentations, gas production, urease and citrate tests, and Voges-Proskaure (VP) were positive.

The antibiotic susceptibility of isolated *K. pneumoniae* assessed by the disc agar diffusion is shown in Table 4. The highest antibiotic resistance in the isolates was found for ceftriaxone (48%) and cefotaxime (46%), while tetracycline was the most effective antibiotic against the isolates showing 85% sensitivity. Thereafter, tobramycin and ampicillin were the most effective antibiotics against the isolates showing 81% and 73% sensitivity, respectively. Among the 100 *K. pneumoniae* isolates, 17, 13, 12, 11, 7, 3, 2 and 1 isolates were resistant to 1, 2, 5, 3, 7, 6 ,8 and 9 antibiotics respectively.

Table 3. The biochemical and microbiological test results for *K. pneumoniae* isolates

Biochemical tests	Results	Biochemical tests	Results
Oxidase test	-	Catalase test	+
Lactose fermentation	+	Sucrose fermentation	+
D-sorbitol fermentation	+	Glucose fermentation	+
Indole	-	Methyl red	-
Sulfide	-	TSI	A/A/gas
Motility	-	Spore production	-
Vogesproskaure	+	Gas production	+
Citrate	+	Urease	+

Table 4. Antimicrobial resistance rates of *K. pneumoniae* isolates

Antibiotics	Resistant (R)	Semi-sensitive (I)	Sensitive (S)
Ampicillin	22%	5%	73%
Amoxicillin	39%	6%	55%
Cefotaxime	46%	10%	44%
Ceftazidime	36%	22%	42%
Tetracycline	9%	6%	85%
Ciprofloxacin	27%	15%	58%
Tobramycin	11%	8%	81%
Co-trimoxazole	27%	4%	69%
Ceftriaxone	48%	10%	42%

The ESBL test analysis was positive for 40 *K. pneumoniae* isolates (40%). Molecular analysis of bla_{TEM} gene by PCR method in 20 ESBL-producing *K. pneumoniae* isolates showed that 11 isolates (55%) were positive (Fig. 1).

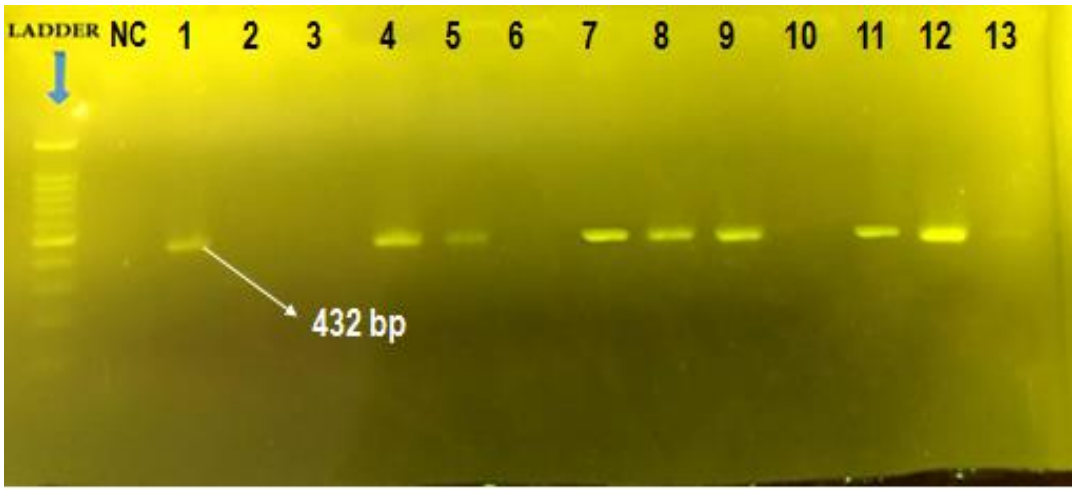

Fig. 1. Agarose gel electrophoresis of PCR-amplified bla_{TEM} gene. Lanes 1, 4, 5, 7, 8, 9, 11, 12 and 13: *K. pneumoniae* isolates showing 432 bp bla_{TEM} amplicon. Lanes 2, 3, 6 and 10: *K. pneumoniae* isolates without bla_{TEM} amplicon. NC: negative control.

There was a significant correlation between ESBL production and *K. pneumoniae* isolates resistant to cefotaxime ($p = 0.000$), ceftazidime ($p = 0.001$), ciprofloxacin ($p = 0.001$), tobramycin ($p = 0.044$), and ceftriaxone ($p = 0.000$). We also found a significant relationship between age and *K. pneumoniae* isolates resistant to Tetracycline ($p = 0.003$) and Ciprofloxacin ($p = 0.036$). There was no significant correlation between gender and antibiotic resistance.

DISCUSSION

Multi drug resistant (MDR)- *K. pneumoniae* has become a universal important problem and a large number studies from different parts of world have been conducted in recent years (Rezaee *et al*. 2011). Similarly, MDR-*K. pneumoniae* strains have become a major treating infection in tertiary care hospitals in Iran. So, information about

the incidence of MDR *K. pneumoniae*, particularly ESBL-producing isolates, in a hospital or society and its treatment has been of great concern for public health. In the present study, we investigated the prevalence of ESBL-producing *K. pneumoniae* isolates and also bla$_{TEM}$ gene in three hospitals, Mazandaran Province, Iran. By following the CLSI screening criteria, 40% of isolates were positive in ESBL production. In contrast to CLSI phenotypic confirmatory test for ESBL production, the genotypic detection test for bla$_{TEM}$ gene was positive for only 55% of isolates. This result suggests the high prevalence of ESBL-producing *K. pneumoniae* in this geographical region. We also found that tetracycline, tobramycin, and ampicillin were the most active antibiotics against the isolates showing 85%, 81% and 73% sensitivity, respectively. In contrast, the highest antibiotic resistance of the isolates was found for ceftriaxone (48%) and cefotaxime (46%). Interestingly, we found a significant relationship between ESBL production and *K. pneumoniae* isolates resistance to cefotaxime, ceftazidime, ciprofloxacin, tobramycin, and ceftriaxone antibiotics. We also found a significant relationship between age and the isolate resistance to tetracycline and ciprofloxacin, while there was no significant correlation between gender and antibiotic resistance. Therefore, by increasing the resistance of MDR-*K. pneumoniae* clinical isolates to these antibiotics and their spread in Iranian hospitals, the government and regulatory agencies should impose strict statutory guidelines for rational use of antibiotics. There are many studies on the prevalence of bla$_{TEM}$ gene in ESBL-producing *K. pneumoniae* isolates in different hospitals. For example, Shakib *et al*. (2018) considered the prevalence of bla$_{TEM}$, bla$_{SHV}$, bla$_{CTXM}$, *bla$_{CTX\text{-}M\text{-}15}$*, *bla$_{PER}$* and *bla$_{VEB}$* and antibiotic-resistance patterns in *K. pneumoniae* isolated from 52 clinical specimens in west of Iran. They showed that the highest and lowest rates of resistance were related to co-trimoxazole (67.3 %) and amikacin (30.7 %) respectively. 55.7% of isolates were multidrug-resistant and 69.23% were positive for ESBL-production, while in our study, 40% of isolates were positive for ESBL production. They also found that bla$_{\text{TEM}}$, bla$_{CTXM}$, and *bla$_{CTX\text{-}M\text{-}15}$* -producing *K. pneumoniae* strains had higher rates of antibiotic resistance compared to negative strains. However, In the present study, only 27% of clinical isolates were resistant to co-trimoxazole which was lower than that reported by Shakib *et al*. (2018).

In another study conducted in Iran, Maleki *et al*. (2018) investigated the prevalence of *bla$_{TEM}$* in 98 *K. pneumoniae* isolates from patients with urinary tract infection in Isfahan, Iran. They found that 25.5% of isolates were ESBL-producing, while 44.9% were multidrug-resistant. In Addition, 19 (76%) isolates were *bla$_{TEM}$* positive. There was a high resistance rate for cefotaxime and ceftazidime which is in agreement with the results of the present study. Interestingly, all ESBL-producing isolates were resistant to cefotaxime (Maleki *et al*. 2018). More recently, Mahmoudi *et al*. evaluated the antimicrobial resistance and the prevalence of *bla$_{TEM}$* in 30 *K. pneumoniae* clinical isolates in an Iranian referral hospital (Mahmoudi *et al*. 2019) reporting a high rate of resistance to cefotaxime (97%), cefazolin (97%), cefexime (83%) and gentamicin (77%) concluding that a total of 29 isolates (97%) were ESBL-producing. The frequency of bla$_{TEM}$ gene in these isolates was 57%. There are also several reports from different countries. For instance, in a study in Northeast India, Bora *et al*. (2014) considered the prevalence of *bla$_{TEM}$* gene in clinical isolates of *K. pneumoniae*. The phenotypic confirmatory test identified ESBL production in 67.24% of clinical isolates. Molecular test analysis revealed that *bla$_{TEM}$* was the most prevalent gene (77.58%) in ESBL-producing *K. pneumoniae* isolates. In another study in India, Parveen *et al*. (2011) reported the prevalence of ESBL-producing status in 37 *K. pneumoniae* isolates collected from clinical specimens of blood cultures. They found that 36 isolates (97.2%) were ESBL positive upon phenotypic testing. In addition, the majority of ESBL-positive isolates were MDR exhibiting 95%, 87% and 92% resistance to gentamicin, ciprofloxacin and ceftriaxone, respectively. Only 21% and 5% of these isolates showed resistance to amikacin and meropenem, respectively. The prevalence of *bla$_{TEM}$* -positive strains was 82%. There was another study in China on the prevalence of ESBL-producing *K. pneumoniae* in clinical isolates in 31 hospitals (Zhang *et al*. 2016), reporting that 31.8% of isolates carried ESBL genes and also bla*CTX-M-14*, bla*CTX-M-15*, and bla*CTX-M-3* were the most prevalent ESBL genes in these isolates. dos Santos *et al*., (2008) evaluated the incidence of ESBL-producing *K. pneumoniae* isolates in two hospitals in Brazil reporting high prevalence of ESBL-producing *K. pneumoniae* (25% in hospital A and 66.7% in hospital B), with high rates of antimicrobial resistance. Imipenem was the most active antibiotic (with 100% susceptibility) against these isolates.

Therefore, the prevalences of ESBL-producing *K. pneumoniae* in clinical isolates from different geographical localities are variable. More importantly, the prevalence rate of ESBL-producing *K. pneumoniae* strains is increasing throughout the world, which raises concerns regarding the treatment and appropriate use of antibiotics for MDR strains.

CONCLUSION

Our findings showed that the prevalence of ESBL-producing *K. pneumoniae* in clinical isolates in Mazandaran Province hospitals is high and emerging which can lead to various health difficulties. More importantly, the frequency of bla_{TEM} gene in these isolates, particularly in MDR isolates, is high which may be a reason for its pathogenesis and multiple antibiotic resistant. Therefore, there is a need to develop the strategies to manage antibiotic resistance in these isolates.

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