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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 treatmentas 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.

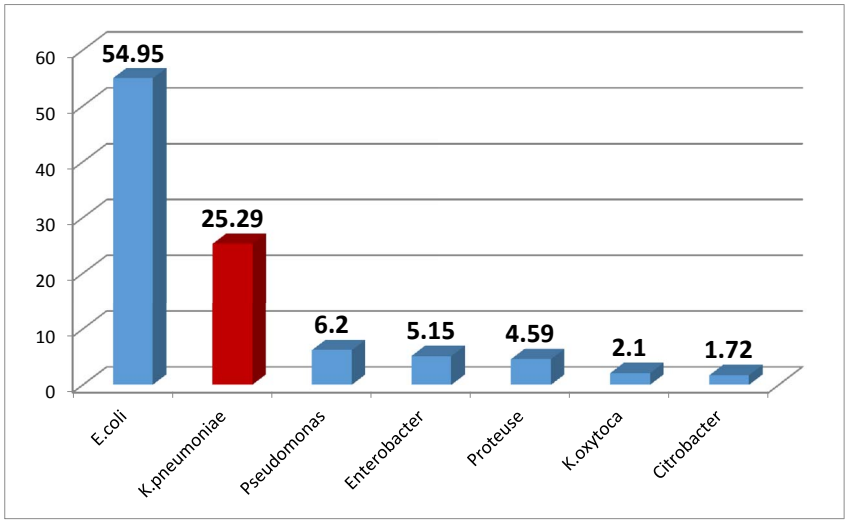

Fig. 1. The percentage of bacteria isolated from urine samples from hospitals in Qom during 2014–2015.