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Original Research

The prevalence of *CMY-2*, *OXA-48* and *KPC-2* genes in clinical isolates of *Klebsiella* spp.

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Abstract: *Klebsiella pneumoniae* is a Gram-negative bacterium which causes several human infections. Treatment of infections related to *K. pneumoniae* has become problematic, because of increasing trend of extended spectrum β-lactamases producing (ESBLs) strains. The present study was aimed to detect the prevalence of ESBL-producing *Klebsiella* spp. and *KPC-2*, *CMY-2* and *OXA-48* β-lactamase encoding genes in clinical isolates of *Klebsiella* spp. isolated from hospitalized patients. In this cross-sectional study carried out from February to August 2014, 144 isolates of *Klebsiella* spp. were collected from different clinical specimens in hospitals in the North of Iran. *Klebsiella* isolates were identified using standard microbiological procedure. Antimicrobial susceptibility patterns were determined using disk diffusion method in accordance with CLSI recommendation. The presence of antibiotic resistance genes were investigated by PCR method. Among 144 *Klebsiella* spp., 118 isolates were identified as *K. pneumoniae* and 26 isolates as *Klebsiella oxytoca*. Antibiotic susceptibility test showed the most resistance rates were against amoxicillin (96.5%) and ampicillin (95.8%). On the other hand, the most effective antibiotic was revealed to be imipenem with resistance rate of 4.9% (Table 2). Among 144 isolated *Klebsiella* strains, 57 cases (39.6%) were ESBL producers. The *OXA-48* and *KPC-2* genes were not detected among *Klebsiella* spp. during the present study, but 21.1% of isolates contained *CMY-2* gene. This is the first report of *CMY-2* gene detection in *Klebsiella* spp. in Iran. The homology between *CMY*-2 genes identified in isolates from Northern Iran and in other countries showed the wide dispersion of this gene around the world.

Key words: *Klebsiella pneumoniae*; Antibiotic resistance; ESBL; CMY-2 gene.

Introduction

Hospital-acquired infections have turned into a global health care concern due to their significant role in extending the normal hospitalization time and increasing the mortality and morbidity rates (1). The multidrug resistant Gram-negative bacteria belonging to the *Enterobacteriaceae* family are known as the most common nosocomial infection causing agents (2,3). *Klebsiella pneumoniae* is a Gram-negative bacterium which causes several infections, such as urinary tract infection (UTI), abscesses, wound infections, pneumonia and diarrhea (4).

Treatment of infections related to *K. pneumoniae* has become problematic, because of increasing trend of extended spectrum β-lactamases producing (ESBLs) strains. ESBLs are a group of enzymes capable of hydrolyzing penicillins, the third-generation cephalosporins, and the monobactams; thus causing resistance to these antibiotics in ESBL-producing bacteria. The majority of ESBL strains are also resistant to other antibiotics such as fluroquinolones and aminoglycosides (5,6). These enzymes are classified into four main groups including A, B, C, and D based on their inhibitory mechanism, type of substrate, and physical characterization such as molecular weight and isoelectric point (7,8). The mentioned phenotypic characteristics differentiate ESBLs from AmpC type β-lactamases, which comprise another group of enzymes commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria. AmpC β-lactamases are typically encoded on the chromosomes of many Gram negative bacteria including *Escherichia coli*, *Citrobacter freundii* and *Enterobacter* spp., but also may be found on plasmids (9). Despite ESBLs, AmpCs are not inhibited by clavulanic acid or other β-lactamase inhibitors. The most frequent gene existing in AmpC β- lactamase is bla_{CMY-2} (10).

On the other hand, additional enzymes called carbapenemases have been detected in *K. pneumoniae* isolates. Carbapenems stimulate the most consistent *in vitro* activity against ESBLs producing *K. pneumoniae*. Despite its being still rare in *Enterobacteriaceae*, resistance to carbapenems is escalating, thus this issues poses a major threat to the management of multidrug-resistant isolates (11,12). The carbapenemases detected in *K. pneumoniae* isolates are metallo-β-lactamases (13), plasmid-mediated clavulanic acid-inhibited β-lactamases (11,12), and the expanded-spectrum oxacillinase OXA-48 (14,15). The bla_{OXA-48} gene is plasmid borne and encodes a carbapenem-hydrolyzing class D β-lactamase which was first identified in a *K. pneumoniae* clinical isolate in Turkey in 2004 (14). This enzyme confers resistance to penicillins and reduced vulnerability to carbapenems, but spares expanded-spectrum cephalosporins (14).

Few efforts have been taken in detecting β-lactamases such as AmpC and carbapenemases enzymes in Gram-negative rods in the North of Iran. The present study

was aimed to detect the prevalence of ESBL-producing *Klebsiella* spp. and *K. pneumoniae* carbapenemase (*KPC-2*), cephamycinase (*CMY-2*) and *OXA-48* β-lactamase encoding genes through screening 144 clinical isolates of *Klebsiella* spp. isolated from hospitalized patients.

Materials and Methods

Clinical samples and bacterial isolation

In this cross-sectional study carried out from February to August 2014, 144 non-duplicated isolates of *Klebsiella* spp. were collected from clinical specimens including wounds, supra pubic, blood, sputum, catheter tips, urine, CSF, skin lesion, tracheal, throat and eye from infected patients admitted to hospitals in the North of Iran. All samples were cultured on EMB agar and Blood agar supplemented with 5% sheep blood (Merck, Germany), then incubated at 37 ºC for 24-48 h. Gram staining and standard biochemical tests including Triple Sugar Iron agar, Simmons' citrate agar, Christensen's urea agar, Indole test, Methyl red and Voges-Proskauer tests were performed on grown colonies to identify *Klebsiella* spp.

Antibiotic susceptibility test

A suspension of 1.5×10^8 CFU/ml (0.5 McFarland standard) of all *Klebsiella* strains was prepared and cultured on Mueller Hinton agar (Merck, Germany) separately, then 19 different antibiotics (MAST, UK) including Cotrimoxazole (25µg, TS25), Ciprofloxacin (5µg, CIP5), Ofloxacin (5µg, OFX5), Nalidixic acid (30µg, NA30) Nitrofurantoin (300µg, NI300), Gentamicin (30µg, GM30), Tetracycline (30µg, T30), Amoxicillin (25µg, A25), Ampicillin (10µg, AP10), Imipenem (10µg, IMI10), Aztronam (30µg, ATM30), Cephalothin (30µg, KF30), Cefoxitin (30µg, FOX30), Cefotaxime (30µg, CTX30), Cefixime (5µg, CFM5), Ceftriaxone (30µg, CRO30), Ceftazidime (30µg, CAZ30), Cefepime (30µg, CPM30), and Augmentin (30µg, AUG30) were placed on plates and incubated for 24h at 37 °C. The results were interpreted according to the CLSI (Clinical Laboratory and Standards Institute) guideline. *Escherichia coli* ATCC 25922 was used as quality control strain for antibacterial susceptibility testing.

Detection of ESBL-producing isolates

During the study, double disk synergy test (DDST) (30 mm) was performed to detect the ESBLs-producing *Klebsiella* spp. Briefly, a suspension (1.5×10^8 CFU/ml) of all *Klebsiella* spp. was cultured on Muller Hinton agar and Ceftriaxone (30µg), Augmentin (30µg), then ceftazidime (30µg), and ceftazidime/clavulanate (30µg/10µg) (MAST, UK) were placed on cultured plate and incubated for 24 hours at 37°C. CLSI guideline was used for interpreting the results. This was a combinational test for phenotypic confirmatory test to detect ESBLs (16, 17). Phenotypic detection of ESBLs was determined by an increase of ≥5 mm in the inhibition zone around clavulanic acid disk compared to the zone around the disks with no clavulanic acid. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive control strains, respectively.

Plasmid extraction

From fresh colonies, plasmid DNA of all *Klebsiella* strains was extracted using gene JET plasmid miniprep Kit (Fermentas, Lithuania) according to its instructions. Concentration of DNA for each extracted plasmid DNA was assessed using Nanodrop.

Amplification of genes by PCR

PCR was implemented via specific primers (Table 1) in a final volume of 25 µL containing 2.5 µL 10x buffer, 0.75 µL $MgCl_2$ (50 mM), 2 unit Taq DNA polymerase (Cinnaclon CO., Iran), 0.5 µL dNTP (10mM) (Cinnaclon Co., Iran), 10 Pico mole from each primers and 0.5 µL from plasmid DNA as a template. Amplification technique was carried out as follows: for bla_{CMY-2}, pre-incubation (94º C for 2 min), followed by 30 cycles of denaturation (94º C for 30 sec), annealing (47º C for 20 sec), extension (72º C for 40 sec) and a final extension (72º C for 10 min). For bla_{OXA-48} gene, pre-incubation (94º C for 2 min), followed by 34 cycles of denaturation (94º C for 30 sec), annealing (48º C for 20 sec), extension (72º C for 40 sec) and a final extension (72º C for 10 min). For bla_{KPC-2} (94º C for 2 min), followed by 34 cycles of denaturation (94º C for 30 sec), annealing (47º C for 30 sec), extension (72º C for 80 sec) and a final extension (72º C for 10 min). All PCR products were subjected to electrophoresis in 1.5% agarose gel (Roche, Germany) containing Sybrsafe. The molecular weight of observed bands under U.V. lamp was detected using DNA size marker (Roche Co, Germany). PCR products were sent to Source Bioscience Company for sequencing. Sequence analysis and comparisons were carried out via programs available at the NCBI server [http://www.ncbi.nlm.nih.gov].

Phylogenetic analysis

The phylogenetic analyses were carried out using MEGA6 (18). The nucleotide sequences from *CMY-2* and 22 *CMY-2* genes selected through BLASTN by applying a 0.001 cut-off for e-value were aligned by Clustal Omega (19) and a maximum parsimony tree was generated with a confidence assigned to the nodes which was then evaluated in a bootstrap analysis with

Table 1. The list of used primers for amplification of resistance genes.

Targeted gene	Primer designation	Primer sequences	Size of Product (bp)
KPC-2	KPC-2-F	5' TAGTTCTGCTGTCTTGTC 3'	375
	KPC-2-R	5' GCGTTATCACTGTATTGC 3'	
OXA-48	OXA-48-F	5' CCGAATAATATAGTCACCATTG 3'	476
	OXA-48-R	5' ATGAGAATAAGCAGCAAGG 3'	
CMY-2	CMY-2-F	5' GCACTTAGCCACCTATAC 3'	200
	CMY-2-R	5' CCTCTTCGTAACTCATTCC 3'	

100 replicates.

Statistical analysis

Data were analyzed using SPSS21 software. Kolmogorov-Smirnov test was applied to assess normal distribution. Prevalence rate and qualitative variables were assessed by percentage, and quantitative variables were reported as the mean ± SD. Fisher exact test & χ^2 was applied for qualitative variables. And finally, P values < 0.05 was considered to be statistically significant.

Results

Among the total 144 clinical isolates of *Klebsiella spp.* (73 isolates from men and 71 isolates from women, mean age 32.86 ± 26.59), 106 cases collected from urine, 9 cases from tracheal, 8 cases from blood, 7 cases wounds, 3 cases from catheter tips, 3 cases from eye, 2 cases from sputum, two cases from anus, one case from supra pubic, one case from CSF, one case from skin lesion, and one case from throat were isolated. Among 144 *Klebsiella* spp., 118 isolates were identified as *K. pneumoniae* and 26 isolates as *Klebsiella oxytoca.* Antibiotic susceptibility test showed the most resistance rates were against amoxicillin (96.5%) and ampicillin (95.8%). On the other hand, the most effective antibiotic was revealed to be imipenem with resistance rate of 4.9% (Table 2). Among 144 isolated *Klebsiella* strains, 57 cases (39.6%) were ESBL producers. The majority of ESBL positive isolates showed high resistance to most of the tested antibiotics with the most resistance rate against ampicillin, amoxicillin, and cefixime (100%) and the most effective antibiotic was imipenem with resistance rate of 8.8%. The *OXA-48* and *KPC-2* genes were not detected among *Klebsiella* spp. during the present study, but 21.1% of isolates contained *CMY-2* gene (GeneBank accession number: KU985243). This is the first report of *CMY-2* gene detection in *Klebsiella* spp. in Iran.

Table 2. Antibiotic resistance pattern of isolated *Klebsiella spp.*

Antibiotic	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	2.1	2.1	95.8
Ciprofloxacin	66	10.4	23.6
Cefoxitin	79.2	4.2	16.7
Aztreonam	57.9	2.1	43.1
Amoxicillin	3.5	-	96.5
Tetracycline	56.9	1.4	41.7
Cefotaxime	51.4	3.5	45.1
Ofloxacin	77.8	1.4	20.8
Cephalotin	47.2	4.9	47.9
Gentamicin	71.5	2.1	26.4
Nalidixic acid	27.1	32.6	40.3
Co-trimoxazole	56.3	2.1	41.7
Cefixime	50.0	2.1	47.9
Nitrofurantoin	41.7	16.0	42.4
Imipenem	93.1	2.1	4.9
Augmentin	44.4	8.3	47.2
Ceftriaxone	54.2	2.1	43.8
Ceftazidime	52.8	3.5	43.8
Cefepime	61.8	11.8	26.4

There was a significant association between ESBL producing *Klebsiella* spp. and resistance to antibiotics except for cefepime, aztreonam, gentamicin, imipenem, augmentin, ceftriaxone, cefixime and ceftazidime ($p < 0.05$). During the present study, the relationship between resistance to non β-lactam antibiotics and ESBL-producing *Klebsiella spp.* which possessed *CMY-2* gene was assessed. According the results of the study, the highest resistance rate was against tetracycline (66.6%) and the lowest was found to be against ofloxacin (33.3%).

According to the results of Fisher's exact test, there was no significant association within *Klebsiella* spp. strains with/without *CMY-2* gene in view of resistance to β-lactam and non β-lactam antibiotics, but in case of cephalothin the association appeared to be significant ($p=0.051$).

CMY-2 showed high similarity to many genes from beta-lactamase superfamily, which were isolated from different species of *Enterobacteriaceae* family with domination of *E. coli*. These genes showed high geographical diversity; were detected from three continents (Asia, Europe and America). *CMY-2* showed less than 40% similarity to the genes *OXA-1* and *KPC-2* (encoding different classes of beta-lactamase); the low similarity could be partly due to the partial sequencing of these two genes (Figure 1).

Discussion

During the past decade, ESBL producing Gram-negative bacilli, especially *Klebsiella* spp. have emerged globally as serious pathogens both in hospital- and society-acquired infections. The prevalence of strains possessing numerous resistant enzymes concurrently has caused severe troubles in treatment and identification of such strains (20). Totally, 39.6% of *Klebsiella* spp. isolates of our study were identified as ESBL producers, which is similar to the findings of Martinez et al. on

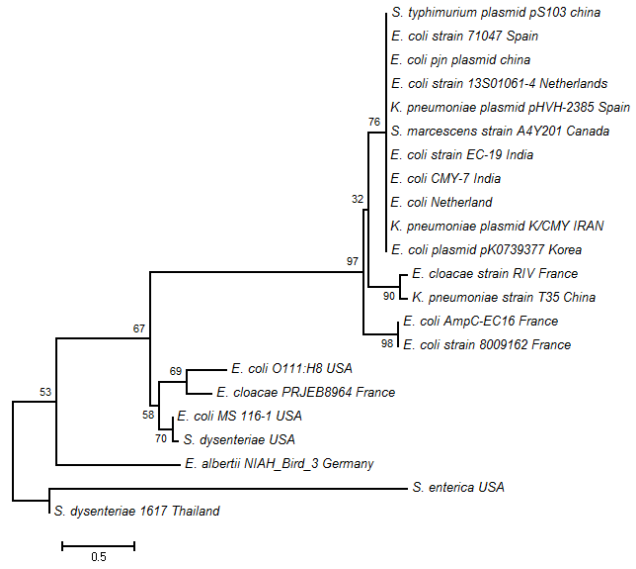

Figure 1. Phylogenetic analysis of *CMY-2* gene. The tree was built by Maximum Likelihood method using MEGA6. *CMY-2* gene; it shows the high similarity of this gene to other beta-lactamase genes isolated from different species and diverse geographical regions.

hospital isolates (21).

K. pneumoniae is the main ESBL-producing organism in the world today (22). In Latin American countries, community-acquired ESBLs are beginning to appear with rates up to 28.7% among *Klebsiella* spp. isolates (23), while the reported rate in Europe is only 5.6% (24). The prevalence of ESBLs-producing *K. Pneumonia* was reported from other cities in Iran such as Ilam, Tehran and Tabriz as 39.4%, 50.7% and 45.8%, respectively, which are higher than our findings (25).

Our findings showed that antibiotic resistance rate was higher in ESBL-producing *Klebsiella* spp. than non-ESBL strains. Among ESBL-producing *Klebsiella* spp., the most resistance rate was against ampicillin, amoxicillin, and cefixime (100%). These results were similar to Amirmozafari et al. findings; however, the resistance rates to tetracycline, nalidixic acid, co-trimoxazole, imipenem and ciprofloxacin, found in our study were lower than Amirmozafari results (26). The study by Mansury et al. showed that the resistance rates against the most of antibiotics were higher than those found in our results (27). Among all available anti-microbial agents, carbapenem is the most effective and reliable treatment option for infections caused by ESBL producing *K. pneumoniae*. The options available for treatment of *K. pneumoniae* are very limited. Therefore, prevention remains as the priority in controlling the development and spread of ESBL-producing organisms.

The results based on PCR and nucleotide sequence analysis revealed that 12 (21.0%) of *Klebsiella* spp. isolated in our study, contained *CMY-2* gene. It was the first report of *cmy-2* gene detection in *K. pneumoniae* in Iran. Several other studies from different parts of the world have reported the presence of these subtypes in isolates of *E. coli* and *Klebsiella* spp. (28-30). No signs of *OXA-48* and *KPC-2* genes were found during this research. The frequency of *CMY-2* gene in the surveyed *Klebsiella* spp. had a lower rate in comparison to another study. The findings of two similar studies carried out in Spain and Singapore showed a 66.7% and 79.0% presence of *CMY-2,* respectively, in tested samples (31,32). Our findings showed that the presence of *CMY-2* gene did not significantly induce resistance to β-lactam and non β-lactam antibiotics.

As the main limitations of the present study, the lack of phenotypic detection for AmpC production, and investigating more antibiotic resistance determinants conferring resistance to β-lactam antibiotics can be mentioned.

The widespread use of drugs might facilitate the rise of antibiotic resistance; thus controlling ESBL-producing *K. pneumoniae* within inpatients should be considered as a high priority. World population growth, high rate of international travels and overuse of antibiotics have led to the wide geographical dispersion of antibiotic resistance genes. None of the patients participating in the present study had visited the hospitals because of infections; this shows the high frequency of hospital-acquired infections happening in health care centers of northern Iran. The homology between *CMY-2* genes identified in isolates from northern Iran and in countries thousands miles away shows the wide dispersion of this gene around the world. This is probably due to the parallel evolution under selective pressure made by overuse of antibiotics in these countries rather than horizontal gene transfer.

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Conflict of Interest Statement
None declared.

Author's contribution
Study concept and design: T. Tavakoly, A. Mojtahedi; acquisition of data and sampling: T. Tavakoly, S. Jamali, M. Khan Mirzaei; analysis and interpretation of data: T. Tavakoly, M. Shenagari; drafting of the manuscript: T. Tavakoly; critical revision of the manuscript for important intellectual content: A. Mojtahedi; study supervision: A. Mojtahedi.

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