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Effect of Zinc Oxide Nanoparticle on the Expression of *mrkA* and *fimA* in Drug-Resistant *Klebsiella pneumoniae*

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ABSTRACT

Background: Nanoparticles are a new generation of antimicrobials. Zinc oxide nanoparticles have attracted a great deal of interest in their medical applications. The aim of the present study was to investigate the effect of zinc nanoparticles on the expression of *mrkA* and *fimA* genes in drug-resistant *K. pneumoniae.*

Methods: A total of 30 clinical isolates of *K. pneumoniae* were collected from Sina hospital and all the isolates were identified by biochemical tests. Antimicrobial resistance pattern was determined by disk diffusion method. PCR method was used to investigate the presence of *mrkA* and *fimA* genes. Biofilm phenotypic test was performed and after conducting MIC test by micro dilution method, real-time PCR was used to study the effect of zinc oxide nanoparticle on the expression of *fimA* and *mrkA* genes.

Results: The highest resistance rate was against cefotaxime and ceftazidime antibiotics (67%). Twenty seven isolates harbored *fimA* gene while 24 isolates harbored *mrkA* gene. Five isolates were identified as strong biofilm producers. MIC values for zinc oxide was 2500 µg/ml in all five isolates. Results of real-time PCR showed that the expression levels of *mrkA* and *fimA* genes in isolates treated with zinc oxide decreased 8.5 and 9 fold, respectively, compared with the control.

Conclusion: This study suggests that zinc oxide can be a suitable candidate for the inhibition of the two studied virulence genes in *K. pneumoniae*.

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Introduction

The emergence of drug resistance is a major problem in the treatment of patients with infectious diseases worldwide. Among hospital-acquired infections and long-term health-related infections, carbapenem-resistant Enterobacteriaceae (in particular, *Klebsiella pneumoniae*), are considered as important causes of mortality. Significantly, there is a very high mortality rate (30-70%) among patients with bacteremia or respiratory infections caused by carbapenamase-producing *K. pneumoniae*. Recent reports indicate the high prevalence of multi-drug resistant *K. pneumoniae* (1). High rates of infections caused by this bacterium is also due to the high potential of this bacterium to spread in the intensive care units as well as the acquisition of antibiotic resistance. Mortality rate of infections caused by *K. pneumoniae* is due to the failure in the selection of appropriate antibiotics (2). Adhesin proteins such as pili are one of the virulence factors in this bacterium with a wide range of molecular motifs with host-specific interactions. *K. pneumoniae* can produce two types of adhesins including type 1 and 3 fimbriae (3). Type I fimbriae is one of the most common adhesion organelles in *K. pneumoniae*. This fimbriae consists of cylinder strands made up of FimA pilin protein subunits. Type III pili is one of the most important adhesions on the surface of *K. pneumoniae* consisting of MrkA protein subunits. This fimbriae is produced by most Klebsiella species and is mostly involved in attaching extra cellular matrix proteins. MrkA protein is the main component of type III fimbriae, however, binding collagen molecules is due to the presence of MrkD adhesion protein (4). Three types of *mrk* genes have been reported in *K. pneumoniae* with the ability of binding extracellular matrix components. *K. pneumoniae* isolates lacking MrkA and MrkD lack binding abilities due to the absense of fimbriae and adhesion proteins (5).

In recent years, using organic antimicrobial agents have become a great concern for controlling pathogenic microorganisms (6). Recent studies suggest the antibacterial features of sulfide nanoparticles and metal oxides. Some nanoparticles made of metal oxides are stabilized in the processing conditions, and in addition to being toxic for bacteria, they have very little toxic effects on human and animal cells (7). Currently, zinc nanoparticle is approved by food and drug administration and is used as a drug additive. The emergence of nanotechnology and the development of materials with new properties at nanoscale has led to the use of zinc oxide nanoparticles as serious antimicrobial agents (8).

Owing to the importance of the antimicrobial properties of nanoparticles on different bacteria (9,10), this study aimed to investigate the effect of zinc nanoparticle on the expression of *mrkA* and *fimA* genes in drug-resistant *K. pneumoniae* isolates to shorten the period of treatment and improve the outcomes.

Materials and Methods

Collection and confirmation of the isolates

In this cross-sectional study, a total 30 *K. pneumoniae* isolates were obtained from different clinical samples of patients referring to Sina Hospital, Tehran, Iran from January 2018 to June 2018. Clinical samples included urine, stool, blood, and sputum. Isolates were identified using standard microbiological tests including culture on MacConkey agar and Gram staining. Biochemical tests for the confirmation of the isolates included endol production, simmon citrate, urease, MR/VP, TSI, and SIM. Finally, isolates were stored in

Brain Heart Infusion (BHI) broth containing 15% glycerol at -20 °C.

Antibiogram assay

Antibiotic sensitivity testing was performed by the standard Kerby Bauer disc diffusion method (11). The antibiotics (Hi-media, Mumbai, India) and their antimicrobial content/disc (µg) included ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), cefoxitin (30 µg), cetotaxime (30 µg), Amikacin (10 µg), and Ciprofloxacin (10 µg). Bacteria showing resistance to three or more antibiotics were labeled as multidrug resistant. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 were used as negative and positive controls, respectively.

Biofilm formation assay

The quantitative microtiter plate (MTP) methods was applied to evaluate the ability of biofilm formation (12). In MTP assay, the specimens were cultured in a solid medium and incubated for 23 hours at 37 °C. Single bacterial colonies were then inoculated into 13 ml tryptic soy broth (TSB) medium (Merck, Darmstadt, Germany) containing 1% glucose (Merck, Darmstadt, Germany) and incubated for 20 hours at 37 °C. In the next step, the turbidity was brought to half McFarland and 200 µl of the suspension was poured into the 96-well plates and incubated at 38 °C for 20 hours. After 24 h of growth, the plates were washed vigorously with 1X phosphate buffer saline (PBS) 4 times, dried for 60 min at 65 °C, and stained with 0.5% (w/v) crystal violet solution. After 15 min staining, the plates were washed with 1X PBS. Then, 100 µl of the mixture containing 83% ethanol and 13% isopropyl was added to the wells and Optical density (OD) at 570 nm of the samples was measured using an ELISA Microplate Readers (Thermo Fisher Scientific, Lenexa, KS, USA). Samples with OD of less than 0.1 were considered as none-adherent, isolates with an OD of 0.1-0.2 were weak biofilm formers, samples with an OD of 0.2-0.3 were considered as moderate biofilm formers, and isolates with an OD of >0.3 were evaluated as strong biofilm producers. Each assay was repeated six times, and the mean biofilm absorbance values were calculated. The results were analyzed using a one-way ANOVA with a Tukey's test.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of zinc oxide nanoparticle against *K. pneumoniae* ATCC 13883 and strong biofilm producers was determined using microdilution method. First, turbidity of microbial broth was prepared with 0.5 McFarland standard. Primary stock was diluted 1:20 to prepare the final bacterial suspension. In order to determine the MIC of zinc oxide nanoparticle, it was mixed with propylene glycol and sonicated for 30 min at 20000 Hz. Then, different concentrations (5000, 2500, 1250, 625, 312, and 156 µg/ml) of this nanoparticle were prepared. 10 µl of bacterial suspension was then added to the 96-well plate and the plate was incubated at 37 ºC for 24 h.

DNA extraction and polymerase chain reaction (PCR) for amplification of mrkA and fimA genes

Bacterial DNA was extracted using a Roche DNA isolation kit (Roche, Basel, Switzerland) based on manufacturer's instruction. Quality and quantity of extracted DNA were measured using Nanodrop and the integrity of DNA samples was assessed through 1% agarose gel electrophoresis. Genomic DNA was used as templates for the amplification of *mrkA* and *fimA* genes with

primers described in Table1. The PCR reaction mixture with the final volume of 20 µl was prepared and DNA amplification was performed in a thermal cycler with denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing temperature (T_m for each primer is shown in Table1) for 45 s, and an extension at 72 °C for 60 s with a final extension at 72 °C for 5 minutes. *K. pneumoniae* ATCC 13883 was used as a control. The PCR products were analyzed on agarose gels.

RNA extraction and Quantitative real-time polymerase chain reaction

Total RNA was isolated using the QIAGEN RNeasy Mini kit (Hilden, Germany). Extracted RNA was analyzed using a Nanodrop ND1000 (Thermo Fisher Scientific, USA) and running on a denaturing 1.5% TAE-agarose gels (80 V for 1 h) to assess its concentration, quality, and integrity. The RNA was DNase treated with Promega RNAse-free DNase (Wisconsin, USA) (at 37°C for 1 h). RNA was precipitated with 1 volume isopropanol and 0.1 volume of 3 M NaOAc (pH 4.6). The suspension was incubated on ice for 20 min and centrifuged at high speed for 30 min at 4°C. The RNA pellet was dried and re-suspended with RNase-free MilliQ H_2O. According to the manufacturer's instructions 500 ng-1µg RNA was converted into cDNA using AccuPower CycleScript RT PreMix (Bioneer, Korea). Quantitative Real-Time PCR was performed on *mrkA* and *fimA* genes in a Rotor-Gene thermal cycler (Corbett 6000; Australia) using SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer; Korea). A total volume of 20 µl reaction containing 2 µl of cDNA, 12.5 µl SYBR Green master mix, 4.5 µl nuclease-free water and 1 µl of each primer (5 pmol) was run according to following program: an initial activation step at 94 °C for 2 minutes, 40 cycles of denaturation at 94 °C for 15 s, annealing (according to Table 1) for 20 s and extension at 72 °C for 20 s. *16s* rRNA gene was used as an internal control to normalize target gene expression measurements. . Real-Time PCR results were analyzed using the 2 (-Delta Delta C(T)) method .

Statistical analysis

Shapiro-wilk and One sample T-test were employed to analyze the data to find out the significant associations amongst the various phenotypic and molecular characteristics of the isolates using SPAA version 19.0 (IBM, Chicago, USA). P-value<0.05 was considered as significant.

Results

Antibacterial susceptibility testing

Results of the antibacterial susceptibility testing by kirby-bauer method on 30 *K. pneumoniae* isolates are illustrated in Table2. The highest antibacterial resistance was for cefotaxime and ceftazidime (67%). Twenty two *K. pneumoniae* isolates were identified as multi-drug resistant isolates.

Expression levels of mrkA and fimA genes

In real-time PCR assay, the studied genes showed different expression levels before and after treatment of strong biofilm-producing *K. pneumoniae* isolates with zinc oxide nanoparticles. After the treatment of isolates with zinc oxide nanoparticles, real-time results showed 8.5 and 9 fold change decrease in the expression of *mrkA* and *fimA* genes, respectively. Table 4 shows the results of antibiotic susceptibility test,

Table 1. Primer sequences designed for amplification of *mrkA* and *fimA* genes.

Genes	Primers (5'→3")	TM	PCR Product Size	Reference
mrkA	F= TGAACTGGACTGGCGGTAAC R= CTTTAGGCTGGGTGGCATCA	60	168 bp	This study
fimA	F= GCTCTGGCCGATACCGTTAC R= AACCTGGCCTAACTGAACGG	60	120 bp	This study
*16s*rRNA	F= GTAGCTGGTCTGAGAGGATGATCAG R= CAAAAGGGCTTTACAACCCGAAGGC	61	156 bp	This study

Table 2. Antibacterial susceptibility testing results.

Antibiotic disk	No. (%)			
	S	I	R	Total
Cefepime	10 (33%)	2 (7%)	18 (60%)	30`
Cefotaxime	8 (27%)	2 (6%)	20 (67%)	30
Cefoxitin	14 (47%)	1 (1%)	15 (51%)	30
Ceftazidime	10 (33%)	0	20 (67%)	30
Meropenem	17 (57%)	2 (4%)	11 (39%)	30
Imipenem	19 (61%)	1 (1%)	10 (37%)	30
Ciprofloxacin	13 (41%)	3 (10%)	14 (49%)	30
Amikacin	13 (44%)	2 (4%)	15 (51%)	30

Table 3. Antibacterial susceptibility testing results.

		MTP results		
Source	*K. pneumoniae* isolate	Weak No. (%)	Moderate No. (%)	Strong No. (%)
Urine	15	3 (20%)	9 (60%)	3 (20%)
Stool	6	3 (50%)	3(50%)	0
Blood	1	0	0	1 (100%)
Sputum	8	2 (25%)	5 (62.5%)	1 (12.5%)
Total	30	8 (27%)	17 (56.6%)	5 (16.66%)

Table 4. The results of antibiotic susceptibility test, MTP results, and expression levels (2^ΔΔCt) of the five studied strong biofilm producing isolates.

Strain No.	Antibiotic susceptibility test*								MTP result (OD value)	Gene expression level (2^ΔΔCt)	
	fep	ctx	fox	cza	cip	amk	ipm	mem		mrkA	fimA
1	R	R	R	R	R	I	R	I	0.251 (Strong)	0.20166044	0.188155843
2	R	R	I	R	R	R	I	R	0.276 (Strong)	0.094732285	0.093428078
3	I	R	R	R	I	R	R	R	0.261 (Strong)	0.10153155	0.087171479
4	R	I	R	R	R	I	R	S	0.265 (Strong)	0.113439894	0.111105335
5	I	R	S	R	R	R	R	R	0.243 (Strong)	0.07588718	0.075362989

*Fep=cefepime; ctx=cefotaxime; fox=cefoxitin; cza= ceftazidime; cip= ciprofloxacin; amk=amikacin; ipm-imipenem; mem= meropenem

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MTP results, and expression levels (2^ΔΔCt) of the five studied strong biofilm producing isolates.

Biofilm formation

In the MTP test, 30 isolates of *K. pneumoniae* were evaluated of which eight of the isolates were identified poor biofilm producers, 17 were moderate biofilms producers, and five were strong biofilm producers. More details of MTP assay findings are described in Table3. *K. pneumoniae* isolates from urine samples were mostly strong biofilm producers.

Determination of the MIC of zinc oxide nanoparticle

Minimum inhibitory concentration of zinc nanoparticle was determined on five *K. pneumoniae* isolates using microdilution broth method. All the studied isolates were strong biofilm producers and they all showed the same MIC of 2500 µg/ml.

PCR amplification

Among 30 *K. pneumoniae* isolates, 27 isolates harbored *fimA* gene and 24 isolates harbored *mrkA* gene. All biofilm-producing isolates harbored these virulence genes.

Discussion

Klebsiella pneumoniae is one of the most common nosocomial infection. It is necessary to find the appropriate therapeutics due to the increase in the prevalence of multi-drug resistant isolates (13). Using novel materials with antibacterial properties have recently somehow solved the problem of antibacterial resistance. One of these materials is metal nanoparticles whose use has increased significantly in medical sciences. Currently, zinc oxide is approved by Food and Drug Administration as a food additive (14). The emergence of nanotechnology and development of materials with new properties at nanoscales has

led to the use of zinc oxide nanoparticles as antimicrobial agents (15).

In this study, antibiogram test was carried out on 30 clinical isolates of *K. pneumoniae*. The highest resistance rate was against cefotaxime (67%) and ceftazidime (67%), whereas the lowest resistance rate was against meropenem (39%) and imipenem (37%), respectively.

According to the antimicrobial pattern of isolates, of 30 studied isolates, 22 (74%) were determined as multi-drug resistant. Similarly, Opondi et al. showed that among clinical *K. pneumoniae* isolates from 2002-2013, more than 80% were multi-drug resistant (16). This high prevalence of multi-drug resistance necessitates finding novel therapeutic approaches and continuous monitoring of antibacterial resistance patterns which help us predict an epidemic nosocomial infection.

In this study, biofilm-producing *K. pneumoniae* showed the highest resistant rate against antibiotics. Similar studies showed higher resistance rates in biofilm-producing *K. pneumoniae* compared to non-biofilm producers. In 2013, Singla et al, showed that the resistance rate against ciprofloxacin and amikacin was higher in biofilm-producing *K. pneumoniae* compared to non-biofilm producers (17). Bellifa et al. also showed that biofilm-producing *K. pneumoniae* isolates were 10-12 times more resistant against gentamycin, cefotaxime, and ciprofloxacin compared to the planktonic isolates (18). However, in 2004, Cernohorska et al. showed that antibiotic resistance in biofilm-producing *K. pneumoniae* was lower against cefepime, ceftazidime, meropenem, ciprofloxacin, and amikacin (19). This discrepancy could be explained by difference in sample population.

In the current study, among 30 *K. pneumoniae* isolates, five (16.6%) were strong biofilm producers, while 17 (56.6%) and eight (26.6%) isolates were intermediate and weak biofilm producers, respectively. Similar studies have been conducted to determine the ability of biofilm formation among *K. pneumoniae* isolates. In 2012, Niveditha et al. showed that 63% of *K. pneumoniae* isolated from urine samples of patients suffering from urinary tract infection had the ability of biofilm formation (20). Results of the study of Yang et al. also showed 62.5% of the *K. pneumoniae* isolated from urine, sputum, wound swabs, and blood generated biofilms (21), a prevalence higher than the results obtained in our study that may be due to difference in geographical locations and difference in sample size.

We also investigated the presence of *fimA* and *mrkA* virulence genes in five strong biofilm producing *K. pneumoniae* isolates. Results showed that all five strong biofilm-producing isolates harbored *fimA* and *mrkA* genes. The prevalence of these virulence genes was compatible with the study of Cordova et al. who showed that among 50 biofilm-producing *K. pneumoniae* isolates, 84% and 98% of isolates carried *mrkA* and *fimH* genes, respectively (22). These results show the importance of these virulence genes in biofilm formation among *K. pneumoniae* isolates. However, another study by Alcantar-Curiel et al. showed a lower prevalence of *mrkA* gene among biofilm-producing *K. pneumoniae* isolates. They studied 69 *K. pneumoniae* isolates among which 55 had the ability of biofilm formation. Among these 55 biofilm-producing isolates, 57% carried *mrkA* gene (23). This difference in prevalence of *mrkA* gene among biofilm-producers may be because of the difference in the isolation site and geographical regions.

Considering the high resistance rate in strong biofilm producers and high prevalence of virulence genes in these isolates, we evaluated the effect of zinc oxide nanoparticles on *K. pneumoniae* isolates with high ability of biofilm formation. To do so, we evaluated the MIC levels of zinc oxide nanoparticles on five strong biofilm producers which was 2500 µg/mL for all five isolates. Moreover, the effect of these nanoparticles was evaluated on expression levels of *mrkA* and *fimA* genes. Expression levels of *fimA* and *mrkA* genes showed 8.5 and 9 fold decrease after exposure to 2500 µg/ml zinc oxide nanoparticles, respectively, compared to the control group (p-value<0.05). Similar studies also indicate the potential effect of zinc oxide nanoparticles on inhibiting the expression of virulence genes in different microorganisms. In 2014, Lee et al. evaluated the effect of zinc oxide on the expression levels of genes involved in biofilm formation in *P. aeruginosa* including *czcS, czcR, czcB, pvdA, phzE, phzC, rhlR, ptrA, opdT,* and *oprD*. Their results showed the significant effect of zinc oxide nanoparticle on decreasing the expression levels of *opdT, oprD, sczS, czcR,* and *czcB* (24).

In 2012, Azam et al. evaluated the antibacterial activity of zinc, copper, and iron oxide nanoparticles on Gram positive bacteria including *S. aureus* and *B. subtilis* an on Gram negative bacteria including *E. coli* and *P. aeruginosa*. Among these compounds, zinc oxide nanoparticle had the highest effect against both Gram-negative and Gram-positive bacteria (25). These results show that due to the clinical importance of infections caused by *K. pneumoniae*, using zinc oxide nanoparticles can be useful in treating these infections.

Conclusion

A high percentage of *K. pneumoniae* isolates are resistant to most of the common antibiotics and multi drug resistant isolates are increasingly emerging. Therefore, it is necessary to monitor the changes in sensitivity of *K. pneumoniae* to different antibiotics to choose the most suitable antibiotic for treatment and also to properly predict the prevalence of infections caused by this microorganism. This helps us adopt novel strategies to decrease or prevent the emergence of antibacterial resistance. The ability of *K. pneumoniae* isolates in biofilm formation leads to high resistance rate to different antibiotics. Owing to the inhibitory effect of zinc oxide nanoparticles on the expression of virulence genes in *K. pneumoniae* isolates, this compound may be useful in controlling the infections caused by MDR *K. pneumoniae* isolates.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare that there is no conflict of interest.

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