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Occurrence of *Klebsiella pneumoniae* in Hospitalized Patients in South of Iran (2014-2016): Assessment of Antibiotic Non-susceptibility and Quinolone Resistance Markers

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Abstract

Objectives: The purpose of the current research was to investigate the existence of *aac(6′)-Ib-cr* and *qnr* (*qnrS, qnrD, qnrC, qnrB, and qnrA*) genes and determine antibiotic sensitivity patterns in *Klebsiella pneumonia* strains, recovered from hospitalized patients in Shiraz, southern Iran.

Materials and Methods: In the current cross-sectional study, the strains isolated from the clinical specimens of hospitalized individuals were tested during 2014-2016 in Shiraz, Iran. The culture was done on microbiological media, and the strains were recognized as *K. pneumonia* based on biochemical tests in the analytical profile index-20E diagnostic strip. All the strains were investigated for antibacterial sensitivity using the CLSI standard guidelines. Gene-encoding antibiotic resistance markers consisting of *aac(6′)-Ib-cr*, *qnrS, qnrD*, *qnrC*, *qnrB*, and *qnrA* were evaluated by polymerase chain reaction (PCR) assay.

Results: Ninety-six strains of *K. pneumoniae,* isolated from hospitalized patients, were entered into the research. Based on the results, 51.04% of strains were recovered from females, and 35.66% of patients were hospitalized in the intensive care unit. Sixty-two strains were isolated from the urine. Imipenem was the most efficient drug against the strains (81.52%). Less than 50% were sensitive to the new generation of quinolones. All isolates harboring antibiotic resistance genes were non-susceptible or at the intermediate level of resistance to ciprofloxacin (CIP). Most *aac(6′)-Ib-cr*$^{+}$ strains were non-susceptible or at the intermediate level of resistance to piperacillin/tazobactam. In PCR assay, 27.08% of isolates had antibiotic resistance traits (*aac(6′)-Ib-cr*, *qnrS, qnrD*, *qnrC*, *qnrB*, and *qnrA*). Finally, *aac(6′)-Ib-cr*, *qnrS*, and *qnrB* markers were found in 7.3%, 10.4%, and 7.3%, respectively.

Conclusions: In this research, the emergence of potentially virulent and carbapenem non-susceptible cases in conjugation with a quinolone-resistant genotype is alarming, thus vigorous clinical manifestations, along with drastic constraints in therapy would be a possible result in this regard.

Keywords: Hospitalized patients, *Klebsiella pneumoniae*, Antibiotic resistance, Quinolone resistance genes

Introduction

Klebsiella pneumoniae, an opportunistic Gram-negative pathogen in the Enterobacteriaceae family, is identified in a wide range of diseases from the infection of the soft tissue, urinary system infection, bacteremia, and pneumoniae in hospitalized patients in developed and developing countries to pyogenic liver abscess and sepsis in community settings in the developing world (1,2).

Hospital-acquired *Klebsiella* diseases are principally created by *K. pneumoniae*, the medically most significant member of the genus (3). It causes severe endemic and epidemic infections in hospitalized patients (4). As an opportunistic microorganism, *K. pneumoniae* mainly attacks immunodeficient persons who suffer from serious underlying illnesses (3). Nosocomial infections are an important difficulty in the delivery of intensive care units (ICUs). Each hospital-acquired infection prolongs an infected individual's stay in the hospital by 5-10 days. This is a significant preventable cause of raised cost, mortality, and morbidity among hospitalized persons.

The emergence of antimicrobial-resistant organisms in individuals with fatal complications such as nosocomial infections has commonly been ascribed to the overuse of drugs. Thus, it is increasingly common to encounter persons infected with pathogenic bacteria that are non-susceptible to nearly all presently existing antimicrobials. The development of non-susceptible pathogens such as *K. pneumoniae* is a potential fear in hospital units (5,6).

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Key Messages

- The emergence of significantly virulent and carbapenem-resistant strains in conjugation with a quinolone-resistant genotype in our research is alarming.

Fluoroquinolone antibiotics are applied for the nosocomial and community-acquired illness treatment caused by *K. pneumoniae*. However, resistance to fluoroquinolones in this bacterium is increasing (7). The mechanisms of plasmid-mediated resistance play a crucial role in non-susceptibility to fluoroquinolones, and its rate is rising in different geographical areas. The *qnr* gene was the first identified plasmid-mediated-quinolone-resistance mechanism. Today, five types of such determinants have been characterized (i.e., *qnrC*, *qnrS, qnrD*, *qnrB*, *qnrA*) in this regard (8).

Another resistance mechanism is mediated by the *aac(6′)-Ib-cr* (plasmid-associated) gene, which encodes an aminoglycoside acetyltransferase AAC(6′)-Ib enzyme conferring reduced susceptibility to ciprofloxacin (CIP) by modifying it. In addition, this resistance gene confers diminished sensitivity to some aminoglycosides (9).

Accordingly, it is of critical importance for institutions to recognize the regional drug non-susceptibility profiles to develop appropriate infection control guidelines and expand a logical drug management with regional protocols for antimicrobial use (10). Such stewardship information is also applied for evaluating the efficiency of the followed guidelines and recognizing new ways for bacterial antibiotic resistance control. Given the great dissemination of multiresistance over the last few years, the objective of the current investigation was to assess the existence of *aac(6′)-Ib-cr,* and *qnr* (*qnrS, qnrD*, *qnrC*, *qnrB*, *qnrA*) genes and determine antibiotic sensitivity patterns in the strains of *K. pneumoniae* recovered from hospitalized patients in Shiraz, the south of Iran.

Materials and Methods

Isolation and Confirmation of the Isolates

The current cross-sectional investigation tested the strains that were recovered consecutively from different samples (i.e., blood, tracheal tube, urine, wound, and sputum) of hospitalized patients between March 2014 and September 2016 in Shiraz, Iran. Briefly, the aerobic blood culture was done using a manual blood cultivator tube. The aerobic culture of other specimens was done on common bacteriological culture media such as MacConkey agar, chocolate agar, and blood agar. All cultures were kept under incubation at 35°C±2 for 24 hours. Bacterial colonies were recovered and subculture was performed on MC agar plates in addition to Gram-staining. The strains were recognized as *K. pneumoniae* according to microscopic morphology, colony morphology, catalase and oxidase tests, motility, pigment production, as well as the fermentation of triple-sugar iron, hydrogen sulfide production, urease, indole production, and citrate utilization. Then, various biochemical tests located in analytical profile index-20E diagnostic strips (bioMérieux Company, France) were applied for the final confirmation of strains (7,11). The isolated strains were stored in the brain heart infusion broth (BHI) medium (Merck Company, Germany) with 20% (v/v) glycerol at -20 °C until the antibacterial susceptibility assay was conducted in the laboratory of clinical microbiology. *Eventually, K. pneumoniae* ATCC 700603 was applied for the quality control of identification.

Antibiotic Susceptibility

Klebsiella pneumoniae strains were cultured from BHI broth-glycerol stocks on MacConkey medium and grown overnight at 35°C±2. All strains were examined for their antibiotic sensitivity profiles using the standard protocols presented by the CLSI (11). Sensitivity testing to 24 antibiotics (MAST Company, UK) was performed by the disc-diffusion technique on cation-adjusted Mueller-Hinton agar plates based on the CLSI measures (16). The antibiotics were aztreonam (ATM, 30 µg), tigecycline (TGC, 15 µg), imipenem (IMP, 10 µg), amikacin (AK, 30 µg), CIP (5 µg), meropenem (MEM, 10 µg), gentamicin (GM, 10 µg), and piperacillin-tazobactam (PTZ, 100/10 µg).

Polymerase Chain Reaction Assay

Bacterial DAN Extraction

A single pure colony of each isolate was inoculated from a MacConkey agar plate into 5 mL of Muller-Hinton broth and incubated for 16-18 hours at 35°C±2. Subsequently, 1.5 mL of the isolate overnight culture was harvested by centrifugation at 7000 g for 5 minutes. After the supernatant was decanted, the pellet was resuspended in 500 µL of deionized water. The bacterial cells were lysed by heating at 95°C for 10 minutes. Then, the debris of the bacterial cells was deleted by centrifugation at 13000 g for 5 minutes. The supernatant of the total sample was applied as the main stock of DNA for polymerase chain reaction (PCR) (15).

Assessment of Antibiotic-non-susceptibility Markers in the Strains by the Molecular Method

Gene-encoding antibiotic resistance markers consisting of *aac(6')-Ib-cr, qnrB*, *qnrC*, *qnrS*, *qnrA*, and *qnrD* were investigated using specific primers in PCR assay (12). The performed PCR assays were previously described by other researchers (Table 1). The expected sizes of amplicons, annealing temperatures, and primer sequences for each PCR are presented in Table 1 (16-18). PCR was carried out and the products were surveyed by electrophoresis in 1% agarose gels by the Tris-Borate-EDTA buffer. Subsequently, the agarose gels were stained with ethidium bromide and analyzed on an ultraviolet transilluminator.

Table 1. Primers and Conditions Applied for Amplifying Resistance Traits in Klebsiella pneumoniae Strains

Target Gene	Primer Name	Sequence (5' to 3')	Length (base)	Annealing (°C)	Amplicon Size (pb)	References
qnrA	qnrA-F	TTCTCACGCCAGGATTTGAG	20	56	468	12
	qnrA-R	TGCCAGGCACAGATCTTGAC	20			
qnrB	qnrB-F	TGGCGAAAAAATTGAACAGAA	21	56	264	13
	qnrB-R	GAGCAACGATCGCCTGGTAG	20			
qnrC	qnrC-F	GGGTTGTACATTTATTGAATC	21	56	307	12
	qnrC-R	TCCACTTTACGAGGTTCT	18			
qnrD	qnrD-F	CGAGATCAATTTACGGGGAATA	22	56	582	14
	qnrD-R	AACAAGCTGAAGCGCCTG	18			
qnrS	qnrS-F	GACGTGCTAACTTGCGTGAT	20	53	428	13
	qnrS-R	AACACCTCGACTTAAGTCTGA	21			
aac(6′)-Ib-cr	aac(6′)-Ib-cr-F	TTGCGATGCTCTATGAGTGGCTA	23	56	482	12
	aac(6′)-Ib-cr-R	CTCGAATGCCTGGCGTGTTT	20			

The expected size of the amplification was determined by comparison with a related gene (DNA) ruler (Figures 1-6). *K. pneumoniae* ATCC 13883 was applied as quality control.

Statistical Analyses

The analysis of data was done by Pearson's chi-square test and using SPSS 19 statistical software. *P* values <0.05 were considered statistically significant.

Results

Patients' Data

Ninety-six clinical strains of *K. pneumonia,* recovered from hospitalized patients, were examined during the above-mentioned period. The strains were isolated from 47 males (48.95%) and 49 females (51.04%). Most patients (35.66%) were hospitalized in the ICU. Most specimens (64.13%) were isolated from the urine (Table 2).

Phenotypic Data and Antibiotic Susceptibility Patterns

Based on the *in vitro* antimicrobial sensitivity testing, IMP was the most efficient drug against strains (81.52% strains were sensitive) while ATM had the least effective antimicrobial sensitivity (30.43% of isolates were resistant). As revealed, 75% of *K. pneumoniae* isolates were sensitive to MEM. Less than 50% of the strains were sensitive to the new generation of the quinolone class of antimicrobial agents (i.e., levofloxacin, ofloxacin, norfloxacin,). Gentamicin non-susceptibility was observed in 42.4% of strains (Figure 1).

In this research, all strains harboring antibiotic resistance determinants (*aac(6')-Ib-cr*, *qnrS, qnrD*, *qnrC*, *qnrB*, and *qnrA*) were non-susceptible or at the intermediate level of resistance to CIP. One strain had both *aac(6')-Ib-cr*$^{+}$ and *qnrA*+ genes with an antibiotic resistance pattern of MEM_I - AK_R - CIP_R - GM_R - ATM_R - PTZ_I. Table 3 presents the characteristics of the positive *K. pneumoniae* strains of antibiotic resistance determinants. Most *aac(6')-Ib-cr*$^{+}$ strains were insensitive or at an intermediate level of resistance to PTZ. Among *qnrS* positive isolates, 3 strains were resistant to MEM.

Detection of Resistance-Associated Genes

Among 96 *K. pneumoniae* isolates for which we performed PCR assay, 27.08% had antibiotic resistance determinants (*aac(6')-Ib-cr*, *qnrS, qnrD*, *qnrC*, *qnrB*, and *qnrA*). Strains harboring the *qnrA* gene were only detected in 8 isolates (8.30% of the strains). The *aac(6')-Ib-cr*, *qnrS*, and *qnrB* genes were found in 7.3%, 10.4%, and 7.3%, respectively. Among the surveyed markers, *aac(6')-Ib-cr* was the most common determinant among the studied isolates. Only 6 isolates had two of the surveyed antibiotic resistance markers. The *qnrD* and *qnrC* determinants were not distinguished in the strains (Figures 2-5).

Discussion

Hospital-acquired infections with non-susceptible Gram-

Table 2. Demographic Traits of Patients Infected With *Klebsiella pneumoniae* (N = 96)

Category	Result (%)
Gender	
Male	48.91
Female	51.08
Ward	
ICU	35.66
Transplant	16.20
Internal	18.37
Surgery	6.96
Neurology	1.08
Other	21.73
Specimen	
Urine	64.13
Blood	7.60
Sputum	4.34
Pleural fluid	2.17
Wound	5.43
CSF	1.08
Stool	2.17
Abdominal fluid	1.23
Discharge	11.93

Note. ICU: intensive care unit; CSF: cerebrospinal fluid.

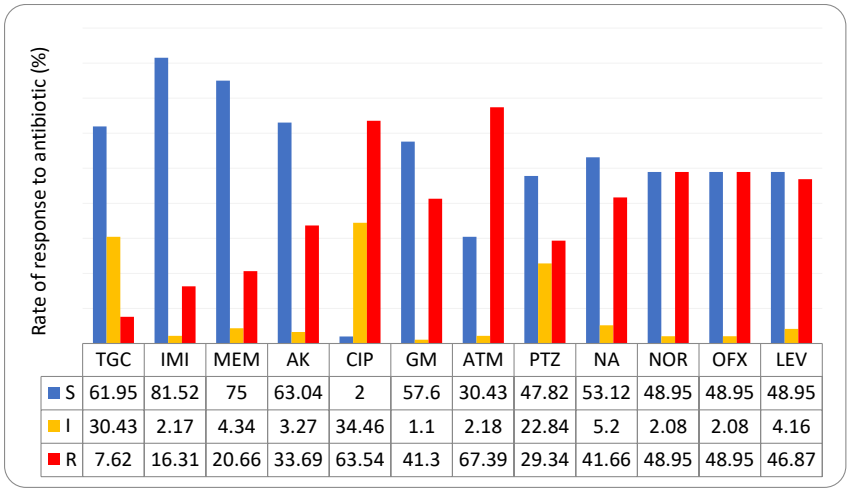

Figure 1. Drug Sensitivity Profile of *K. pneumoniae* Recovered From Hospitalized Patients (Percentages). TGC, tigecycline; IMI, imipenem; MEM, meropenem; AK, amikacin; CIP, ciprofloxacin; GM, gentamicin; PTZ, piperacillin/tazobactam; ATM, aztreonam; NA, nalidixic acid, NOR, norfloxacin; OFX, ofloxacin; LEV, levofloxacin; S, sensitive; I, intermediate; R, resistant.

Table 3. Features of *qnr*+ or *aac(6′)-Ib-cr*$^{+}$ *Klebsiella pneumoniae* Isolates

Isolate No.	Source	Hospital Ward	Relevant Marker	Antibiotic Resistance Pattern
23	Sputum	ICU	*qnrS*$^{+}$	IMI_R, MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_R
15	Urine	ICU	*qnrS*$^{+}$	TGC_I, AK_I, CIP_R, GM_R, ATM_R, PTZ_I
31	Urine	Central laboratory	*aac(6′)-Ib-cr*$^{+}$	AK_R, CIP_R, GM_R, ATM_R, PTZ_I
127	Urine	Transplantation	*qnrA*$^{+}$	MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_R
148	Urine	Clinical treatment	*qnrA*$^{+}$	TGC_R, CIP_R, ATM_R, PTZ_I
41	Stool	ICU	*qnrS*$^{+}$	IMI_I, MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_R
63	wound	Internal	*qnrS*$^{+}$	MEM_I, AK_R, CIP_R, GM_R, ATM_R, PTZ_I
27	Urine	Internal	*qnrA*$^{+}$	IMI_R, MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_R
53	Stool	Internal	*qnrS*$^{+}$	IMI_R, MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_R
89	Urine	Treatment evaluation	*qnrA*$^{+}$	MEM_R, AK_R, CIP_R, GM_R, ATM_R, PTZ_I
25	Wound	Surgery	*qnrS*$^{+}$	CIP_R, ATM_R, PTZ_I
3	Urine	Internal	*qnrS*$^{+}$	CIP_R, ATM_R, PTZ_R
69	Urine	ICU	*aac(6′)-Ib-cr*$^{+}$	CIP_I
134	Wound	Surgery	*qnrB*$^{+}$,	TGC_I, CIP_R, GM_I
147	Urine	Internal	*qnrS*$^{+}$	TGC_I, CIP_R, GM_R, ATM_I, PTZ_I
14	Urine	Neurology	*aac(6′)-Ib-cr*$^{+}$	TGC_R, CIP_R, ATM_I, PTZ_I
61	Blood	Internal	*aac(6′)-Ib-cr*$^{+}$	CIP_I, ATM_R
38	Urine	Transplantation	*qnrA*$^{+}$,	CIP_I, ATM_R
214	Urine	Central laboratory	*qnrA*$^{+}$,	CIP_R
65	Urine	Endocrinology	*qnrB*$^{+}$,	MEM_R, CIP_R, ATM_R, PTZ_R
11	Sputum	ICU	*qnrS*$^{+}$	TGC_I, CIP_R, ATM_R, PTZ_R
45	Urine	Transplantation	*aac(6′)-Ib-cr*$^{+}$	TGC_I, CIP_R, GM_R, ATM_R, PTZ_I
106	Urine	ICU	*qnrA*$^{+}$,	CIP_I, GM_R, ATM_R
112	Urine	Internal	*qnrB*$^{+}$,	AK_R, CIP_R, GM_R, ATM_R, PTZ_R
107	Urine	Internal	*qnrB*$^{+}$,	CIP_I, GM_R, ATM_R
97	Urine	Internal	*qnrB*$^{+}$,	TGC_R, CIP_R, ATM_R, PTZ_R
74	Urine	Transplantation	*aac(6′)-Ib-cr*$^{+}$	TGC_I, CIP_R, GM_R, ATM_R, PTZ_I
91	Urine	Central laboratory	*qnrB*$^{+}$,	CIP_I, GM_R, ATM_R
95	Urine	Endocrinology	*qnrB*$^{+}$,	AK_R, CIP_R, GM_R, ATM_R, PTZ_R
80	Urine	ICU	*qnrS*$^{+}$	CIP_I, GM_R, ATM_R
113	Sputum	ICU	*qnrA*$^{+}$,	TGC_R, CIP_R, ATM_R, PTZ_R
11 (99)	Sputum	ICU	*aac(6′)-Ib-cr*$^{+}$	TGC_R, CIP_R, ATM_R, PTZ_R

Note. ICU: Intensive care unit; TGC_R: Tigecycline resistance; IMI_R: Imipenem resistance; MEM_R: Meropenem resistance; AK_R: Amikacin resistance; CIP_R: Ciprofloxacin resistance; GM_R: Gentamicin resistance; ATM_R: Aztreonam resistance; PTZ_R: Piperacillin/tazobactam resistance; CIP_I: Intermediate level ciprofloxacin resistance; PTZ_I: Intermediate level piperacillin/tazobactam resistance; TGC_I: Intermediate level tigecycline resistance; ATM_I: Intermediate level aztreonam resistance.

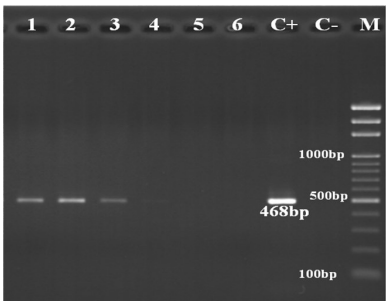

Figure 2. PCR Assay for the Identification of the *qnrA* Gene in *K. pneumoniae* Strains. *Note.* PCR: Polymerase chain reaction; *K. pneumoniae*: *Klebsiella pneumoniae*; **Lanes: M:** 100-bp DNA ladder; **1-3:** Positive samples; **4-6:** Negative samples; **7:** Positive control (*K. pneumoniae* ATCC 10031); **8:** Negative control.

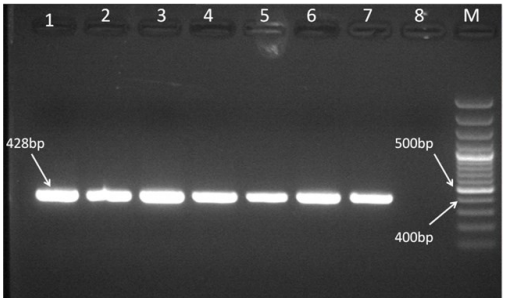

Figure 4. Detection of the *qnrS* Gene in *K. pneumoniae* Isolates. *Note.* *K. pneumoniae*: *Klebsiella pneumoniae*; **Lanes 1-6:** Positive samples; **Lane 7:** Positive control; **Lane 8:** Negative control; **Lane M:** 100-bp DNA ladder.

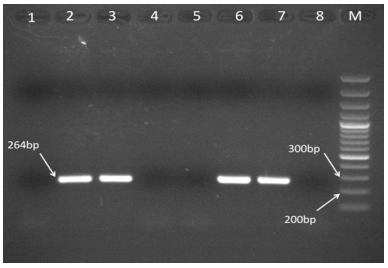

Figure 3. Agarose Gel Electrophoresis of the Amplified *qnrB* Gene by the PCR Assay. *Note.* PCR: Polymerase chain reaction; Lanes: M: 100-bp DNA ladder; **1, 4,** and **5:** Negative samples; **2, 3,** and **6:** Positive samples; **7:** Positive control (264 bp); **8:** Negative control.

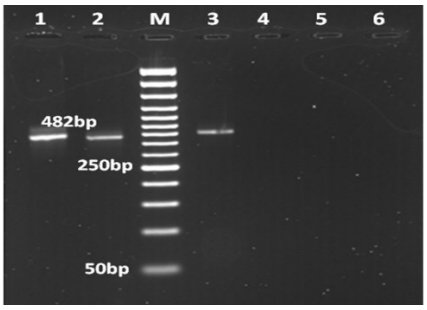

Figure 5. Agarose Gel Electrophoresis of the Amplified *aac(6′)-Ib-cr-R* Gene by the PCR Assay. *Note.* PCR: Polymerase chain reaction; **Lanes 1-2:** Positive samples; **M:** 50-bp DNA ladder; **3:** Positive control; **4:** Negative control; **5-6:** Negative samples.

negative bacteria, especially the isolates of *K. pneumoniae* have become a major health concern when there is a lack of promising new effective antibiotics on the horizon. Although antibiotic non-susceptibility was formerly noted mainly in hospital settings, it is nowadays also frequently revealed in community-acquired infections (15).

Resistance markers including *aac(6)-Ib-cr* and *qnr* may facilitate propagation and raise the rate of quinolone-non-susceptible isolates. To date, *qnr* determinants have been extensively identified in eastern and southern Asia, south, and north Europe and America. The current research showed a rate of 27.08% for *aac(6')-Ib-cr* and *qnr* markers among 96 strains of *K. pneumoniae* collected during 2014-2016 in Shiraz, Iran.

The rate of IMP-non-susceptible *K. pneumoniae* in this study (18.48%) is higher than the rate reported in Iran (2%). Kaczmarek et al demonstrated the trends of the emergence of carbapenem-non-susceptible *K. pneumoniae* in the United States (New York City) hospital settings (16). In this research (17), antibacterial non-susceptibility to gentamicin (42.4%) displayed by clinical *K. pneumoniae* strains was lower compared to the one demonstrated in Sanandaj, Iran (53.30%).

Among the tested *K. pneumoniae* isolates, 38.05% showed resistance (resistance and at an intermediate level of resistance) to TGC, which may suggest the emergence of strains resistant to relatively new drugs. The progressive increase in the TGC non-susceptible rate in Iran is alarming, hence, it cannot serve as the antibiotic of choice for therapeutic goals in the near future. Non-susceptibility to TGC in *K. pneumoniae* is on the rise according to reports from different regions worldwide, and its efficiency as a therapeutic drug is unclear, and the affected persons treated by TGC exhibit continuous bacteremia caused by this bacterium. Non-susceptibility to TGC in *K. pneumoniae* results from the upregulation of the *ramA* gene that causes the efflux-pump (AcrAB-TolC) overexpression.

In the current investigation, the frequency of *qnrA* and *qnrB* among the isolated strains of *K. pneumoniae* was 8.30% (8 of 96) and 7.30% (7 of 96), respectively. The rate of *qnrS* was more among the tested isolates, as noted by other researchers. However, the two important *qnr* markers (i.e., *qnrB* and *qnrA*) were observed with the same prevalence despite the low frequencies of *qnrC* and *qnrD* observed in our research. In this study, the frequency of *aac(6)-Ib* carriage among the strains (7.40%) was higher than that among *K. pneumoniae* strains in other studies (17,19,20). Furthermore, among *aac(6)-Ib-cr* positive strains, 3 cases were MEM non-susceptible. In the study by Xue et al (18)

in China, the most frequently indicated quinolone non-susceptible markers was *qnrS* (13.2%), followed by *aac(6')-Ib-cr* (6.2%) and *qnrB* (3.7%). In another study by Heidary et al in Iran, 85% of *K. pneumoniae* strains recovered from hospitalized individuals carried the *aac(6')-Ib-cr* marker (19).

Quinolones are the drugs which are widely used in the community, hospital settings, and structures in aquatic environments. Exposure to lower concentrations of these antibiotics raises the chance for the selection of resistance. Hence, they can be the stock of a potential progressive force for the selection of non-susceptibility to quinolones. In addition, the aquatic environment is a significant source of new drug resistance markers (20).

The emergence of significantly virulent and carbapenem-resistant strains in conjugation with a quinolone-resistant genotype in our research is alarming. Serious clinical manifestations together with drastic restrictions in treatment would be a possible result in this respect. Antimicrobials are introduced into diets in the agriculture industry as a veterinary medicine and as growth-inducing to gain adequate amounts of food (21-24). Amazingly, until lately, about 70% of antimicrobials in the animal food chain was for non-therapeutic goals such as growth induction. Such utilization is usually done via feeding at extremely low concentrations over long periods. This is a highly perilous work that could increase the populations of non-susceptible organisms.

Many developed countries manage the Antimicrobial Stewardship Programs to standardize antibiotic therapy, diminish treatment-associated costs, reclaim clinical manifestations and safety, and prevent antimicrobial resistance. So far, antimicrobial surveillance in Iran has been limited to a few research centers. Initial studies indicate that there has been a significant rise in the frequency of multidrug-resistant bacteria.

Conclusions

The updates of trends in the usage of antibiotics in hospital units are vital for the clinical care design and improvement of antibiotic prescription. Antibiotic resistance trends from large multicenter and national surveillance research can serve as foundational information for more efficient treatments.

Authors' Contribution

All listed authors have equally contributed to the project.

Conflict of Interests

The authors have no conflict of interests.

Ethical Issues

The project has been approved by ethics committee of Yazd University of Medical Sciences.

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