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Presence of *qacEΔ1* and *cepA* genes and susceptibility to a hospital biocide in clinical isolates of *Klebsiella pneumoniae* in Iran

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Abstract. The aim of this study was to evaluate the susceptibility of *Klebsiella pneumoniae* clinical isolates to antibiotics and to a quaternary ammonium compound (QAC) disinfectant as the concentrations used clinically and to determine the presence of the *qacEΔ*1 and *cepA* genes for the first time in Iran. In total, 85 *K. pneumoniae* isolates were randomly collected from hospitalized patients at the general hospitals in Lorestan, Iran. Antibiotic and antiseptic susceptibility testing was performed according to Clinical and Laboratory Standards Institute recommendations. *K. pneumonia* isolates were screened by PCR amplification of *qacEΔ*1 and *cepA* genes using specific primers and sequence analysis of the amplified regions were also performed. From 85 isolates of *K. pneumoniae*, 34 (40%) isolates were multidrug resistance (MDR). The evaluation of the susceptibility to the QAC disinfectant revealed that 51 (60%) isolates had reduced susceptibility to QAC disinfectant. The *qacEΔ1* gene was detected in 26 isolates (30.6%). While *cepA* gene was found in 19 isolates (22.3%) of *K. pneumonia*. Seventy-three percent (19/26) *qacEΔ1*-positive isolates were detected in the biocide-resistant isolates. Whereas, 63.1% (12/19) *cepA*-positive isolates were found in the biocide-resistant isolates. Out of *qacEΔ1* and *cepA*-positive isolates, 65.4% (17/26) and 42.1% (8/19) were among MDR isolates, respectively. No significant association of biocide resistance with the presence of *qacEΔ1* and *cepA* genes was observed ($P>0.05$). The results of present study shows that there was a close link between *qacEΔ1* gene and antibiotic resistance, but no significant association of biocide resistance with the presence of *qacEΔ1* and *cepA* genes was observed in *K. pneumoniae* in Iran.

INTRODUCTION

Klebsiella pneumoniae (family Enterobacteriaceae) is an opportunistic pathogen that colonizes >75% of hospitalized patients (Podschun & Ullmann, 1998). Moreover, it has been identified as important common pathogens for nosocomial pneumonia (7 to 14% of all cases), septicaemia (4 to 15%), urinary tract infection (6 to 17%), wound infections (2 to 4%), intensive care unit (ICU) infections (4 to 17%), and neonatal septicaemias (3 to 20%) (Janda & Abbott, 2006). In recent years, emergence of multidrug-resistant *K. pneumoniae* isolates is becoming a serious antibiotic management problem and result in the great concern around the world (Paterson *et al*., 2002; Strahilevitz *et al*., 2009). Recently, biocides as disinfectants and antiseptics are widely used to improve infection control standards in hospitals (Romao *et al*., 2011). However, a particular concern is that repeated usage of disinfectants may lead to emergence of resistance with reduced susceptibility not only to the antiseptics but possibly to antibiotics as well (Randall *et al*., 2004). It has been previously shown that expression

of efflux systems is one of the main mechanisms of biocide resistance (Paulsen *et al.*, 1993; Poole, 2005). *K. pneumoniae* similar to the other Gram-negative bacteria may harbor multi-drug transporters efflux systems including *cepA*, *qacE* and *qacEΔ1* proteins (Paulsen *et al.*, 1993, 1996; Kazama *et al.*, 1998; Kücken *et al.*, 2000). The *qacEΔ1* gene was described first by Paulsen *et al.* (1993) which is included in the 3'conserved segment of class I integron as a defective version of the *qacE* gene, being known as a quaternary ammonium compound (QAC) resistance determinant (Herruzo-Cabrera *et al.*, 2004; Kohlenberg *et al.*, 2010).

At present, there are few studies on determinants of resistance to biocides and susceptibility to biocides in *K. pneumoniae*. In a study conducted by Abuzaid *et al.* (2012), it has been proven that in *K. pneumoniae* clinical isolates, there was a close correlation between carriage of efflux pump genes, *cepA*, *qacEΔ1* and *qacE* genes and decreased biocide susceptibility, but not antibiotic resistance. To the best of our knowledge and according to a survey of the literature, there is no study of prevalence of *qacEΔ1* and *cepA* genes and susceptibility to hospital biocides in clinical isolates of *K. pneumoniae* in Iran. Therefore this study was aimed to evaluate the susceptibility of *K. pneumoniae* clinical isolates to antibiotics and to a QAC disinfectant as the concentrations used clinically and to determine the presence of the *qacEΔ1* and *cepA* genes.

MATERIALS AND METHODS

Study area

This descriptive study was performed from December to September 2012 on hospitalized patients at the general hospitals of Lorestan province, located between valleys of Zagros Mountain in the west of Iran, bordering with the provinces of Markazi, Hamedan, Kermanshah, Khuzestan, Ilam, and Isfahan. Lorestan covers an area of 28.294 km^2 and its population is approximately 2 million people. The major cities in this province are Khorramabad, Borujerd, Aligoodarz, Dorood, Koohdasht, Azna, Alashtar, Noor Abad and Pol-e-Dokhtar (Figure 1) (Kheirandish *et al.*, 2013).

Bacterial isolates

In total, 85 *K. pneumoniae* isolates were randomly collected over the period between December and September 2012 from hospitalized patients at the general hospitals of Lorestan, Iran. The isolates were collected from different specimens, including urine, sputum, lesion, blood and other specimens. All isolates were routinely cultured on

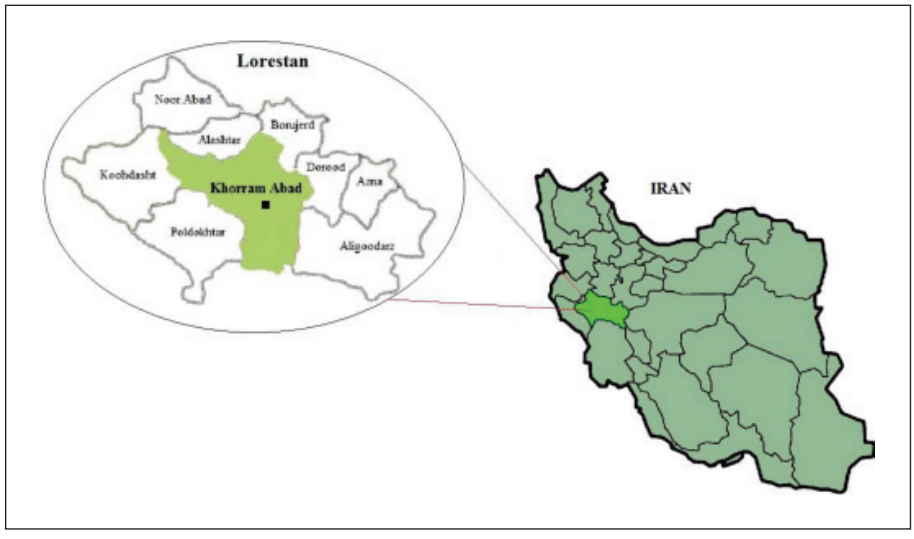

Figure 1. Geographical location where this study was carried out

Mueller-Hinton (MH) agar plates, and typical colonies were picked up, identified by biochemical tests using the API®-20E test kits (bioMérieux, Lyon, France). The bacteria were grown at 37°C for 18–24 h for preparation of bacterial suspension and DNA extraction.

Antimicrobial Susceptibility test

The antimicrobial susceptibility of the isolates to amikacin (10 µg), ampicillin (10 µg), meropenem (10 µg), nalidixic acid (30 µg), cefotaxime (30 µg), ceftazidime(30 µg), cefteriaxone (30 µg), cephalexine (5 µg), cefexime (30 µg), gentamicin (10 µg) and imipenem (10 µg) (all antibiotics were purchased from Oxoid, UK) was determined by disk diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI) on Mueller-Hinton agar plates (CLSI, 2012). The quality control was carried out by using standard strains of *K. pneumoniae* ATTC BAA-1705. Multidrug resistance (MDR) was defined as isolates being resistant to 2 or more different classes of antibiotics.

Disinfectant Susceptibility Test

Disinfectant susceptibility of *K. pneumoniae* clinical isolates was found by measuring the minimum inhibitory concentration (MIC) of QAC disinfectant (Didecyl dimethyl ammonium chloride – Borer chemic AG, Switzerland). MICs were determined using the micro-broth dilution method according to the guidelines of the CLSI (CLSI, 2012)

Polymerase Chain Reaction (PCR) for detection of *qacEΔ1* and *cepA* genes

The 85 clinical isolates of *K. pneumoniae* were screened by PCR amplification of the antiseptic resistance genes of *qacEΔ1* and *cepA*, as previously described by Abuzaid *et al*. (2012). The *qacEΔ1* primer pairs F5'GCCCTACACAAATTGGGAGA3', R5'CTGCGGTACCACTGCCACAA3' were designed to amplify 370 base pair (bp), with annealing temperature of 49°C for 40 seconds. In addition, the *cepA* primer pairs were designed to amplify 1051 bp F5' CAACTCCTTCGCCTATCCCG3', R5'TCAGGTCAGACCAAACGGCG3' with annealing temperature 66°C for 30 seconds.

For preparation of DNA templates, colonies were transferred to an Eppendorf tube and DNA was extracted using DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's protocol. Positive (containing strains with known *qacEΔ1* and *cepA* genes) and negative (without DNA template) controls were included in each run. The PCR products were separated by electrophoresis on 1.5% agarose gel in TBE (Tris-borate EDTA) and visualized with GelRed staining.

DNA sequencing

DNA sequence analysis was performed by direct sequencing of both strands using an automated sequencer. The DNA sequences obtained were compared and analyzed using the BLAST online search engine from GenBank at the National Center for Biotechnology Information website.

Statistical analysis

Data analysis was carried out by using SPSS statistical package (version 17.0) (SPSS Inc., Chicago, IL, USA). Significant differences between susceptible and less susceptible isolates to the disinfectant and multi-resistance to antibiotics, and presence of *qacEΔ1* and *cepA* genes were evaluated using the chi-square test. In addition, $P<0.05$ was considered statistically significant.

RESULTS

Antibiotic and QAC Susceptibility

From 85 isolates, 34 (40%) isolates were MDR. The highest rate of resistance was observed in cefotaxime (85%) and ceftazidime (68%). Moreover, the lowest rate (≤10%) of resistance was seen in imipenem, meropenem and amikacin, respectively. The evaluation of the susceptibility to the QAC disinfectant by micro-broth dilution method revealed that 51 (60%) isolates had reduced susceptibility to QAC disinfectant with MICs ranging from 32 to 256 mg/L, while 34 isolates (40%) were susceptible to the QAC disinfectant and showed MICs of lower than 4 mg/L.

Detection *qacEΔ1* and *cepA* genes

PCR was performed to determine the correlation between reduced susceptibility with *qacEΔ1* and *cepA* genes as specific resistance genes to biocides. The *qacEΔ1* gene was detected in 26 isolates (30.6%), while the *cepA* genes were found in 19 isolates (22.3%) of *K. pneumoniae*. In addition, 6 (7%) isolates had both *qacEΔ1* and *cepA* genes. From 26 *qacEΔ1*-positive isolates, 27% (7/26) of the isolates were those susceptible to the QAC disinfectant, whereas 73% (19/26) of the *qacEΔ1*-positive isolates were found in the less susceptible isolates (Table 1). Among the *cepA*-positive isolates, 36.9% (7/19) were among the isolates susceptible to the QAC disinfectant. Whereas 63.1% (12/19) of the *cepA*-positive isolates were detected in the less susceptible isolates of *K. pneumoniae* to the disinfectant (Table 2). Among the *qacEΔ1* and *cepA*-positive isolates, 65.4% (17/26) and 42.1% (8/19) were MDR isolates, respectively.

Chi-square test was performed to compare between susceptible and less susceptible isolates to the QAC disinfectant and the presence or absence of *qacEΔ1* and *cepA* genes revealed reduced susceptibility to the disinfectant was independent of presence of *qacEΔ1* (P=0.102) and *cepA* (P= 0.75) genes. We also compared multi-resistant and non-multiresistant isolates, concerning the presence or absence of *qacEΔ1* and *cepA* genes. In this case, presence of *qacEΔ1* gene was well correlated with multi-resistance (P<0.0001), while it was independent of presence of *cepA* (P= 0.457).

Table 1. Clinical characteristics of *qacEΔ1* positive *K. pneumoniae* isolates in Lorestan, Iran

Susceptibility to Antiseptic	Susceptibility to Antibiotic	Diagnoses	Age	Sex	Specimen	No. Strains
R[d]	MDR[b]	Pneumonia	12 Months	Male	Sputum	Kp5
S[e]	NM[c]	UTIa [a]	6 years	Male	Urine	Kp 7
R	MDR	Pneumonia	23 years	Female	Sputum	Kp13
R	MDR	UTI	9 years	Female	Sputum	*Kp16
R	NM	Pneumonia	3 years	Male	Sputum	Kp18
S	NM	Pneumonia	51 years	Male	Tracheal	Kp19
R	MDR	Pneumonia	13 years	Male	Sputum	Kp22
S	MDR	Pneumonia	6 Months	Male	Sputum	*Kp27
R	MDR	Septicemia	33 years	Female	Blood	Kp30
R	MDR	Pneumonia	14 Months	Male	Sputum	Kp32
R	NM	UTI	52 years	Female	Urine	*Kp33
S	MDR	Pneumonia	12 Months	Female	Sputum	Kp39
R	MDR	UTI	4 years	Male	Urine	Kp41
R	MDR	Pneumonia	19 Months	Female	Sputum	Kp46
S	NM	Pneumonia	2 years	Male	Sputum	Kp52
R	MDR	Pneumonia	11 years	Male	Sputum	Kp53
R	NM	Pneumonia	3years	Female	Sputum	*Kp57
R	MDR	Pneumonia	47 years	Male	Sputum	Kp60
R	MDR	Pneumonia	8 Months	Male	Sputum	Kp62
S	MDR	Pneumonia	5 years	Female	Sputum	Kp64
R	NM	Pneumonia	18 Months	Female	Sputum	Kp69
R	MDR	UTI	20 years	Male	Urine	*Kp71
S	NM	UTI	6 years	Female	Urine	Kp73
R	MDR	Pneumonia	14 Months	Male	Sputum	Kp77
R	MDR	Pneumonia	27 years	Male	Sputum	*Kp82
R	NM	Pneumonia	12 years	Male	Sputum	Kp84

* : positive for both genes (*qacEΔ1* and *cepA*); a: Urinary tract infection; b: Multidrug resistance; c: Non-multidrug resistant; d: Resistance; e: Susceptible.

Table 2. Clinical characteristics of *cepA* positive *K. pneumoniae* isolates in Lorestan, Iran

Susceptibility to Antiseptic	Susceptibility to Antibiotic	Diagnoses	Age	Sex	Specimen	No. Strains
R[d]	MDR[b]	Pneumonia	5 years	Male	Sputum	Kp8
S[e]	NM[c]	UTIa [a]	11 years	Male	Sputum	Kp 12
R	NM	Pneumonia	18 years	Female	Sputum	Kp18
R	MDR	UTI	9 years	Female	Sputum	*Kp16
R	NM	Pneumonia	4 years	Male	Sputum	Kp20
S	NM	Pneumonia	42 years	Male	Tracheal	Kp19
R	NM	Pneumonia	13 years	Male	Sputum	Kp24
S	MDR	Pneumonia	6 Months	Male	Sputum	*Kp27
R	NM	Septicemia	33 years	Female	Blood	Kp31
R	MDR	Pneumonia	14 months	Female	Sputum	Kp32
R	NM	UTI	52 years	Female	Urine	*Kp33
S	MDR	Pneumonia	12 months	Female	Sputum	Kp38
S	NM	UTI	4 years	Male	Urine	Kp43
R	NM	Pneumonia	3years	Female	Sputum	*Kp57
S	NM	Pneumonia	2 years	Male	Sputum	Kp62
R	MDR	UTI	20 years	Male	Urine	*Kp71
R	NM	Pneumonia	3years	Male	Sputum	Kp74
S	MDR	Pneumonia	47 years	Male	Sputum	Kp80
R	MDR	Pneumonia	27 years	Male	Sputum	*Kp82

* : positive for both genes (*qacEΔ1* and *cepA*); a: Urinary tract infection; b: Multidrug resistance; c: Non-multidrug resistant; d: Resistance; e: Susceptible.

DNA sequencing

In this study, PCR products of *qacEΔ1* and *cepA* genes were sequenced by direct sequencing of both strands using an automated sequencer. The complete sequences of positive *qacEΔ1* and *cepA* isolates were deposited in GenBank database and assigned the accession numbers AB894355, AB894356.

DISCUSSION

K. pneumoniae is an opportunistic pathogen that usually causes hospital and community acquired bacterial infections in humans (Podschun & Ullmann, 1998). Currently, emergence of multidrug resistant *K. pneumoniae* isolates is becoming a serious antibiotic management problem and result in the great concern around the world (Paterson *et al.*, 2002; Strahilevitz *et al.*, 2009). Although biocides have been widely used as a tool to minimize the spread of such resistant bacteria especially in hospitals, very little is known about the decreased susceptibility to these biocides and its relationship with resistance to antibiotics (Randall *et al.*, 2004). Thus, the aim of this investigation was to evaluate the susceptibility of *K. pneumoniae* clinical isolates to antibiotics and to a QAC disinfectant commonly used in health-care systems, at concentrations used clinically and to determine the presence of the *qacEΔ1* and *cepA* genes, as QAC resistance determinants. Our findings revealed a high rate of disinfectant decreased susceptibility (60%) among clinical isolates of *K. pneumoniae*. Moreover, antibiotic susceptibility tests indicated that the highest rate of resistance was observed in ceftazidime and carbencillin, whereas the lowest rate of resistance was seen in imipenem, meropenem and amikacin, respectively. In this survey, the presence of *qacEΔ1* gene was detected in 30.6% of the clinical isolates of *K. pneumoniae*. The *qacEΔ1* gene was present in 50% (17/34) of the multi-resistance isolates, and it was present in only 17.7% (9/51) of those considered as non-multiresistance isolates.

Various studies have proven that the presence of *qacEΔ1* gene was well correlated with multi-resistance isolates (Kazama *et al*., 1998; Kücken *et al*., 2000; Wang *et al*., 2008; Romao *et al*., 2011). These studies also showed that *qacEΔ1* gene and several genes that encoded resistance to some antimicrobial drugs are located on a mobile genetic element (integron). In the present study, the higher percentage (73%) of *qacEΔ1*-positive isolates (19/26) was found among less susceptible isolates to the biocide. Nevertheless, less susceptibility of isolates to the biocide was independent of presence of *qacEΔ1* gene. In addition, the *cepA* gene was found in 22.4% of the clinical isolates of *K. pneumoniae*. The *cepA* gene was also detected in 23.5% (8/34) of the multi-resistance isolates and 23.5% (12/51) of the less susceptible isolates to the disinfectant. Therefore, our results indicated that reduced susceptibility to the disinfectant and multi-resistant was independent of presence of *cepA* gene. Our findings were in agreement with Romao *et al*. (2011), who investigated the presence *qacEΔ1* in *Pseudomonas aeruginosa* isolates. They demonstrated that this gene probably does not play an important role in biocide resistance in *P. aeruginosa*. In contrast to our results, Abuzaid *et al*. (2012) showed that there was a close link between carriage of efflux pump genes, *cepA*, *qacEΔ1* and *qacE* genes and reduced biocide susceptibility, but not antibiotic resistance in *K. pneumoniae* clinical isolates. However, it should be noted that *qacEΔ1* gene is a defective gene and various mechanisms are involved in biocide resistance (Paulsen *et al*., 1993; Poole, 2005).

In conclusion, the proper use of biocide is a foundation of any effective program of prevention and control of healthcare associated infections. Meanwhile, our study showed high frequency of *qacEΔ1* in MDR *K. pneumoniae*, therefore improper usage of biocide can probably lead to biocide resistance and higher antibiotic resistance in *K. pneumoniae*.

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