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Detection of VIM-1 and IMP-1 genes in *Klebsiella pneumoniae* and relationship with biofilm formation

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ABSTRACT

Klebsiella pneumoniae is an important human pathogen that is considered in recent years due to nosocomial infections resistant to treatment as well as the ability to form biofilms particularly in patients with urinary tract infection in ICU or hospital. The aim of this study was to evaluate the prevalence of VIM1, IMP1 genes and their ability to form biofilm in *K. pneumoniae* strains isolated from patients with urinary tract infection. In the study, using culture and biochemical methods, 1807 *K. pneumoniae* samples were isolated from patients with urinary tract infection hospitalized or referred to hospitals in Qom in 2013–2014. For isolation of MBL producing isolates, Double Disk Synergy Test (DDST) was used. Then MBL positive isolates were examined for the presence of VIM1, IMP1 genes using PCR method. Furthermore, all strains were investigated for biofilm formation by phenotypic microplate method. From 3165 urine samples cultured, 1807 isolates of *K. pneumoniae* were isolated and 109 strains (93.2%) were positive for MBL enzymes production. PCR results showed that the prevalence of VIM1 and IMP1 genes are 15.6 and 6.4%, respectively. The Phenotypic method indicated that 91.2% of isolates formed biofilm. Biofilm formation in *K. pneumoniae* isolates is high and there is a significant relationship between strong biofilm formation and prevalence of VIM1 and IMP1 genes. Also due to the presence of MBL genes in *K. pneumoniae* and horizontal transfer of genes to other bacteria, and to control the indiscriminate use of antibiotics, the hospital infection control methods must be considered.

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacilli that belongs to the *Enterobacteriaceae* family, which are part of the natural microflora of the human gut that can cause infections such as cystitis, pyelonephritis, septicemia, pneumonia, peritonitis, meningitis, diarrhea, osteomyelitis, and ulcers infections [1]. These bacteria can easily spread among people as nosocomial infections [2]. Urinary tract infection is a one of the largest bacterial infection that occurs in the community and in the hospital [3]. It is estimated that urinary tract infection includes25-40% of nosocomial infection [4] and each year more than 8.3 million people see a physician due to urinary tract infection. The UTI infection of this bacteria are seen in patients with urinary catheters, diabetes or immunosuppressed patients [5] and it is able to produce biofilm. Biofilms are bacterial populations that are connected by exopolysaccharides matrix on the surfaces. A matrix of extracellular polymeric substances (EPS) essentially consists of polysaccharides, proteins, lipids and nucleic acids in various amount. Moreover, Extracellular biofilm matrix, and oxygen gradient exist in biofilm architecture prevents the action of some antibiotics [6,7]. Biofilm bacteria are more resistant to antibiotics than other bacteria, so biofilms are a major cause of resistance to antimicrobial substances. Some studies reported that more than 65% of hospital-acquired infections are caused by biofilm-producing strains which are resistant infections with high healthcare costs. These biofilm infections are 10–1000 times more resistant to the effects of antimicrobial agents and antibiotics [6,7].

Klebsiella spp generates fimbriae, which facilitate bacterial binding to host mucosal surfaces and with capsules and can exhibit anti-phagocytic activity. One and three pili-types of *Klebsiella spp* play a role in the colonization of urinary tract infection. Organisms that are present in the biofilm use one or more mechanisms of drug resistance [8]. Biofilm formation ability in bacteria leads to chronic urinary tract infections [8]. Biofilms physically protect bacteria against the host immune system and antibiotics. This phenomenon is one of the causes of infections disease recurrence [9]. Eighty percent of infections are associated with the use of urinary catheters where *K. pneumoniae* plays an important role in the formation of biofilm on urinary catheters [10].

Increase in antibiotic resistance among *Enterobacteriaceae* family is a

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cause of major concern. Beta-lactam is an important antibiotic for the treatment of *K. pneumoniae* infections, but multidrug-resistant strains of ESBL producing strains in recent decades, have caused severe infections, which are resistant to treatment and even lead to death [6].

Carbapenem is the most effective drug for the treatment of ESBL producing *K. pneumoniae* infections. However, clinical use of carbapenema reaffected by carbapenemase enzymes produced by resistant bacteria (basically MBLs). These enzymes (MBLs) have been reported in many parts of the world and have very high resistance to all beta-lactams except aztreonam [11].

Class B of lactamases includes VIM (Verona integron-encoded metallo-β-lactamase) and IMP (Imipenemase) and most recently (New Delhi metallo-β-lactamase-1) NDM-1. These enzymes are able to hydrolyze penicillins, cephalosporins, Monobactams and carbapenems except aztreonam [12].

The aim of this study is to examine the relationship between biofilms and the presence of carbapenem resistance genes (VIM and IMP).

2. Materials and methods

2.1. Isolation and identification

In this study, *K. pneumoniae* were isolated from patients with urinary tract infection referred to Shahid Beheshti, Nekoyi, Kamkar, Vali Asr and Gulpaigani hospitals of Qom between 2013 and 2014. Out of 3165 urine samples, 1807 cultured plates were positive and bacteria were isolated as described in (Fig. 1). Patients with WBC ≥5/hpf and $\geq 10^4$ CFU/ml in urine cultures were selected for this study.

For this purpose, Midstream Specimen of Urine was taken for cultivation and were incubated for 18 to 24 h on conventional microbiology environments such as eosin methylene blue, MacConkey and blood agar at 37 °C. Identification of *K. pneumoniae* and other factors causing urinary infection was done by microbiological methods, including Gram stain, biochemical tests such as oxidase, catalase, IMVIC and urease test. The bacteria isolated were examined by API20E kit for final approval (Fig. 2).

2.2. MBL-producing strain identification

Imipenem-resistant strains were identified by antibiotic susceptibility test using Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany) and 10 µg imipenem disc (MAST, England) according to CLSI 2013 instructions. *E. coli* ATCC25922 strain and *K. pneumoniae* ATCC 700603 strain were used as a negative and positive control, respectively.

Strains resistant to imipenem were used to identify MBL-producing strains using DDST (Double disk synergy test) on Mueller Hinton agar medium. For this purpose, a 10 µg imipenem disc alone and an imipenem disk with 10 µl EDTA (0.5 mM) at distance of 20 mm were used on Mueller-Hinton agar. After 18 h of incubation at 37 °C inhibition zone of imipenem alone, they were compared with imipenem and EDTA. An increase of 7 mm or more on imipenem and EDTA compared to imipenem alone indicated the presence of MBL indicating that the test is positive [13].

2.3. Identifying the biofilm forming strains

For testing biofilm formation, using phenotypic microplate, isolates were incubated in Luria broth (LB) at 37°Cfor 18 h. Then 200 µl of TSB was transferred to every well of sterile microplate (96 cells), then 10 µl of bacterial suspension of incubated isolates (24 h) at opacity of half McFarland was added into the wells and was incubated at 37 °C for 24 h and then discharged from the wells and was washed 3 times with saline to keep the plate completely dry.

Then 200 µl of 1% crystal violet was poured for 20 min into the wells and was washed 3 times with saline and dried. Finally, 200 µl of DMSO was added to each well and the plate was examined by ELISA Plate Reader at 595 nm wavelength. High absorption is indicative of biofilm formation. Each test was repeated three times [14].

The ability to produce biofilm was considered in four categories:

Group 1: strong biofilm OD > 0.5.

Group 2: middle biofilm 0.5 > OD > 0.3.

Group 3: weak biofilm OD < 0.3.

Group 4: Lack of biofilm OD < 0.15.

To control the test, an environment without adding bacterial suspension was used as a negative control. Samples that generated biofilm phenotypically were examined in terms of Photo Electron FE-SEM (Field Emission Scanning Electron Microscopy.

2.4. PCR reactions and identification of VIM and EMP genes

DNA extraction was done by PP-214SBacteria Genomic DNA Extraction Kit (BioNeer Korea). PCR reaction was considered at 25 µl final volume containing 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 10 mMTris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1U Top DNA polymerase. Table 1 shows the sequence of used primers and PCR conditions for both VIM and IMP genes.

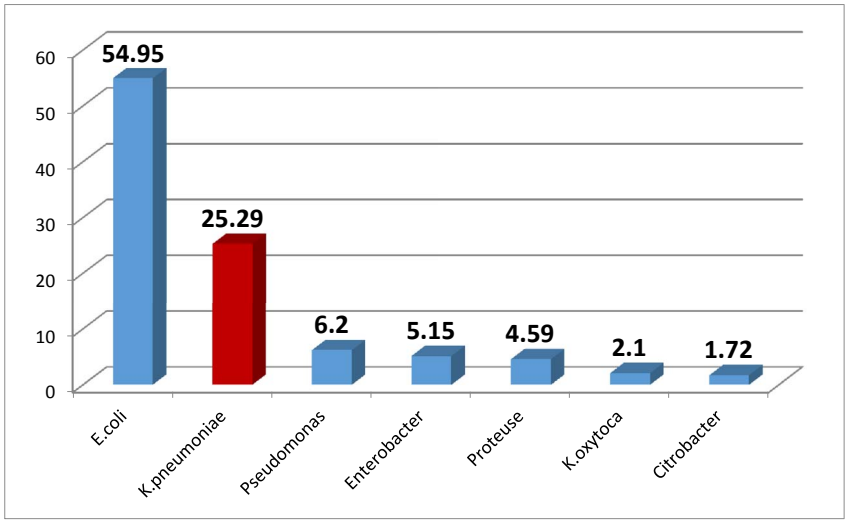

Fig. 1. The percentage of bacteria isolated from urine samples from hospitals in Qom during 2014–2015.

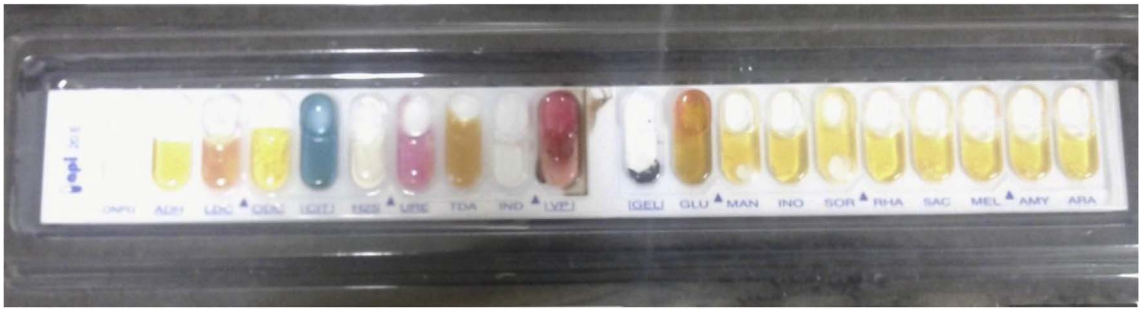

Fig. 2. API test results to confirm the identification of the isolates of *K. pneumoniae*.

2.5. Electrophoresis of PCR products

PCR products undergone electrophoresis on 1.5% agarose gel. Agarose gel was stained with ethidium bromide and was photographed by UV trans-illuminator (INGENIUS, SYN GENE). A DNA 100bpladder (BioNeer, Korea) was used as the size of marker.

2.6. Data analysis and statistical analysis

For plotting of graphs, Excel was used. Statistical analysis was performed with SPSS version 18.0 software (SPSS Inc. Chicago, IL, USA). To study the presence of VIM-1 and IMP-1 genes and biofilm formation chi-square test was used. P-value < .05 were considered as statistically significant.

3. Result

This cross-sectional study was performed on urine samples collected from hospitalized patients as well as outpatient suspected of *K. pneumoniae* in Shahid Beheshti, Kamkar, Nekoyi, Gulpaygani and Valiasr hospitals in Qom during the one-year period from January 2014 to January 2015.

During January 2014 to January 2015, 3165 urine samples were cultured and 57.1% or 1807 samples were positive. Out of 1807 urine samples, 993 *E. coli*, 457 *K. pneumoniae*, 112 *Pseudomonas spp*, 93 *Enterobacter spp*, 83 *Proteus spp*, 38 *Klebsiella oxytoca* and 31 *Citrobacter spp* samples were separated (Fig. 1).

From 457 *K. pneumoniae* isolates, 259 samples (56.7%) were patients who were hospitalized two or more days in the hospital with no symptoms of infection with *K. pneumoniae* in the initial hospitalization and 198 samples (43.3%) were outpatient. The largest and the smallest number of isolates of *K. pneumoniae* which are related to ICU and CCU respectively, are shown in Table 2.

The urinary tract infections in women were 286 cases (62.6%) and 171 cases were seen in men (37.4%). The age range of patients was 1–89 years. The average age of women and men were 41.95 and 37.96years, respectively.

In examining the frequency of MBL enzymes of *K. pneumoniae* isolates by phenotype method, 117 of 457 *K. pneumoniae* isolates (25.6%) were resistant to imipenem and 109 cases (93.2%) were positive in terms of MBL enzymes in Double Disk Synergy Test (imipenem disk alone and imipenem with- EDTAFig. 3).

PCR results showed that VIM gene frequency is 17 (15.6%) and VIM-1 gene electrophoresis gel image is shown in (Fig. 4). The frequency of the IMP gene is 7 (6.4%) and IMP-1 gene electrophoresis gel image is shown in (Fig. 5).

Using phenotype microplate method, biofilm formation showed that 417 isolates of *K. pneumoniae* (91.2%) have formed biofilm (Fig. 6) and samples that generated biofilm phenotypically were examined in terms of Photo Electron FE-SEM (Field Emission Scanning Electron Microscopy (Fig. 7).

The **Relationship** between biofilm formation and MBL genes are

Table 2
Frequency of *K. pneumoniae* isolates in different units of the hospital.

Unit	No	Percent
ICU	109	35.7
Surgery	90	29.5
Internal	58	19
Obstetrics and Gynecology	32	10.3
CCU	17	5.5

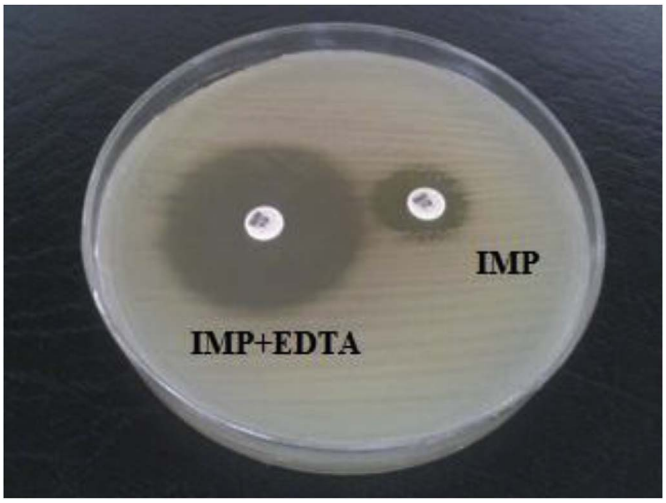

Fig. 3. MBL-producing *K. pneumoniae*. The right one is imipenem and the left is imipenem and EDTA. An increase of 7 mm or more in the zone of inhibition as compared with imipenem-EDTA and imipenem alone indicates the positivity of test and the presence of MBL.

Table 1
Primers for Amplification of Genes From *K. pneumoniae* isolates.

Primer Name	Primer Sequence (5′ to 3′)	Cycle	Initial Denaturation	Cycling	Final Extension	Length (bp)
IMP-1	F:AAAAAAGACGGTAAGGTTCAAGC R:ACCAGTTTTGCCTTACCATATTTG	33	5 min, 94 °C	30 s, 95 °C; 30 s,56.9 °C; 1 min,72 °C	7 min, 72 °C	260
VIM-1	F:CAAGTCCGTTAGCCCATTCC R:GGCACAACCACCGTATAGCAC	33	5 min, 94 °C	30 s, 95 °C; 30 sec,58 °C; 1 min,72 °C	10 min, 72 °C	539

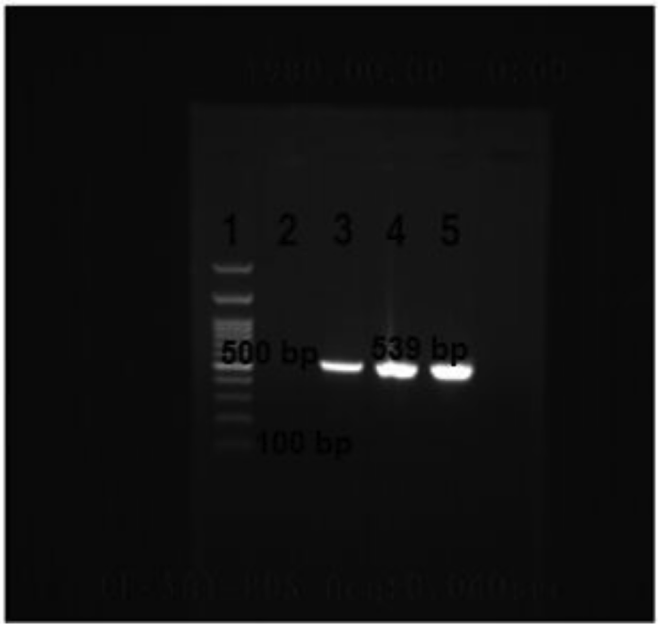

Fig. 4. PCR product for detection of VIM-1 gene in *K. pneumoniae.* **Lane1:** DNA ladder 100bp: **Lane2:** negative control (E.coli [ATCC25922]). **Lane3:** positive control (*K.pneumoniae* [ATCC 700603]): **Lane 4 and 5**: samples with VIM-1gene.

Fig. 5. PCR product for detection of IMP-1 gene in *K. pneumoniae.* **Lane1:**DNA ladder 100bp **Lane2:** positive control (*K.pneumoniae* [ATCC 700603]): **Lane 3 and 4**: samples with IMP-1 gene: **Lane5:** negative control (E.coli [ATCC25922]).

summarized in Table 3 and Fig. 8.

The relationship between the ability to form biofilm and MBL genes are summarized in (Table 3). There is a significant relationship between presence of VIM and IMP genes and biofilm formation (chi-square test [P value < .05]).

4. Discussion

K. pneumoniae causes 5 to 7.5% of all nosocomial infections and is one of four common pathogens in the intensive care unit [15–17]. Replacement, growth, and reproduction of pathogens in the kidneys, bladder, and urinary tract is called UTI or (Urinary Tract Infection). In this infection, there are clinical symptoms such as dysuria, urinary frequency, pain and sometimes fever [18].

Urinary tract infections are one of the problems millions of people are facing. The most common type of urinary tract infection is

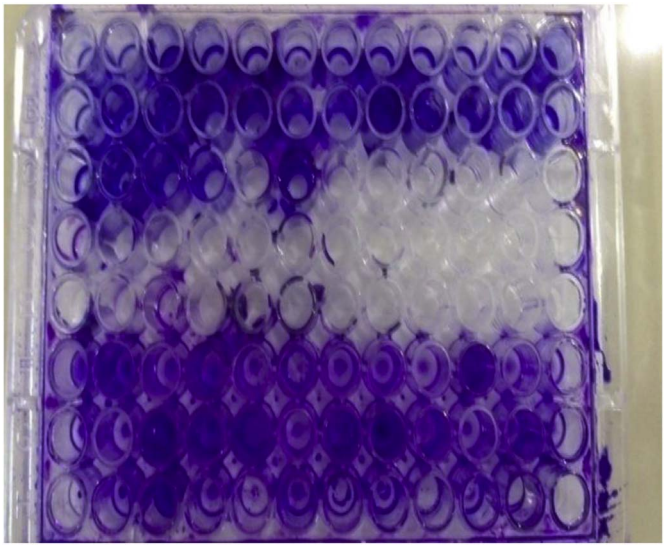

Fig. 6. Image of microplate in phenotype microplate method biofilm formation with crystal violet stain.

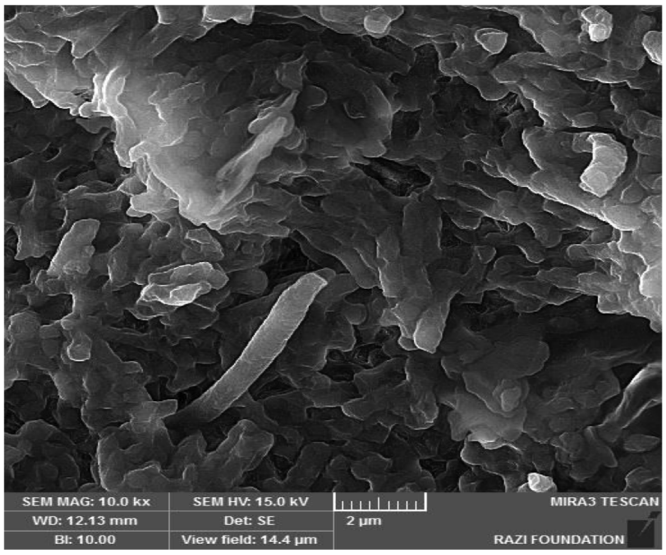

Fig. 7. Image of electronic microscopy (FE-SEM) of *K. pneumoniae*. formed biofilm.

asymptomatic bacteriuria. In the world, 150 million people suffer from urinary tract infection annually [19].

Epidemiological studies have shown that urinary tract infections are not limited to a specific age, but can also be seen in all age groups including infants, children and adults. The prevalence of this infection in women is 10 times more than men [20,21].

The presence of beta-lactamase genes such as TEM, SHV, CTX in the bacteria led to resistance to beta-lactams (penicillins and cephalosporins), but today, the presence of carbapenem resistance genes in these bacteria is important. Carbapenemase-producing *Enterobacteriaceae* (CPE) have a potential for severe infections resistant to conventional treatment, because they are resistant to antimicrobial drugs. In the US, KPC (*Klebsiella Pneumoniae* Carbapenemase) is the most common and important resistance mechanism to carbapenem in *Enterobacteriaceae*, while MBLs such as VIM, IMP and NDM are more common in Asia [22].

Another problem that makes *Klebsiella spp* to be an important pathogen that causes urinary tract infection is the formation of biofilm. One and three pili-types a role in the colonization of urinary tract infection [23]. Biofilms are bacteria mass connection that have the ability to accumulate and bind to the surface. This causes more bacterial

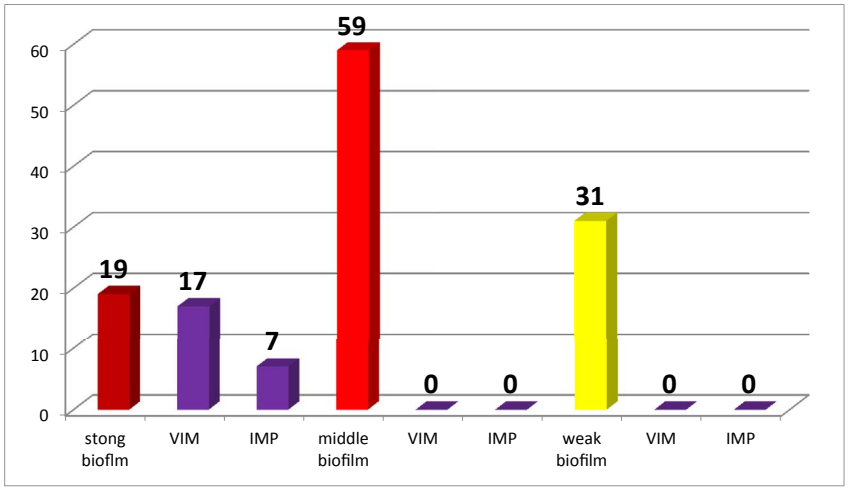

Fig. 8. Distribution of VIM and IMP genes and its relation with biofilm formation in *K. pneumoniae* isolates.

Table 3
Relationship between isolates for biofilm producer or biofilm non producer and VIM1, IMP1 genes in 109 MBL of *K. pneumoniae* isolates.

Methallo beta lactamase genes	Number of Isolates No.109	Strong Biofilm producer No.19	Middle Biofilm producer No. 59	WeakBiofilm producer No. 31	P value
VIM 1					
Positive	17	17	0	0	< .05
negative	92	2	59	31	
IMP 1					
Positive	7	7	0	0	< .05
negative	102	12	59	31	

resistance to antimicrobial agents and low permeability of drugs to biofilm layer leads to their ineffectiveness [24]. On another hand, One of the most important antibiotic tolerance mechanisms in biofilm is a slow growth rate of bacteria deactivates cell wall targeting antibiotics which need fast growth. Moreover, Extracellular biofilm matrix and oxygen gradient exist in biofilm architecture prevents the action of some antibiotics. In addition, persister cells, that is dormant cells have altered metabolism and can survive antimicrobial treatment [25,26].

In this study, from total urine samples,57.1% was positive and *K. pneumonia*e prevalence was 25.3%, which is in line with a study in India in 2013 [27]. However, it is lower compared to the study in Sao Paulo, Brazil in 2015 [28] and a study in Nigeria (2015) (32.4%) [29].

The results of the study regarding the resistance to imipenem are in line with the studies accomplished in Shahrekord [30], Zanjan [31] and a study in Brazil in 2015 [28]. However, the resistance is higher compared with one study in India in 2013 [27] and in Hamadan [32], which seems to be due to differences in geographic location and pattern of antibiotic use.

In terms of MBL enzymes, this study (93.2%) is close to a study in 2015 in India [33], but it was higher compared to a study in 2014 in Malaysia [34] and Nigeria [35] as well as a study performed in 2007 in Kerman [36]. It could be due to genetic differences between strains in different regions.

VIM genes prevalence in this study was 15.6% which matched the results achieved in India in 2013 [4] as well as the study published in 2009 in the US [37], but it was higher compared to a study in Nigeria in 2015 [29] and one study in 2010 [32] in Hamadan which may be due to genetic differences between the strains and increase in resistance genes.

In terms of biofilm formation in *K. pneumoniae* isolates, this study matched the results of the US in 2013 [38] and India (2012) [14], as well as the other study, was conducted in 2013 [38]. In terms of correlation between MBL genes and biofilm formation, no similar study has been done so far, and in this study, there was a significant relationship between VIM and IMP and biofilm formation.

5. Conclusion

The results of this study showed that the prevalence of MBL-producing *K. pneumoniae* in patients in hospitals in Qom is high (93.2%). Given that MBL-producing strains can become resistant to all beta-lactam antibiotics, attention to urinary tract infection (UTI) is of noticeable issue. Therefore, identification of *K. pneumoniae* which is capable of producing MBL was essential and can help physicians in prescriotion of antibiotics for the treatment of patients with infections caused by these bacteria.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

We certify that all data collected during the study is presented in this manuscript and no data from the study has been or will be published separately.

Authors' contributions and article approval

All authors were involved in study design, data collection.

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