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Antibiotic susceptibility pattern and identification of quinolone-resistant strains of *Klebsiella pneumoniae* clinical specimens from Khorramabad, Iran

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Abstract. The present investigation is designed to evaluate antibiotic susceptibility pattern and identification of qnr strains of *Klebsiella pneumoniae* in clinical specimens isolated from general hospitals in Khorramabad, Iran. Total of 107 *K. pneumoniae* isolates were randomly collected since December 2011 until September 2012 from hospitalized patients at general hospitals in Khorramabad, Iran. The isolates were collected from different clinical samples including urine, sputum, lesion, and blood. Biochemical tests were performed for identification of isolates. Antibiotic susceptibility test was performed using disc diffusion method according to recommendations of Clinical and Laboratory Standards Institute using 13 antibiotic disks. *K. pneumoniae* isolates were screened by multiplex PCR amplification of *qnrA*, *qnrB* and *qnrS* using specific primers and sequence analysis of amplified regions of the isolates was also performed. Chi-square test was used to analysis and P value of < 0.05 was considered statistically significant. All clinical isolates were confirmed as *K. pneumoniae* by complete biochemical identification (gram staining, oxidase negative, indole positive, Simon's citrate positive and urease positive). Forty-three (40.2%) out of 107 isolates were multidrug-resistant (MDR). Ciprofloxacin (quinolone) susceptibility testing showed that 34 isolates (31.8%) were resistant, 7 isolates (6.5%) were intermediately resistant and 66 isolates (61.7%) were sensitive. Eighteen (16.8%) out of 107 *K. pneumoniae* clinical isolates were positive for *qnr* genes. Among all the *qnr*-positive isolates, 16 isolates (88.9%) carried *qnrB*, 1 isolate (5.55%) carried *qnrS* and the rest (5.55%) carried both *qnrB* and *qnrS* genes while no *qnrA* was detected in these clinical isolates. *Qnr* determinants were detected in 8 (23.5%) of the ciprofloxacin-resistant isolates as well as 1 (14.3%) and 9 (13.6%) intermediate and sensitive isolates, respectively. No significant association was observed between ciprofloxacin resistance and presence of *qnr* genes ($P>0.05$). Findings of the present study indicated high frequency of *qnr*-positive *K. pneumonia*e in Lorestan province, Iran. However, there is no association between quinolone resistance and presence of *qnr* genes in isolates of *K. pneumonia*e.

INTRODUCTION

Klebsiella pneumoniae are oppotunitistic pathogens from the enterobacteriaciae family that caused hospital and community acquired bacterial infections such as neonatal enteritis, meningitis, urinary tract infections, soft tissue infections, bacteremia, and septicemiain humans (Paterson *et al.*, 2002). It has been identified as an important cause of 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 has become a serious antibiotic management problem and led to great concern around the world (Strahilevitz *et al.*, 2009). Quinolones (fluoroquinolones such as ciprofloxacin) are broad-spectrum

antibacterial agents, commonly used for treatment of infections both in human and veterinary medicine (Raei *et al.*, 2009). At present, quinolone resistance is a widespread phenomenon among the Enterobacteriaceae (Robicsek *et al.*, 2006; Strahilevitz *et al.*, 2009). Main mechanisms of resistance to quinolones in this family including mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase and topoisomerase IV, efux pumps enhancement or decreased accumulation mediated by reduce in permeability of bacterial cell wall are chromosomally mediated (Poole 2005; Kim *et al.*, 2010; Hooper 2003; Nikaido 2003; Martinez-Martinez *et al.*, 1998; Pakzad *et al.*, 2011). Recently, mechanisms of plasmid-mediated quinolones resistance (PMQR) by *qnr* genes have been reported (Martinez-Martinez *et al.*, 1998). These *qnr* genes (*qnrA*, *qnrB* and *qnrS*) encode proteins of the pentapeptide repeat family that interfere with the action of quinolones on bacterial DNA gyrase and topoisomerase IV (Martinez-Martinez *et al.*, 1998; Tran *et al.*, 2005; Jacoby *et al.*, 2006). Although the *qnr* gene indicates a low level of resistance to quinolones, its attendance aids the selection of chromosomal mutations, facilitating increased resistance in the host strain (Martinez-Martinez *et al.*, 1998; Tran *et al.*, 2005; Jacoby *et al.*, 2006).

Frequency of *qnr* genes PMQR associated with the *qnr* genes in different human clinical enterobacterial isolates was determined first in the USA in 1994 using a *K. pneumoniae* isolate (later termed *qnrA1*) (Martinez-Martinez *et al.*, 1998), which was then widely reported worldwide (Wang *et al.*, 2004, 2009; Minarini *et al.*, 2008; Yang *et al.*, 2004; Wu *et al.*, 2007; Robicsek *et al.*, 2006; Teo *et al.*, 2009; Saiful Anuar *et al.*, 2013). However, no study has been done on prevalence of *qnr* genes among enterobacterial clinical isolates in Iran. The present investigation designed to evaluate antibiotic susceptibility pattern and identification of qnr strains *of K. pneumoniae* in clinical specimens from general hospitals in Khorramabad, Iran.

MATERIALS AND METHODS

Bacterial isolates

A total of 107 *K. pneumoniae* isolates were randomly collected since December 2011 until September 2012 from hospitalized patients at general hospitals of Shohadaye Ashayer (54 isolates), Tamin Ejtemaei (31 isolates) and Shahid Madani (22 isolates) in Khorramabad, Iran. The isolates were collected from different specimens, including urine, sputum, lesion, blood and other specimens. All the isolates were routinely cultured on Mueller-Hinton (MH) agar (Merck, Germany) plates and typical colonies were picked up and identified by biochemical tests using the API®-20E test kits (bioMérieux, Lyon, France). The bacteria were grown at 37°C for 18–24 h in order to prepare bacterial suspension and DNA extraction.

Antibiotic Susceptibility Pattern

Antibiotic susceptibility of the isolates to amikacin (10 µg), meropenem (10 µg), kanamycin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefteriaxone (30 µg), co-amoxiclav (10 µg), tetracycline (10 µg), ciprofloxacin (5 µg), cefexim (30 µg), gentamicin (10 µg), sulfamethoxazole-trimethoprim (10 µg) and imipenem (10 µg) (all the antibiotics were purchased from Mast, UK) was determined by disk diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI, 2009) on Mueller–Hinton agar plates. In this study, multidrug-resistance (MDR) isolates were defined as isolates with resistance to ≥4 different antibiotic classes. In addition, *K. pneumoniae* ATTC BAA-1705 was used as a quality control strain.

Screening for qnr genes

All of the clinical isolates were screened by multiplex PCR ampli-fication of *qnrA*, *qnrB* and *qnrS* using specific primers as previously described by Robicsek *et al.* (2006). For preparation of DNA templates, colonies were transferred to an Eppendorf tube and DNA was extracted using cina pure

DNA extraction Kit (Cinagen, Iran) in accordance with the manufacturer's protocol. PCR reactions were performed in a total volume of 25 µl, including 2 µL of DNA template, 1.5 mM MgCl2 (Thermo Scientific, USA), 200 µm of each dNTP (Thermo Scientific, USA), 2.5 µL of 10x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 10 pmol of each of the six primers and 1 U of Taq polymerase (Thermo Scientific, USA). Target fragments were amplified under PCR conditions of 94°C for 45 s, 53°C for 45 s and 72°C for 60 s with cycle number of 32. The PCR product sizes were 516 bp, 469 bp and 417 bp for *qnrA*, *qnrB* and *qnrS*, respectively. Positive (containing strains of *qnrB* (AB894352) and *qnrS* (AB894353) and negative (without DNA template) controls were included in each run. Amplification products were provisionally identified according to their sizes in gel red (Sigma-Aldrich, St Louis, USA) electrophoresis.

DNA sequencing

In this study, DNA sequence analysis was carried out direct sequencing of both strands using an auto-sequencer. The obtained DNA sequences were compared and analyzed using BLAST online search engine from GenBank in website of National Center for Biotechnology Information (http://www.ncbi.nlm. nih.gov/blast).

Statistical analysis

Data analysis was carried out using SPSS statistical package (version 17.0) (SPSS Inc., Chicago, IL, USA). In addition, Chi-square test was used for compari-son and P value of < 0.05 was considered statistically significant.

RESULTS

Antibiotic Susceptibility Pattern

All clinical isolates were confirmed as *K. pneumoniae* by complete biochemical identification (gram staining, oxidase negative, indole positive, simon's citrate positive and urease positive). Out of 107 isolates, 43 isolates (40.2%) were MDR (Table 1). The highest rate of resistance was observed in ceftazidime and cefotaxime. Moreover, the lowest rate of resistance was seen in imipenem, meropenem and amikacin, respectively. Ciprofloxacin (quinolone) susceptibility testing showed that 34 isolates (31.8%) were resistant, 7 isolates

Table 1. Antibiotic Susceptibility Pattern of *K. pneumoniae* Strains

Resistance to Antibiotics	Number of Isolates (%)
Resistance to 0 antibiotic (Sensitive)	40 (35.5)
1 Antibiotic	12 (11.2)
2 Antibiotics	11 (10.3)
3 Antibiotics	1 (0.93)
4 Antibiotics	2 (1.9)
5 Antibiotics	2 (1.9)
6 Antibiotics	3 (2.8)
7 Antibiotics	6 (5.6)
8 Antibiotics	4 (3.7)
9 Antibiotics	9 (8.5)
10 Antibiotics	5 (4.7)
11 Antibiotics	4 (3.7)
12 Antibiotics	4 (3.7)
13 Antibiotics	4 (3.7)
MDR[a]	43 (40.2)

[a] MDR: Multi drug resistance (resistance to ≥4 different antibiotic classes)

(6.5%) were intermediately resistant and 66 isolates (61.7%) were sensitive.

Screening for qnr genes

Eighteen (16.8%) out of 107 *K. pneumoniae* clinical isolates screened by multiplex PCR, were positive for the *qnr* genes (Figure 1). Among all the *qnr*-positive isolates, 16 isolates (88.9%) carried *qnrB*, 1 isolate (5.55%) carried *qnrS* and the rest (5.55%) carried both *qnrB* and *qnrS* genes while no *qnrA* was detected in the present clinical isolates. Table 2 shows clinical characteristics of these isolates and distribution of *qnrA*, *qnrB* and *qnrS*. *Qnr* determinants were detected in 8 (23.5%) of the ciprofloxacin-resistant isolates as well as 1 (14.3%) and 9 (13.6%) intermediate and sensitive isolates, respectively (Table 3). No significant association was found between ciprofloxacin resistance and presence of *qnr* genes (P=0.074).

DNA sequencing

Complete sequences of positive *qnrB* and *qnrS* isolates were submitted to the GenBank database and assigned accession numbers of AB894351 and AB894354, respectively. The results of sequence *qnrB* had 98% overlap with genes encoding quinolone resistance in *K. pneumoniae* and *Aeromonas hydrophila* in the gene bank. Whereas, the results of sequence *qnrS* had 94% and 93% overlap with genes encoding quinolone resistance in *A. hydrophila* train 12107 and *Escherichia coli* in the gene bank, respectively.

DISCUSSION

K. pneumoniae causes more than 70% of hospitalized patients and result in about 8% of all nosocomial infections (Azadpour *et al.*, 2015). Right now, emergence of multidrug-resistant *K. pneumoniae* isolates was considered as the main antibiotic management problem and caused major apprehension globally (Strahilevitz *et al.*, 2009). In the current study we evaluated the frequency of *qnrA*, *qnrB* and *qnrS* genes by multiplex PCR in Khorramabad, Iran. In this survey, *qnr* genes were determined in 18 (16.8%) of the isolates. These results were similar to some studies carried out in Taiwan and the USA (Wu *et al.*, 2007; Robicsek *et al.*,

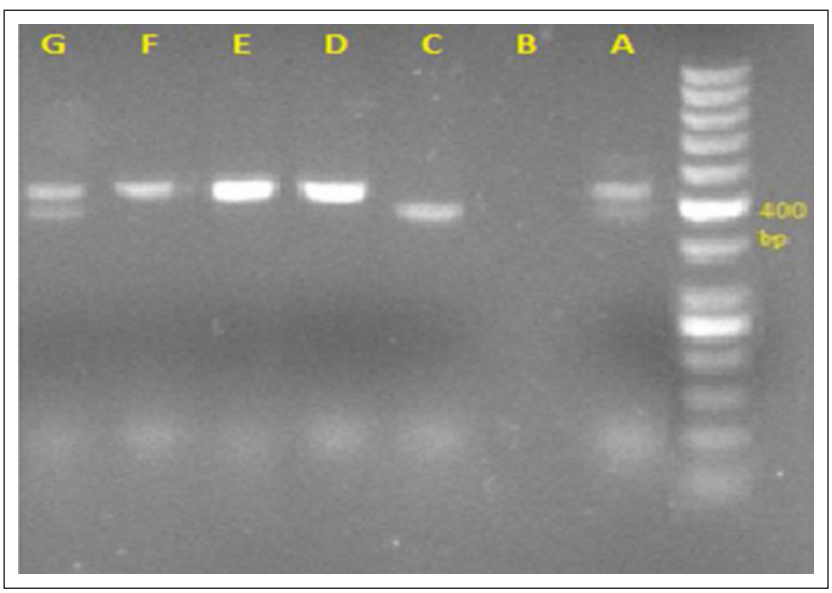

Figure 1. Gel electrophoresis of multiplex PCR products in *K. pneumoniae* isolates from hospitalized patients at the general hospitals of Khorramabad, Iran (2012); Marker- 50 bp, **A, D, E**: Positive for *qnrB* 469 bp, **B**: Negative Control, **C:** Positive for *qnrS* 417 bp, **F**: Positive control, **G**: Positive for *qnrB* and *qnrS*.

Table 2. Clinical characteristics and *qnr* genotype of the *qnr*-positive isolates

Number of Strains	Specimen	Sex	Age
Kp6	Sputum	Male	12 Months
Kp 8	Urine	Male	6 years
Kp13	Sputum	Female	23 years
Kp17	Urine	Female	9 years
Kp18	Sputum	Male	3 years
Kp19	Tracheal	Male	51 years
Kp26	Sputum	Male	13 years
Kp33	Sputum	Female	6 Months
Kp42	Urine	Female	33 years
Kp44	Sputum	Male	14 Months
Kp57	Urine	Female	52 years
Kp68	Sputum	Female	12 Months
Kp73	Urine	Male	4 years
Kp84	Sputum	Female	8 Months
Kp93	Sputum	Male	2 years
Kp97	Urine	Male	19 Months
Kp101	Sputum	Female	11 years
Kp104	Sputum	Male	47 years

[a] : Urinary tract infection

Table 3. Frequency of *qnr* genes based on ciprofloxacin (Quinolone) susceptibility in *K. pneumoniae* isolates from Lorestan, Iran. The obtained results showed there is no significant association between ciprofloxacin resistance and presence of *qnr* genes (P=0.074)

Ciprofloxacin susceptibility	No. (%) isolate with *qnr* determinants				
	qnrA	*qnrB*	*qnrS*	*qnrB* + *qnrS*	Total
Sensitive (n=66)	0 (0)	8 (12.1)	0 (0)	1 (1.5)	9 (13.6)
Intermediate (n=7)	0 (0)	1 (16.6)	0 (0)	0 (0)	1 (14.3)
Resistant (n=34)	0 (0)	7 (20.6)	1 (2.9)	0 (0)	8 (23.5)
Total (n=107)	0 (0)	16 (14.9)	1 (0.93)	1 (0.93)	18 (16.8)

2006). Prevalence of *qnr* in the present study was also higher than that shown in other areas in Brazil (2.3%), Singapore (5.2%) and the USA (11.1%) (Wang *et al.*, 2005; Minarini *et al.*, 2009; Teo *et al.*, 2009). While it was much lower than the prevalence of *qnr* genes detected in Malaysia (48.9%) and China (65.5%) (Yang *et al.*, 2004; Saiful Anuar *et al.*, 2013). This difference between prevalence of *qnr*-positive isolates might be related to some factors such as geographical region of isolates and method of study. The present findings indicated that *qnrB* was the most prevalent one (88.9%), followed by *qnrS* (5.55%), whereas no isolates carried *qnrA* among all the clinical isolates. In the studies conducted by Minarini *et al.* (2008) and Saiful Anuar *et al.* (2013) on clinical isolates in Brazil (2.3%) and Malaysia (31.9%), respectively, *qnrB* has been proven to be

more prevalent than other *qnr* genes among the tested clinical isolates. In contrast, in other investigations carried out in China and the USA, it has been shown that *qnrS* (14.9%) and *qnrA* (14%) are the most prevalent of all *K. pneumoniae* clinical isolates screened by multiplex PCR, respectively (Robicsek *et al.*, 2006; Wang *et al.*, 2008, 20). The present investigation shows no *qnrA* in its clinical isolates; similarly, Minarini *et al.* (2008) and Saiful Anuar *et al.* (2013) in Brazil and Malaysia have indicated no *qnrA* or *qnrS*-positive isolates among their clinical isolates of *K. pneumonia*e, respectively.

Here, *qnr* determinants (*qnrA*, *qnrB* and *qnrS*) were found from 8 (23.5%) ciprofloxacin-resistant, 1 (14.3%) intermediate and 9 (13.6%) sensitive isolates. Similar to these results, Saiful Anuar *et al.* (2013) reported that the highest percentage of *qnr* determinants (47.8%) was found in ciprofloxacin-resistant isolates. It has been previously demonstrated that clinical isolates with *qnr* determinants are known to harbor multiple ciprofloxacin resistance mechanisms such as variations in *gyrA* or reduce the drug permeability and therefore facilitate high resistance to ciprofloxacin (Martinez-Martinez *et al.*, 1998; Tran *et al.*, 2005). However, in the present study, there is no significant association between ciprofloxacin resistance and presence of *qnr* determinants. There are some limitations in this study, for example conjugation and cloning experiments to investigate the plasmids of *qnr* positive isolates which are the topic of our future study. In conclusion, the present study showed that there was high frequency of *qnr*-positive *K. pneumoniae* in Khorramabad, Iran. However, there is no association between quinolone resistance and presence of *qnr* genes in isolates of *K. pneumoniae*.

Conflicts of interest

The authors have no conflicts of interest.

REFERENCES

Azadpour, M., Nowroozi, J., Goudarzi, G.R., Mahmoudvand, H. (2015). Presence of qacEΔ1 and cepA genes and susceptibility to a hospital biocide in clinical isolates of *Klebsiella pneumoniae* in Iran. *Tropical Biomedicine* **32**(1): 109-15.

Clinical and Laboratory Standard Institute (CLSI), (2009). Performance standards for antimicrobial susceptibility testing; 19th informational supplement. Wayne, PA; M100-S19.

Hooper, D.C. (2003). Mechanisms of quinolone resistance. In: Hooper, D., Rubinstein, E. (Eds.), Quinolone Antimicrobial Agents. ASM Press, Washington, DC.

Jacoby, G.A., Walsh, K.E., Mills, D.M., Walker, V.J., Robicsek, A. & Hooper, D.C. (2006). *qnrB*, another plasmid mediated gene for quinolone resistance. *Antimicrobial Agents Chemotherapy* **50**: 1178-1182.

Janda, J.M. & Abbott, S.L. (2006). The Genera *Klebsiella* and *Raoultella*. *The Enterobacteria* (2nd ed, 115-129), Washington, USA: ASM Press.

Kim, M.H., Lee, H.J., Park, K.S. & Suh, J.T. (2010). Molecular characteristics of extended spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* and the prevalence of qnr in Extended spectrum beta-lactamase isolates in a tertiary care hospital in Korea. *Yonsei Medical Journal* **51**(5): 768-74.

Martinez-Martinez, L., Pascual, A. & Jacoby, G.A. (1998). Quinolone resistance from a transferable plasmid. *Lancet* **351**: 797-799.

Minarini, L.A., Poirel, L., Cattoir, V., Darini, A.L. & Nordmann, P. (2008). Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *Journal of Antimicrobial Chemotherapy* **62**: 474-478.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. *Microbiology and Molecular Biology Reviews* **67**: 593-656.

Pakzad, I., Ghafourian, S., Taherikalani, M., Sadeghifard, N., Abtahi, H., Rahbar, M., *et al.* (2011). qnr Prevalence in Extended Spectrum Beta-lactamases (ESBLs) and None-ESBLs Producing *Escherichia coli* Isolated from Urinary Tract Infections in Central of Iran. *Iranian Journal of Basic Medical Sciences* **14**(5): 458-464.

Paterson, D.L., Sagnimeni, A.J., Hansen, D.S., Mohapatra, S., Casellas, J.M., *et al.* (2002). Community-acquired *Klebsiella pneumoniae* bacteremia: Global differences in clinical patterns. *Emerging Infectious Diseases* **8**: 160-166.

Poole, K. (2005). Efux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **56**: 20-51.

Raei, F., Eftekhar, F. & Feizabadi, M.M. (2014). Prevalence of quinolone resistance among extended-spectrum β- Lactamase producing uropathogenic *Klebsiella pneumoniae*. *Jundishapur Journal of Microbiol*ogy **7**(6): e10887.

Robicsek, A., Jacoby, G.A. & Hooper, D.C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infectious Diseases* **6**: 629-40.

Saiful Anuar, A.S., Mohd Yusof, M.Y. & Tay, S.T. (2013). Prevalence of plasmid-mediated *qnr* determinants and gyrase alteration in *Klebsiella pneumoniae* isolated from a university teaching hospital in Malaysia. *European Review for Medical and Pharmacological Sciences* **17**: 1744-1747.

Strahilevitz, J., Jacoby, G.A., Hooper, D.C. & Robicsek, A. (2009). Plasmid-mediated quinolone resistance: a multifaceted threat. *Clinical Microbiology Review* **22**: 664-689.

Teo, J.W., Ng, K.Y. & Lin, R.T. (2009). Detection and genetic characterisation of *qnrB* in hospital isolates of *Klebsiella pneumoniae* in Singapore. *International Journal of Antimicrob*ial *Agents* **33**: 177-180.

Tran, J.H. & Jacoby, G.A. (2005). Hooper, D.C. Interaction of the plasmid-encoded quinolone resistance protein *qnr* with *Escherichia coli* DNA gyrase. *Antimicrobial Agents Chemotherapy* **49**: 118-125.

Wang, A., Yonghong, Y., Quan, L., Wang, Y., Chen, Y., Deng, L., *et al.* (2008). Presence of *qnr* gene in *Escherichia coli* and *Klebsiella pneumoniae* resistant to ciprofloxacin isolated from pediatric patients in China. *BMC Infectious Diseases* **8**: 68-74

Wang, M., Sahm, D.F., Jacoby, G.A. & Hooper, D.C. (2004). Emerging plasmid-mediate quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrobial Agents Chemotherapy* **48**: 1295-1299.

Wu, J.J., Ko, W.C., Tsai, S.H. & Yan, J.J. (2007). Prevalence of plasmid-mediated quinolone resistance determinants *qnrA*, *qnrB* and *qnrS* among clinical isolates of *Enterobacter cloacae* in a Taiwanese Hospital. *Antimicrobial Agents Chemotherapy* **51**: 1223-1227.

Yang, H., Chen, H., Yang, Q. & Chen, M. (2004). Wang, H. High prevalence of plasmid-mediated quinolone resistance genes *qnr* and *aac(6_)-Ib-cr* in clinical isolates of Enterobacteriaceae from nine teaching hospitals in China. *Antimicrobial Agents Chemotherapy* **52**(12): 4268-4273.