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Detection of efflux pump gene *cep*A in *Klebsiella pneumonia* and its effect on resistance to biocides

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Abstract

***Background & Aims*:** *Klebsiella pneumonia (K.pneumonia)* is one of the causative agents of lung infections, wound infections, urinary tract, and bloody diarrhea. One of the most common ways of transmission in neonatal and surgical wards is through hospital staff, nurses, and physicians. It could be transmitted to hospitalized patients and personnel through feces, respiratory secretions, contaminated equipment, and hands. To prevent the transmission of nosocomial infections, hand washing of employees with biocides can be effective.

Materials & Methods: The minimum inhibitory concentration of 65 *K.pneumonia* isolates was determined according to CLSI guidelines compared to common biocides used in educational hospitals in Urmia, Iran, such as benzalkonium chloride and chlorhexidine. PCR was performed to evaluate the presence of *cep*A genes.

Results: The results showed a significant relationship between the presence of *cep*A gene and high MIC compared to chlorhexidine bioside in *K. pneumoniae*. But there was no significant relationship between the presence of *cep*A gene and multidrug-resistant (MDR) isolates.

Conclusion: It is concluded that, detection of *cep*A gene or other genes involving drug resistance should be extended by using another tests with more reliability and reproducibility like gene expressions and gene cloning methods.

***Keywords*:** *K. pneumonia*, Chlorhexidine, MIC, *cep*A

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Introduction

Klebsiella pneumonia is one of the most important opportunistic pathogens in high-risk patients with alcoholism, malnutrition and immune suppression. *K. pneumonia* causes a variety of infections such as pneumonia, meningitis, urinary tract infection (UTI), bacteremia and liver abscess. The important virulence factors of this gram-negative bactria included structural antigens O and K, adhesive molecules (CF29K, KPF 28), cidrophores (Enterobactin, Aerobactin, Yersinobactin), ST and LT toxins, and urease (1, 2). Two of the disinfectants used in hospitals for cleaning the surfaces and hand washing are biocides chlorhexidine and benzalkonium chloride which decrease transmission of Klebsiella infections. Chlorhexidine is active on the anionic surface and cell membrane of microorganisms. It is used as disinfectant for hands, skin, and surgical sites. Benzalkonium

chloride is active on the cell membrane to make failure in molecular interactions and permeability of lipid layers (3, 4). Over dose and exposure of this agents cause resistance in the microorganism. There are various mechanisms of resistance against biocides in Klebsiella and other bacterial agents including broad spectrum beta lactamases, changing target sites, biofilm production and efflux pumps. *cep*A is one of the encoding genes of efflux pumps which lead to biocide resistance. The recent molecular studies on efflux pumps in *K. pneumonia* and other bacteria reached to detection and identification of the efflux pump encoding genes such as *cep*A with comparing the standard susceptibility tests (5). This study aimed detection of *cep*A gene in chromosomal DNA of *K. pneumonia* obtained from clinical isolates collection in Microbiology Department of Urmia University of Medical Sciences.

Material & Methods

Subjects:

Sixty five identified and labeled strains of *K. pneumonia* were obtained from clinical isolates collection (Department of Microbiology, School of Medicine, UMSU). All study strains were harvested on the culture medium using eosin-methylene blue for the susceptibility tests and molecular study.

Susceptibility Test (MIC):

According to CLSI procedures, serial dilutions of two study biocides, benzalkonium chloride and chlorhexidine were prepared. Bacterial cell suspensions were made and justified by using a spectrophotometer system as equal turbidity as the standard McFarland 0.5 (Feingold Diagnostic Microbiology). Separately, the serial dilutions of biocides were treated with the bacterial cell suspension in 96 well micro-plates and incubated at 37 °C for 24 to 48 hours. Minimum inhibitory concentration of the biocides was determined at the cut-off point of bacterial growth.

Molecular Assay:

For the detection of *cep*A gene, the chromosomal DNA extracted by using boiling, phenol-chloroform and colony PCR methods (6, 7). The amplicons were profiled into PCR as following protocol: in a 25 µl total volume, 1µl of each forward and reverse *cep*A primers (F5′CAACTCCTTCGCCTATCCCG3′ and R5′TCAGGTCAGACCAAACGGCG3, respectively) were added along with 12.5µl of premix (containing, Taq DNA polymerase, PCR buffer, nucleotides and $MgCl_2$) and 9.5µl of double deionized water. One microliter of amplicons or small amount of a single fresh colony was inoculated. Thermal cycler program for PCR included: 4 minutes at 95°C for the first denaturize, 35 cycles of denaturizing at 95°C for 30 Sec, annealing at 58°C for 30 Sec, and extension at 72°C for 40 Sec in addition to a final extension at 72°C for 5 minutes. Five microliters of each PCR products profiled into a 1.5% agarose gel electrophoresis and the PCR bands were visualized by UV trans-illuminator (Gel doc System).

Results

MICs of biocides:

The ranges of MIC for chlorhexidine were from 32 µg/ml to 128 µg/ml and for benzalkonium chloride was from 2 µg/ml to 32 µg/ml (Table 1). The minimum inhibitory concentration of benzalkonium chloride is lower than chlorhexidine and can inhibit bacterial growth at lower concentrations.

Detection *cep*A Gene:

From 65 *K. pneumoniae* isolates, 18 (27.6%) had *cepA* gene. The range of MIC for benzalkonium chloride in 8 isolates (44.4%) was from 16 to 32 µg/ml and the range of MIC in 10 isolates (55.5%) was from 4 to 8µg/ml. The MIC for chlorhexidine in 15 isolates (83%) was seen between 64 to 128 µg/ml and the MIC in 3 isolates (16.6%) was 32 µg /ml. (Table 2).

There was a significant relationship between the presence of *cepA* gene and the minimum inhibitory concentration of chlorhexidine (P=0.029), but there was no significant relationship between the presence of *cepA* genes and the minimum inhibitory concentration of benzalkonium chloride (P=0.272).

Table 1: Minimum inhibitory concentration of biocides in 65 *K. pneumoniae* isolates

Biocides	MIC dilution of each biocide										MIC Range (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)
Serial Dilutions	1	2	4	8	16	32	64	128	256	512			
Benzalkonium chloride	-	1	12	16	15	21	-	-	-	-	2-32	24.6	49.2
Chlorhexidine	-	-	-	-	-	4	26	35	-	-	32-128	98.4	196.9

Table 2: Presence of *cepA* gene with MIC of biocides

Biocides	MIC (µg/ml)	cepA
Benzalkoniumchlorid	2	-
	4	3
	8	7
	16	3
	32	5
Chlorhexidine	32	3
	64	7
	128	8

From 65 isolates of *K. pneumoniae*, 18 isolates (27.6%) carried cepA gene and 9 isolates (50%) of them were MDR.There was no significant relationship between the presence of *cep*A gene and multidrug resistance isolates. Also, there was no significant relationship between *K. pneumoniae* MDR isolates and MIC of chlorhexidine and with benzalkonium chloride biocides.

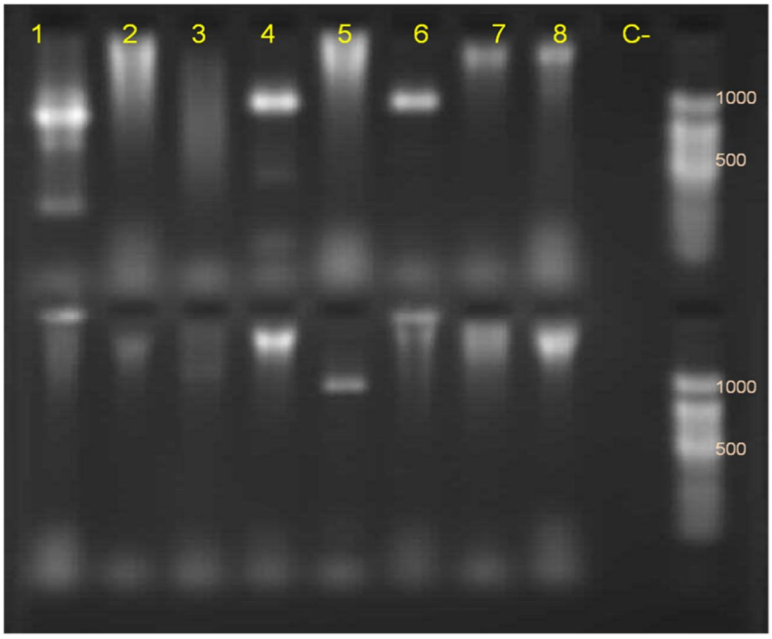

Fig 1: Agarose gel electrophoresis picture of PCR band for *cep*A gene, lanes 1, 4, 6, and 13 shows the amplified gene with 1050 bp band size, lane 9 and 10 show negative control and DNA marker 100 bp, respectively.

Discussion

Basically, evaluation of antibiotic and biocide susceptibility of *K. pneumonia* and other hospital pathogenic bacteria is necessary to be performed periodically at the health care systems. Molecular assays on the genes involved in drug resistance support us in designing new protocols. The present study focused on *cep*A gene, one of the efflux pump genes, by showing the relevance of decrease in susceptibility to biocides benzalkonium chloride and chlorhexidine with presence of genes encoding the efflux pumps in *K. pneumonia.*

Our results of sixty-five clinical isolates of *K. pneumonia* showed 93.8% susceptibility with minimum inhibitory concentration 64-128 µg/ml of chlorhexidine. Among these, 37% of the isolates were multi-drug resistant based on the data information about antibiotic susceptibility testing performed previously on bacterial isolates collection (Reference???). Our findings of molecular study showed that the *cep*A gene was detected in 27.6% of all studied *K. pneumonia* strains. Among those, 44.4% inhibited by benzalkonium chloride (MIC <32 µg/ml)in comparison with that of 83% inhibited by chlorhexidine (MIC>64 µg/ml).

The study of Azadpoor *et. al.* resulted low susceptibility to the same biocides in less than 63% of *K. pneumonia* isolates carrying *cepA* gene (8). In another study, Abuzaed *et. al.* detected the *cepA* gene in all of the studied *K. pneumonia* clinical isolates with low susceptibility to both biocides, chlorhexidine and benzalkonium chloride. In contrast to the findings of Azadpoor and Abuzaed (5, 8), our study data showed no correlation between the resistance or low susceptibility to biocide benzalkonium chloride and detection of *cep*A gene encoding efflux pump in *K. pneumonia*, although, a clear relevance was seen with that of chlorhexidine. As a conclusion, detection of *cep*A gene or other genes involving drug resistance could be an acceptable finding if the study extended by using other tests with more reliability and reproducibility like gene expressions and gene cloning.

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Conflict of interest

The authors have no conflict of interest in this study.

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