

Prevalence of CTX-M and TEM β -lactamases in *Klebsiella pneumoniae* Isolates from Patients with Urinary Tract Infection, Al-Zahra Hospital, Isfahan, Iran

Abstract

Background: Extended-spectrum β -lactamase (ESBL)-producing is a significant resistant mechanism to β -lactams in *Enterobacteriaceae*, especially in *Klebsiella pneumoniae*. The main objectives of this study were to genetically characterize urinary clinical isolates of *K. pneumoniae* through the investigating of *blaTEM*, *blaCTX-M* and using molecular typing by Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) method. We also determined the frequency of antibiotic resistance of *K. pneumoniae* strains to characterize the β -lactamases included. **Materials and Methods:** A cross-sectional study was carried out to evaluate 98 strains of *K. pneumoniae* isolated from urine culture of outpatients referred to Al-Zahra Hospital, Isfahan, Iran. Antibiotic susceptibility testing was performed using Kirby-Bauer's method. Screening of ESBLs was carried out using double-disk screening test. PCR technique was performed to detect *TEM* and *CTX-M* genes. The total DNA of each strain was tested by ERIC-PCR. **Results:** In 98 *K. pneumoniae* studied clinical isolates, 25.5% were ESBL producing and 44.9% multidrug-resistant (MDR). From 25 ESBL isolates, 23 (92%) cases showed MDR phenotype. In ESBL producing isolates, 23 (92%) were *blaCTX-M* and 19 (76%) *blaTEM* positive. The antimicrobial drug susceptibilities of ESBL isolates indicated high resistant rates for cefotaxime and ceftazidime. All 25 ESBL producing isolates were resistant to cefotaxime. Complex patterns of fingerprints isolates showed that 36% of the isolates were belonged to the cluster no 5. **Conclusion:** This study revealed high antimicrobial resistance rates among ESBL isolates which can lead to various health difficulties. Epidemiological data collection from patients is recommended to develop the strategies to manage antibiotic resistance.

Keywords: Antimicrobial resistance, *blaCTX-M*, *blaTEM*, *Klebsiella pneumoniae*, multidrug-resistant

Introduction

Klebsiella pneumoniae is one of the most important human bacterial pathogens, result to the extensive range of community and hospital acquired infections that may lead to morbidity and mortality, particularly in immunocompromised patients.^[1,2] Urinary tract infections (UTIs)-induced by multidrug-resistant (MDR) *K. pneumoniae* isolates are a serious public health problem. The potency of many antimicrobial agents have been limited, also these bacteria are hard and expensive to cure.^[3]

During the past decade, drug companies improved many novel Gram-positive antimicrobial agents to fight MDR bacteria, but unfortunately, the growing problem of MDR in Gram-negative bacteria

was not paralleled by the developing of novel antimicrobials.^[4] Estimation of epidemiological data caused by MDR *K. pneumoniae* in various parts of the world indicated to increased prevalence with distinct variety in many regions. These divergences require geographical surveys due to their resistance patterns and risk factors.^[2]

Recently, the report of the Infectious Diseases Society of America particularly referred extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella* spp. as a category of MDR Gram-negative bacilli.^[5]

β -lactams are the most usual drugs against bacterial infections and extended spectrum β -lactamase (ESBL) producing

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is a significant resistant mechanism to β -lactams in *Enterobacteriaceae*, especially in *K. pneumoniae*.^[6,7]

ESBLs producing isolates mainly exists in hospitals and frequently in Intensive Care Units (ICUs), but recently have been developed in the community.^[8]

ESBLs belong to the Ambler class A, display resistance to β -lactam antibiotics except cephamycins and carbapenems, and are inhibited by clavulanic acid.^[9] A previous similar study in China was shown high rates of MDR, extensively drug-resistant and pandrug-resistant strains among *K. pneumoniae* isolates, also most of these antibiotic resistance genes were transferable.^[1]

TEM and *SHV* types are the first ESBLs have developed by genetic mutation from native β -lactamases. New classes of ESBLs have emerged such as *CTX-M*, *PER*, *VEB*, *TLA-1*, *GES/IBC*, *SFO-1*, and *BES-1*. However, *CTX-M* enzymes have been widely detected worldwide. The prevalence of the different types of ESBLs varies by region and the clinical state and is changing over time.^[6]

Recently, the *CTX-M* enzymes seem to be more prevalent throughout the world, whereas other ESBLs of the *SHV* and *TEM* subgroups appear to decrease. Several nosocomial outbreaks caused by *CTX-M* producing *K. pneumoniae* because of misuse of broad-spectrum antibiotics, especially in ICU or immunodeficient patients.^[7-9]

The *CTX-M* family was grouped based on sequence similarity into four distinct subtypes included *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, and *CTX-M-9*.^[10]

ESBLs derived from *TEM*-and *SHV*-type penicillinases, have been extending in Spain, the United Kingdom, Canada, China, and Korea, especially in the *Enterobacteriaceae* including *K. pneumoniae* and *E. coli*.^[7,10] In contrast with *TEM* and *SHV* type ESBLs, most of the *CTX-M* producers show degrees of resistance to cefotaxime and ceftriaxone.^[10]

The phenotype of β -lactams resistance may propose the existence of *CTX-M* enzymes; therefore, polymerase chain reaction (PCR) has been performed greatly to identify *bla* genes.^[10]

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) produce specific strain patterns by amplification of repetitive DNA elements. ERIC-PCR has demonstrated the microbial variety and functional evolutionary of microorganisms.^[11]

The main objectives of this study were to genetically characterize urinary clinical isolates of *K. pneumoniae* through the investigating of *blaTEM*, *blaCTX-M* (the two most common genes) and using molecular typing by ERIC-PCR. We also determined the frequency of antibiotic resistance of *K. pneumoniae* strains to characterize the β -lactamases included and to highlight the genetic variety of these isolates.

Materials and Methods

Study design and population

This retrospective cross-sectional study was carried out from February 2015 to December 2015 in Al-Zahra Hospital, affiliated to Isfahan University of Medical Sciences.

Subjects and methods

During the study, a total of 98 nonduplicate clinical isolates of *K. pneumoniae* were recovered from urine specimens of patients that were referred to different wards of Al-Zahra Hospital, Isfahan, Iran.

Subsequent taking informed permission from the patients, their demographic information, including age and gender were completed in question sheets. All the parts of the study were supervised and approved by the Ethical Committee of Isfahan University of Medical Sciences (code 394573).

Sample collection and microbial detection

Urine specimens were cultured on blood agar and MacConkey agar plates (Hi-Media, India) at 37°C for 24 h. The grown isolates were recognized by the morphology of colonies, Gram-staining, and biochemical tests properties. *K. pneumoniae* colonies on blood agar medium were large, cupola shape, and mucoid that move to merge. On MacConkey medium, *K. pneumoniae* colonies were large, mucoid dark pink, which specifies the lactose fermentation. The biochemical tests for *K. pneumoniae* included of negative indole, positive urease, positive VP, positive Simmons' citrate agar, variable magnetic resonance, and no motile. Bacterial isolates were refrigerated on nutrient agar plates or frozen (-70°C) in microtubes containing 15% glycerol and tryptic soy broth medium.^[2] Afterward, molecular confirmation was performed by PCR of the *ureD* gene. Analysis for the presence of the *ureD* gene demonstrated that all the isolates confirmed as *K. pneumoniae* were positive for the *ureD* gene.

Antibiotic susceptibility

The estimate of individuals for antibiotic susceptibilities was performed using disk diffusion method (Kirby-Bauer's) as stated by the Clinical and Laboratory Standards Institute (CLSI) guidelines.^[12] The following antibiotics were tested: piperacillin/tazobactam (100/10 μ g), cephalothin (30 μ g), cefotaxime (30 μ g), imipenem (10 μ g), ceftazidime (30 μ g), gentamicin (10 μ g), tetracycline (30 μ g), tigecycline (15 μ g), ciprofloxacin (5 μ g), colistin (50 μ g), trimethoprim-sulfamethoxazole (TS) (1.25/23.75 μ g), and nitrofurantoin (300 μ g). The antibiotic disks were obtained from MAST, UK. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive control strains, respectively.^[12]

Phenotypic extended spectrum β -lactamase recognition

K. pneumoniae isolates were screened for possible ESBL production using ceftazidime and ceftoxitin disks. These strains were further tested for ESBL producing by double-disk synergy test (DDST). This phenotypic assay is based on the exhibition of a synergy image between disks with cefotaxime (30 μ g) and ceftazidime (30 μ g), which were placed about 20–30 mm from a disk with clavulanic acid (10 μ g). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.^[10]

Molecular characterization of resistance genes

Bacterial genomic DNA of suspected ESBLs isolates was extracted directly from 24 h colonies on 5% sheep blood agar plates (Hi-Media, India) by boiling a dense suspension in sterile distilled water for 10 min and centrifugation for 2 min at 13,000 \times g.^[13] ESBL-encoding genes were recognized using specific primers for the *bla*TEM gene (forward primer: 5'AGTATTCAACATTTCCGTGTCG3' and reverse primer 5'GCTTAATCAGTGAGGCACCTATC3') and *bla*CTX-M gene (forward primer: 5'CGTGCTGATGAGCGCTTTGC 3' and reverse primer 5'AGATCACCGCGATATCGTTG 3'). DNA amplification was performed using the following thermal cycling conditions: Initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing temperature of 59°C for *bla* CTX-M and 61°C for *bla*TEM for 25 s and extension at 72°C for 45 s followed by a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis for 1 h at 80 V in 1.5% agarose gel containing 0.05 mg/L safe view to detect specific bands in 0.5X TAE buffer. Primers were purchased from Metabion (Munich, Germany), Master Mix from Amplicon and other molecular materials were bought from Cinnagen, Iran. *K. pneumoniae* ATCC 7881 was used as a positive control strain.

Enterobacterial repetitive intergenic consensus polymerase chain reaction

The total DNA of each strain was tested by ERIC-PCR. DNA was amplified using the primers ERIC-R: 5'-AAGCTCCTGGGGATTCA-3' and ERIC-F: 5'AGTAAGTGACTGGGGTGAGCG-3' with the following program: Initial denaturation at 95°C for 5 min, with the next forty cycles includes a denaturation step at 92°C for 30 s; annealing at 60°C for 45 s; extension at 72°C for 30 s; followed by a final extension step at 72°C for 10 min and final storage at 4°C. The ERIC-PCR reaction was performed in a 20 μ L volumes containing 8 μ L deionized water, 10 μ L Master Mix (Amplicon), 0.5 μ L of each Primer, and 1 μ L of the crude extract DNA.

The ERIC-PCR products were screened by electrophoresis for 45 min in electric field of 100 V on 1.5% agarose gel. The 100 base pair DNA marker (Cinnagen) was used and the gels were subsequently stained for 30 min in a solution containing 0.5 mg of safe view per milliliter.^[14]

Computer-assisted enterobacterial repetitive intergenic consensus polymerase chain reaction DNA fingerprint analysis

ERIC-PCR fingerprints of amplified DNA fragments were visually compared. The difference of the patterns by at least one band was classified as different strains. The 1.0 kb DNA marker was used as an external reference standard to normalize positions of the bands on the different lanes and gels. The existence of a given band was coded as 1 and the lack of a given band was coded as 0 in an information matrix and analyzed using the PyElph 1.4 software (*BMC Bioinformatics U.S*). Dendrograms of differences were set up for each case.

Definitions

MDR was explained as obtained insusceptibility to at least one agent in three or more antimicrobial classes.^[1]

Statistical analyses

Data were entered and analyzed using SPSS software version 20 (IBM U.S 2009). The description of the results was carried out by frequencies. For categorical variables, different groups were compared using the Chi-square test or Fisher's exact test. Logistic regression was used for analyzing independent variables that determine an outcome.

All probabilities were two-tailed and the value of $P = 0.05$ was considered to be statistically significant.

Results

During the period of our study, 98 strains of *K. pneumoniae* were isolated. Thirty-five (35.7%) cases were male and 63 (64.3%) were female. The age range of patients was 11–89 (42.41 ± 18.36) years. The antimicrobial susceptibility profile showed that colistin had the highest level of activity in these isolates, also susceptibility to imipenem was 80%. Carbapenems presented the highest levels of activity; imipenem inhibited from more than 80% of the isolates. More than 50% of the strains were susceptible to piperacillin-tazobactam and TS [Table 1].

From the 98 studied isolates, 25 (25.5%) were ESBL producers by the DDST, and 44 (44.9%) indicated MDR phenotype. Among ESBL producing isolates, 23 (92%) cases examined MDR ($P = 0.001$, odds ratio (OR) =28.47, confidence interval: 6.16–131.65). The prevalence of *bla*CTX-M and *bla*TEM producing *K. pneumoniae* isolates were assessed 24 (24.5%) and 18 (18.4%), respectively. In 25 ESBL producing strains, 23 (92%) *bla*CTX-M and 19 (76%) *bla*TEM were positive [Figures 1 and 2].

The antimicrobial drug susceptibilities of ESBL producing *K. pneumoniae* isolates showed the high resistant rates for cefotaxime and ceftazidime (100% and 88%), respectively, and the high sensitivity rates for colistin and tigeicycline (100% and 92%), respectively [Table 2].

All 23 *blaCTX-M* producing *K. pneumoniae* isolates examined were resistant to cefotaxime, and all but one isolate of *blaTEM* were resistant to cefotaxime.

Significantly, the rates of resistance to most antibiotics were much higher among ESBL positive isolates than ESBL negative isolates ($P = 0.038$). No significant statistical differences were found between gender or age group and MDR, ESBL, and anti-microbial resistant ($P > 0.05$).

The genomic diversity analysis of 25 strains of ESBL strains of *K. pneumoniae* has been carried out using the ERIC-PCR fingerprinting method with ERIC-type primer.

The ERIC-PCR profiles revealed differentiation of the 23 band pattern which grouped into six main cluster with a maximum of 24% similarity. Complex patterns of fingerprints isolates showed that 36% of the isolates were belonged to the five cluster [Figure 3].

Table 1: Percentage of antimicrobial susceptibility of 98 strains of *Klebsiella pneumoniae*

Antibiotic name	%S	%I	%R
Ceftazidime	58.2	3.1	38.8
Cefoxitin	67.3	6.1	26.5
Cefotaxime	53.1	3.1	43.9
Ciprofloxacin	76.5	20.4	3.1
Colistin	98	0	2
Gentamicin	79.6	1	19.4
Imipenem	83.7	5.1	11.2
Nitrofurantoin	50	13.3	36.7
Piperacillin/tazobactam	73.5	10.2	16.3
Tetracycline	59.2	2	38.8
Tigecycline	94.9	0	5.1
Trimethoprim/sulfamethoxazole	56.1	0	43.9
Cefalotin	49	2	49

S: Sensitive, I: Intermediate, R: Resistance

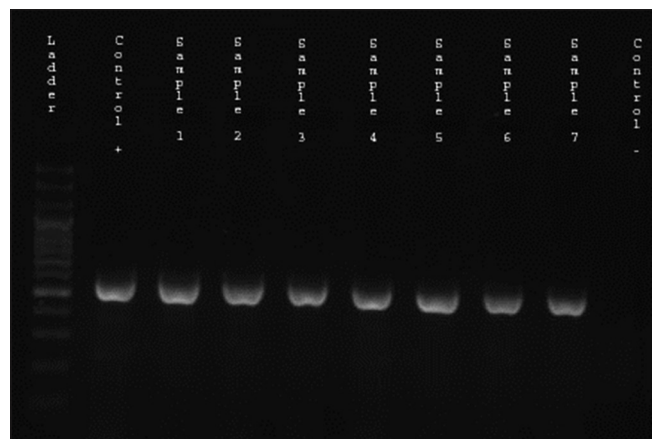


Figure 1: Gel electrophoresis of polymerase chain reaction products following amplification with specific primer for *blaCTX-M* gene (568 bp) Lane 1: The 100 bp ladder, Lane 2: *blaCTX-M* positive control, Lane 3–9: Positive clinical isolates for *blaCTX-M* gene, Lane 10: Negative control

Discussion

UTIs are one of the most common infectious diseases, and *K. pneumoniae* is one of the most prevalent organisms causing MDR UTI. Most of the epidemiologic studies focus on antibiotic resistance patterns in different regions.^[3] Therefore, in this study, we determined the frequency of antibiotic resistance of *K. pneumoniae* strains and the genetic diversity of these isolates in outpatient referred to Al-Zahra Hospital, Isfahan, Iran.

In general, urinary *K. pneumoniae* isolates have high rates of resistance to the commonly used antimicrobial agents.^[15] Our findings showed that colistin had the highest level of activity in these isolates, also susceptibility to imipenem was 80%. Carbapenems presented the highest levels of activity; More than 50% of the strains were susceptible to piperacillin-tazobactam and trimetoprim/sulfamethoxazole. Thus, the cross-resistance with cepheims could be related to the misusing of broad-spectrum antibiotics such as penicillins, cephalosporins, chloramphenicol, tetracyclines, and aminoglycosides.

Other studies that performed in Iran (Tehran, Zahedan) and also in Algeria had shown high rates of resistance to carbapenems, β -lactams, ciprofloxacin, aminoglycosides, but intermediately susceptible to tigecycline and susceptible to gentamicin. In spite of our study, high rates of colistin-resistant strains reported from Algeria.^[5,6,16-18]

The emergence of MDR *K. pneumoniae* is a worldwide important problem and many studies with different results has been conducted in recent years.^[19] In this study, the prevalence of MDR was 44.9% among the *K. pneumoniae* isolates and. In another study that performed in Iran, the prevalence of MDR *K. pneumoniae* isolates was reported 74%, which is more than our results. Furthermore, in other study that was conducted in Kashan, MDR *K.*

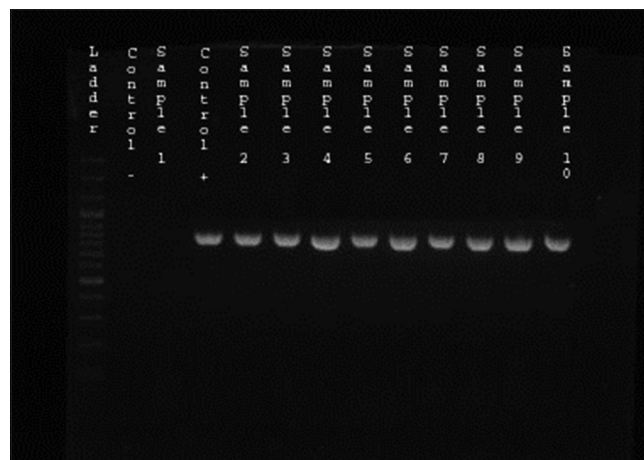


Figure 2: Gel electrophoresis of polymerase chain reaction products following amplification with specific primer for *blaTEM* gene (850 bp) Lane 1: The 100 bp ladder, Lane 2: *blaTEM* negative control, Lane 3: Negative clinical isolate, Lane 4: Positive control, Lane 5–13: Positive clinical isolates for *blaTEM* gene

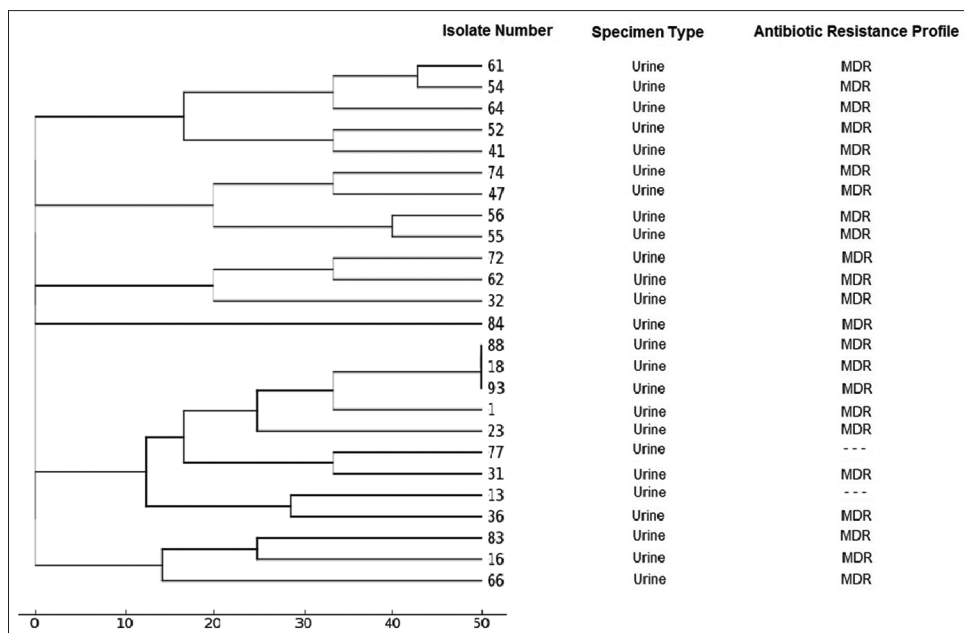


Figure 3: Dendrogram based on the cluster analysis (UPGMA) of the estimate of genetic similarity by ERIC - polymerase chain reaction drawn with PyElph 1.4 software

Table 2: Antibiotic susceptibility pattern of *Klebsiella pneumoniae* extended spectrum β-lactamase producing strains with disk diffusion method

Antibiotic name	Antibiotic class	%S	%I	%R
Cefotaxime	Cephems	0	0	100
Cefoxitin	Cephems	24	12	64
Ceftazidime	Cephems	8	4	88
Cefalotin	Cephems	12	0	88
Ciprofloxacin	Quinolones	68	24	8
Colistin	Lipopeptides	100	0	0
Gentamicin	Aminoglycosides	44	4	52
Imipenem	Penems	84	4	12
Nitrofurantoin	Nitrofurans	48	12	40
Piperacillin/tazobactam	β-lactam + inhibitors	43.8	25	31.2
Tetracycline	Tetracyclines	25	6.2	68.8
Tigecycline	Tetracyclines	75	18.8	6.2
Trimethoprim/sulfamethoxazole	Folate pathway inhibitors	25	0	75

S: Sensitive, I: Intermediate, R: Resistance

pneumoniae rate was 46.6% and no imipenem resistance was reported.^[2] In others studies that were conducted on positive urine cultures for *K. pneumoniae* in China, Pakistan, and Bangladesh the percentage of MDR isolates were 63.8%, 71%, and 85%, respectively.

The probable explanation for this higher rates of MDR and antibiotic resistance, especially in developing countries may be attributable to irregular antibiotic prescription, genetic, geographic, social behaviors and sampling biases and different patients' characteristics.^[2] In this study, the rate of antibiotic resistance in unhospitalized is lower than hospitalized patients that mentioned better condition.

According to the present study, gender or age group were not known as risk factors for MDR strains ($P > 0.05$), despite findings of a similar study that was performed in Sudan showed the male gender as the MDR risk factor, which is inconsistent with our investigation. Moreover, no significant difference was found between MDR rate and age group according to the previous studies in Kashan, Iran, and Sudan, which is compatible with our research.^[2,20]

Recently, many studies have reported epidemic outbreaks of ESBL-producing *Enterobacteriaceae*.^[21,22]

The overall prevalence of ESBL-producing *K. pneumoniae* isolates altered significantly according to geographic districts of countries and the different hospital conditions. ESBL prevalence rate in our study is 25.5% that is upper compared to those reported in Northern Algeria (19.9%), Spain (20.8%), and Tunisia (20.2%). However, higher prevalence rates of ESBL-producing *K. pneumoniae* isolates were recognized in Tehran, Iran (69.7%),^[17] Zahedan, Iran (66.7%),^[16] Ahvaz, Iran (27%),^[23] Isfahan, Iran (40%),^[24] South America (45.4%–51.9%), France (26%) and Saudi Arabia (55%) and 70% in Pakistan.^[3,6] The reason for lower rate of antibiotic, antimicrobial resistance was that isolates were taking only from outpatients referred to hospital.

Antimicrobial susceptibility analysis of the ESBL-producing isolates found highly prevalent resistances to third generation cephalosporins and about 50% to gentamicins. Our results are similar to other study that was performed in Madagascar (100%) to third generation cephalosporins, 87.7% to gentamicins.^[9] It is now verified that the consumption of antibiotics, especially third generation cephalosporins, is the most major risk factor in the development of bacterial resistance.^[6]

From the 25 ESBL studied isolates, 23 (92%) cases examined MDR ($P = 0.001$, OR = 28.47) and this finding was according to many other similar studies. These results indicated that there is a strong association between infection with ESBL-producing *K. pneumoniae* and the use of several different antibiotics. For controlling of outbreaks of infection emphasis must be placed on the logical and sensible use of all antimicrobial agents.^[24]

The prevalence of *blaCTX-M* and *blaTEM* genes in ESBL strains were 92% and 76%, respectively. These results demonstrated a high proportion of *CTX-M* and *TEM* enzymes among ESBL-producing strains in outpatients referred to Al-Zahra Hospital. Our data were in accordance with other studies that performed in different parts of our country and showed the most prevalent genes were *CTX-M* types, *SHV* and *TEM*.^[17] In Morocco, ESBL-producing *Enterobacteriaceae* have been detected in different hospitals and the ESBL genes isolated in Morocco were *blaTEM*, *blaSHV*, *blaDHA*, and *blaOXA* types.^[25] ESBL-producing *K. pneumoniae* isolates are a serious problem and describes the high resistant rates to common antibiotics.^[3] *CTX-M*-producing *K. pneumoniae* isolates demonstrated rapid emergence and spread mid-to late-2000s in the United States.^[26]

The coexistence of *CTX-M* ESBL and *TEM* type β -lactamases in these isolates may have also contributed to the observed high rate of antimicrobial drug resistance.^[26] Most of the *CTX-M* ESBL positive strains in the current study were detected *TEM* positive (18/24). These data have clinical suggestion for selecting empiric antibiotic treatment when infection caused by ESBL-producing *K. pneumoniae* is suspected.^[26] Although pulsed-field gel electrophoresis is evaluated as the gold standard method for genotyping, ERIC-PCR is an alternative method for producing fingerprints of bacterial genomes, especially in *Enterobacteriaceae*.^[18,27] Our findings showed that 92% of the isolates were belonged to five independent clusters, therefore, there is considerable diversity of genetic types of *CTX-M* producers and complexity of the resistance phenotype was found.

There are several potential limitations to our study included failure to distinguish colonization from true infection, lack of a comparison group, small numbers of patients, the inclusion of only specific patient populations, and the limiting of more investigation of patients. It is necessary to mention that we did not perform sequencing as a preferred complementary procedure then we could not see the probable mutations and the mechanism of resistance.

Conclusion

The current study revealed relatively high antimicrobial resistance rates among ESBL isolates. About 11% of imipenem-resistant strains were found. Furthermore, the MDR rate compared with developed countries was high.

We found a diversity of the genotypes and the complexity of the resistance phenotypes. Epidemiological data collection from outpatients is recommended to develop the strategies to manage antibiotic resistance.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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