Research article

Effect of biogenic bismuth nanoparticle on the expression of New Delhi metallo-β-lactamase (NDM) gene in Multidrug-Resistant *Klebsiella pneumoniae*

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A B S T R A C T

Aim and background: The emergence of Multidrug-Resistant *Klebsiella pneumoniae* is a global concern due to high mortality and treatment challenges. One of the most important genes for resistance is NDM, which makes the organism resistant to most antibiotics. Today, the use of nanoparticles as therapeutic options has stimulated researchers around the world to investigate its effects. The aim of this study was to investigate the effects of biosynthesized bismuth nanoparticles (Bi NPs) on the expression of NDM genes in multidrug-resistant *K. pneumoniae*.
Materials and methods: In this study, 5 multidrug-resistant *K. pneumoniae* clinical isolates from patients referred to Afzalipour Hospital in Kerman, Iran, were used. Antibiotic sensitivity test was performed by disc diffusion method. The presence of the NDM gene was checked in isolates using a PCR reaction. The isolates containing the NDM gene were exposed to the biosynthesized and characterized bismuth nanoparticles, and the effects on the expression of the NDM gene was investigated using real-time PCR.
Results: The results showed that 3 isolates of *K. pneumoniae* had NDM genes. In TEM and SEM analysis showed that the nanoparticles had a spherical structure and an average size of 22.36 nm. The investigation of biogenic Bi NPs on the expression of the NDM gene demonstrated that the samples treated with bismuth nanoparticles decreased the expression of the NDM gene by 1.6 times compared to the control group ($p < 0.011$).
Conclusion: Our findings showed that biosynthesized Bi NPs have a high potential to deal with antibiotic resistance genes and can be a promising for treatment.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic, Gram-negative pathogen and a member of the Enterobacteriaceae family

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[1]. This bacterium included in the list of pathogens of the ESKAPE group (*Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *K. pneumoniae*, *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Enterobacter* spp.), which are known as bacteria that cause opportunistic infections and have a high level of antibiotic resistance, due to the acquisition of antibiotic resistance genes [2,3]. *K. pneumoniae* is a capsular bacterium and responsible for 10 % of hospital-acquired infections and causes a wide range of infections including urinary tract infections (UTIs), pneumonia, burn infections, meningitis, and septicemia [4]. New strains of *K. pneumoniae* have become increasingly resistant to antibiotics, making antibiotic treatment difficult worldwide [5–7].

One of the most important antibiotic resistance genes acquired in this bacterium is extended-spectrum beta-lactamases (ESBLs), which cause resistance to cephalosporins and monobactams [8]. Carbapenemases are a type of beta-lactamase enzymes that have limited the use of carbapenem antibiotics such as imipenem and meropenem, which are used as the last line of treatment in severe infections caused by this bacterium [9]. The first carbapenemases were identified in *K. pneumoniae* bacteria in 1996 and were named KPC (*K. pneumoniae* carbapenemase) [10]. According to Ambler's classification, carbapenemases are divided into three categories: carbapenemases belonging to class A β-lactamases that are inhibited by clavulanic or boronic acids, such as KPC, class B or metallo-β-lactamases (MBLs) that are inhibited by EDTA and dipicolinic acid, such as imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM), and class D or oxacilinases that are not inhibited by classical inhibitors such as OXA-48 [11–15].

In the past decades, the blaNDM gene has been spreading at an unprecedented speed, which is why it has attracted worldwide attention. This gene was first reported in 2008 in a strain of *K. pneumoniae* from a patient hospitalized in New Delhi, India. 24 types of NDM genes from blaNDM-1 to blaNDM-24 have been identified in different bacterial species worldwide until 2019. Genes encoding blaNDM-1 to blaNDM-24 are mostly transferred through conjugative plasmids belonging to several incompatibility groups (Inc) [16]. In addition, other mobile genetic elements involved in the transfer of genes include IS elements, integrons, and transposons. The ability to hydrolyze beta-lactam antibiotics by NDM-producing bacterial strains limits the treatment options for infections caused by these bacteria and increases the mortality rate [17].

Nowadays, the use of nanoparticles has started a new way for treatment, medical diagnosis and drug delivery [18–20]. Nanoparticles are synthesized by various methods, including physical, chemical and biological [21]. Physical and chemical methods for the synthesis of nanoparticles have disadvantages, including the use of expensive reagents, hazardous reaction conditions, and time-consuming processes [22]. Since chemical and physical methods require capital and energy expenditure, as well as the use of toxic chemicals and organic solvents in their process, biogenic methods are considered safe and effective alternative methods with minimal waste production [23]. In recent years, several easy, reproducible, scalable, cost-effective, and safe green synthesis methods for nanoparticles have been developed. In biological methods, organisms such as bacteria, yeasts, and fungi are widely used in green synthesis methods to produce nanoparticles [24].

One of the types of these nanoparticles is metal nanoparticles that have high abilities such as disrupting translation and transcription in bacteria as well as affecting gene expression [25]. Biogenic metal nanoparticles are nanostructures that have been synthesized through biological methods, including bacteria, and due to their high surface-to-volume ratio and small size, they have attracted significant attention in terms of biological applications [23]. The aim of this study is to investigate the effects of biogenic bismuth nanoparticles on the expression of the NDM gene in clinical strains of multidrug-resistant *K. pneumoniae* by Real Time PCR method for therapeutic applications in the future.

2. Materials and methods

2.1. Bacterial samples

In this study, 5 samples of clinical *K. pneumoniae* resistant to antibiotics were used from archives of the Medical Microbiology Department of Kerman University of Medical Sciences. This sample was isolated from patients referred to Afzalipur Hospital, Kerman, Iran. To investigate the pattern of antibiotic sensitivity and resistance to carbapenems, the Mueller Hinton agar culture medium and disc diffusion method were used. In this study, imipenem (10 μg), amikacin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), ceftriaxone (30 μg), and ceftazidime (30 μg) antibiotic discs prepared by Padtan Teb company were used and according to the CLSI standard method.

2.2. Detection of NDM gene with PCR

First, clinical isolates of *K. pneumoniae* were cultured in a nutrient agar medium and incubated at 37 °C for 24 h. Then, several colonies of bacteria were removed and dissolved in 100 ml of sterile distilled water inside the microtube. Then the microtube was placed in a bain-marie with a temperature of 99 °C degrees for 15 min. Finally, the microtube was centrifuged at 10,000 rpm for 5 min. Then, the supernatant containing DNA was transferred to a sterile microtube. The PCR reaction was performed using the NDM-specific

Table 1
NDM gene specific primer.

Gene	Product size (bp)	Sequence (5–3)	Ref.
NDM	201	F 5′- TGCCCAATATTATGCACCCGG- 3′ R 5′- CGAAACCCGGCATGTGCGAGA- 3′	[26]

primers in Table 1 and the temperature conditions and master mix composition in Table 2.

2.3. Biosynthesis of bismuth nanoparticles

Bacillus subtilis strain SFTS isolated from copper mine soil was used to synthesize bismuth nanoparticles. Briefly, the desired bacteria were added to nutrient broth culture media containing 0.1 mg/ml bismuth in OD equal to 0.08 to 0.13 and incubated at 30 °C and 150 rpm for 72 h. After 72 h, the bacterial biomass was centrifuged with 10,000 rpm for 10 min and broken several times using liquid nitrogen. After several steps of washing, the nanoparticles were finally purified using a combination of water-*n*-octanol [27]. Also, in order to determine the characteristics of nanoparticles the analysis of FTIR, TEM, and SEM was performed.

2.4. Real-time PCR

The investigation was carried out using the real-time PCR technique by the following steps:

1) Cultivation: First, the bacterial isolate containing carbapenemase genes was cultured in the Muller Hinton broth medium in the presence and absence of the inhibitory concentration of biosynthesized bismuth nanoparticles. 2) RNA extraction: Total RNA was extracted using the RNA extraction kit of Karmania Pars Gene Company and a spectrophotometer was used to check the quality of RNA. 3) Synthesis of cDNA: For cDNA synthesis, the cDNA synthesis kit of Karmania Pars Gene Company was used. 4) Real-time PCR: The frequency of the NDM gene for 3 isolates of *K. pneumoniae* was measured by Real-Time PCR and using a cyber green method in Step one plus device (ABI, USA) using specific primers. To perform the Real-Time PCR technique, a Cyber Green master mix prepared by Ampilicon company (Denmark) was used. The amplified product at the end of each cycle was identified through the fluorescence signal curve, which indicates the reaction and positive amplification, and Ct (Threshold Cycle) was reported using the threshold limit in the exponential phase of the graph [28].

The analysis of gene expression was done using real time PCR and with the temperature conditions and master mix composition in Table 2.

2.5. Statistical analysis

To ensure the accuracy of the primers, a standard curve was drawn for the NDM gene and 16S rRNA gene (Fig. 1A and B). For this purpose, different dilutions (1–10) of cDNA were prepared, then a real-time PCR test was performed for them and the obtained Cts and available concentration were used to draw the standard curve.

A dilution series of 1×10^2 to 1×10^7 CFU/µl was used to prepare the internal standard (Fig. 1C). For each of them, the qPCR test was performed in a series (consecutive) in one run. The resulting Cts and concentrations in each reaction were used to draw the standard graph. Finally, using the Ct data of unknown samples and the standard chart of the amount of bacteria in each sample was obtained.

In order to analyze and investigate the change in NDM gene expression in bacteria treated with bismuth nanoparticles, the Livak method $(2^{-\Delta\Delta ct})$ was used.

Also, SPSS Statistics 24 software was used to analyze the data. Also, for statistical analysis, one-way analysis of variance (ANOVA) and $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Bacterial samples and detection of NDM gene

Out of a total of 5 *K. pneumoniae* clinical isolates 2 isolates were isolated from blood culture, and 3 isolates were isolated from urine, bronchi, and wound cultures and were resistant to all antibiotics used in the antibiogram test. Also, out of a total of 5 *K. pneumoniae* multi-drug resistant isolates, 3 isolates had the NDM gene (Table 3).

3.2. Characterization of bismuth nanoparticles

FTIR analysis revealed the existence of three peaks in the regions of 3396.93 cm^{-1}, 1653.68 cm^{-1}, and 541.56 cm^{-1}. In this

Table 2
Temperature conditions and master mix composition to investigate NDM gene expression.

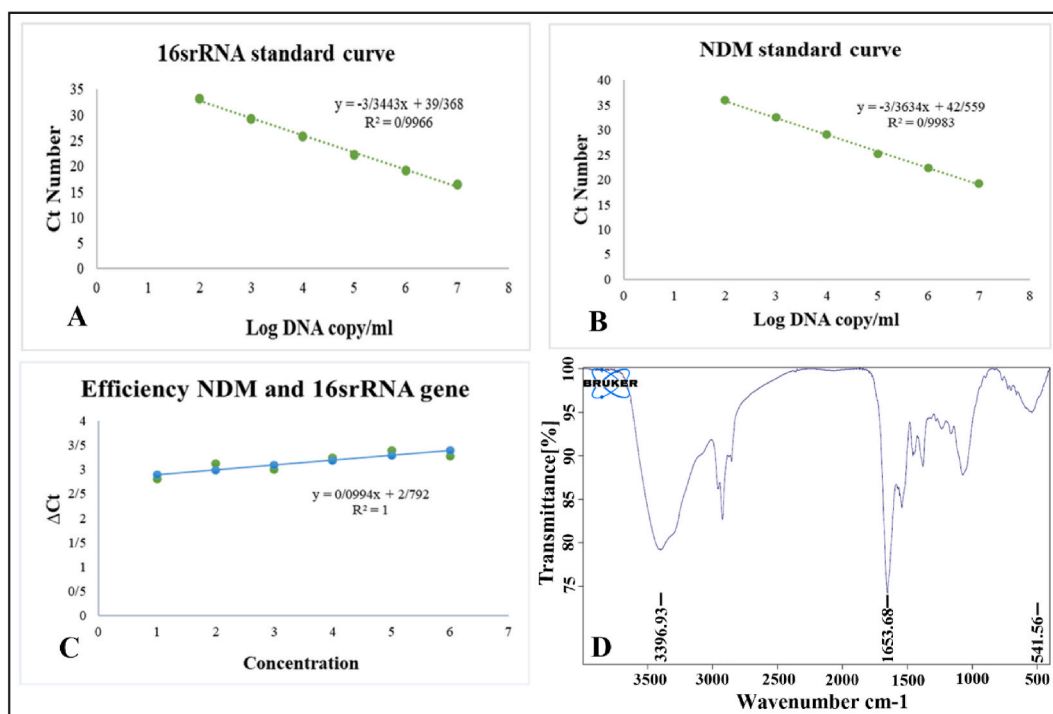| Temperature conditions				master mix composition	
perform the reaction	Temperature (°C)	Time	Number of cycles	Reaction materials	Amount (µL)
Initial denaturation	95	10 min	1	Distilled water	3
Denaturation	95	60 s	30	Master Mix	5
Annealing	59	30 s	30	Forward primer	0.5
Extension	72	30 s	30	Reverse primer	0.5
Final extension	72	10 min	1	Template DNA	1

Fig. 1. 16s rRNA (A) and NDM (B) gene standard curve, Linear regression curve of 16s rRNA and NDM gene (C), FTIR analysis (D).

Table 3
Characteristics of multidrug-resistant *Klebsiella pneumoniae* isolates.

Number	Sample	Imipenem	Amikacin	Nalidixic acid	Ciprofloxacin	Gentamicin	Ceftriaxone	Ceftazidime	NDM
1	Wound	R	R	R	R	R	R	R	+
2	Urine	R	R	R	R	R	R	R	+
3	Bronchus	R	R	R	R	R	R	R	+
4	Blood	R	R	R	R	R	R	R	−
5	Blood	R	R	R	R	R	R	R	−

R: resistant; NDM: New Delhi metallo-β-lactamase.

analysis, the peak at 3396.93 cm^{-1} can be attributed to the O–H stretching vibration, mostly for water molecules adsorbed to the nanoparticle surface. The peak in the region of 1653.68 cm^{-1} can also be described as the C=O stretching vibration, which may be associated with the biologically active groups on the surface of the nanostructures. Furthermore, the absorption peak at 541.56 cm^{-1} is mostly related to the metal-oxygen vibration, which represents Bi–O [29] (Fig. 1D).

The results of TEM imaging showed that the biosynthesized Bi NPs are spherical, and also the results of SEM imaging demonstrated that the average size of nanoparticles was about 22.36 nm (Fig. 2).

3.3. Effects of biosynthesized bismuth nanoparticles on NDM gene expression

Three isolates of *K. pneumoniae* containing the NDM gene were exposed to biosynthesized Bi NPs, and after real-time PCR, the CTs results were recorded (Table 4)., which can be observed in Table 3. Gene expression was reduced in Bi NPs treated strains (green curves) compared to non-treated ones (red and brown curves) (Fig. 3).

The results of the investigation of biogenic Bi NPs on the expression of the NDM gene demonstrated that the samples treated with nanoparticles decreased the expression of the NDM gene by 1.6 times compared to the control group (p < 0.011) (Fig. 4). According to the results of this study, it seems that metal nanoparticles can directly and indirectly affect bacterial DNA by passing through the bacterial membrane and reducing the expression of resistance genes, or through the effect or interaction with the zinc element present in the active site of carbapenemase enzymes can reduce the expression of NDM resistance genes. Several similar studies were reported on the expression of antibiotic-resistance genes in different bacteria by different nanoparticles. In a study, Lotfpour et al. [30] investigated the effect of iron nanoparticles on the expression of the TEM beta-lactamase gene in *Pseudomonas aeruginosa* and reported that the expression of the TEM gene in two clinical isolates of *Pseudomonas aeruginosa* treated with iron nanoparticles decreased by 78 % and 75 % compared to the treated isolates. In another study, Arab et al. [25] examined iron nanoparticles on the expression of the
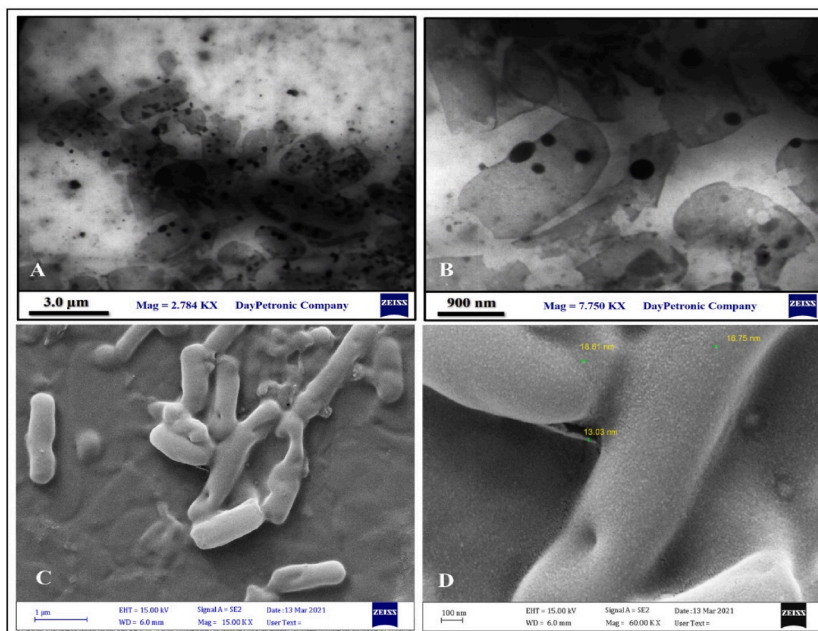
Fig. 2. Bi NPs in TEM (A, B) and SEM (C, D) imaging.

Table 4
Effect of biosynthesized bismuth nanoparticle on NDM gene expression.

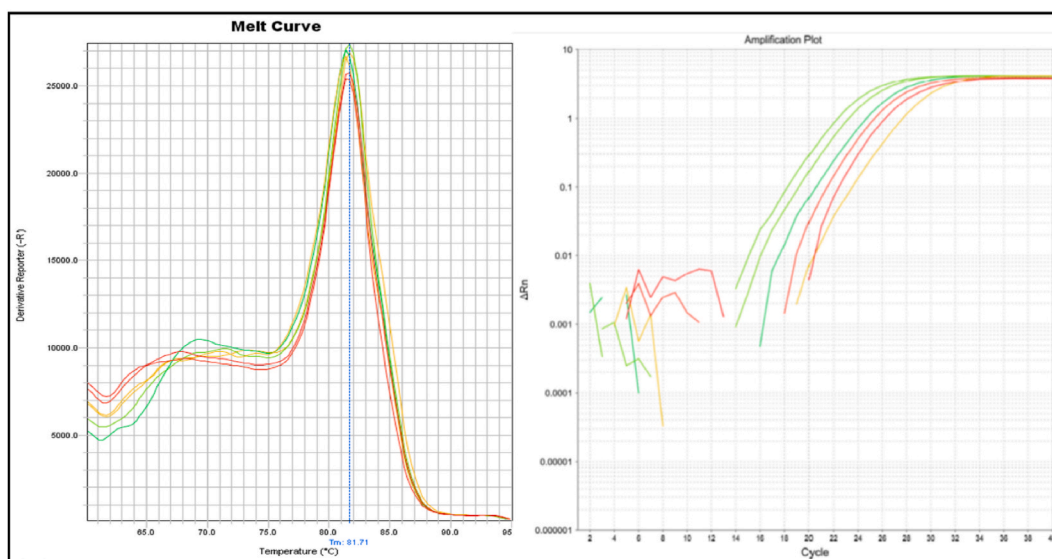| Sample	CT-T-NP + NDM	CT-T-16S	ΔCT- NDM + NP	CT-C-NDM	CT-C-16S	ΔCT- NDM	ΔΔCT	Fold change	Gene expression level
1	25.33	23.2	2.13	24.32	22.79	1.53	0.6	0.66	**−1.51**
2	25.92	25.18	0.74	21.75	21.35	0.4	0.34	0.79	**1.26-**
3	25.77	15.33	10.44	22.52	13.1	9.42	1.03	0.49	**2/02-**
Average gene expression level									**−1.6**

T: test, C: control, NP: nanoparticle.

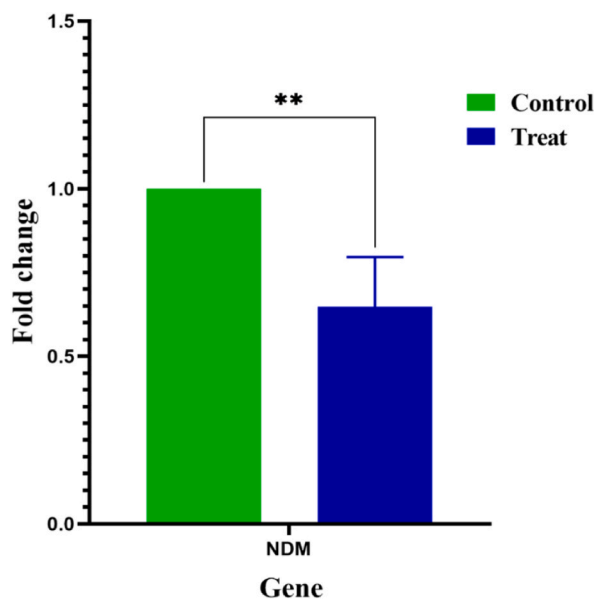**Fig. 3.** Melt curve analysis and observed changes in Ct values.

Fig. 4. Fold change diagram of NDM gene expression treated with bismuth nanoparticles in *Klebsiella pneumoniae* clinical isolates.

tetA gene in clinical isolates of tetracycline-resistant *Staphylococcus aureus* and indicated that iron nanoparticles reduced the expression of the tetA gene in clinical isolates of tetracycline-resistant *Staphylococcus aureus* by 2 times. Furthermore, in a study, Rashid et al. [31] studied silver nanoparticles on MecA gene expression in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and reported that silver nanoparticles reduced MecA gene expression in methicillin-resistant *Staphylococcus aureus* isolates. In a study, albadiri et al. [32] investigated the effect of silver nanoparticles on three isolates of *Escherichia coli* containing the TEM gene. The results of this study demonstrated that silver nanoparticles significantly decreased the expression of the TEM gene compared to the control group ($p < 0.05$). In another study, Alkhikani et al. [33] examined the effect of silver nanoparticles on the expression of the rmtB gene in aminoglycoside-resistant *Pseudomonas aeruginosa* clinical isolates. The results displayed that silver nanoparticles significantly reduce the expression of rmtB gene ($p < 0.01$). The effect of iron oxide nanoparticles on *Pseudomonas aeruginosa* strains containing CTX-M gene isolated from burn patients was investigated. The results showed that iron oxide nanoparticles significantly reduce the expression of the CTX-M gene in isolates treated with these nanoparticles ($p < 0.00$) [34]. The effects of zinc nanoparticles on the expression of Van A, mec A, and cfr resistance genes in vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and linezolid-resistant *Staphylococcus aureus* isolated from burn wound samples were investigated. The results demonstrated that zinc nanoparticles reduced the resistance to methicillin from 81.9 % to 13.30 %, vancomycin from 33.60 to 0 %, and linezolid from 29.30 to 0 % with $P < 0.001$ in the isolates [35]. Piri Gharaghie et al. [36] investigated the effects of silver nanoparticles on the Bla-per1gene (a type of extended-spectrum β-lactamase (ESBL)) in 12 isolates of *Acinetobacter baumannii* isolated from clinical samples. All 12 isolates were resistant to all antibiotics except Cloistin. The results of this study displayed that silver nanoparticles significantly reduced the expression of the Bla-per1 gene in all isolates with $P < 0.05$. Alinaghiyan et al. [37] investigated the effects of biosynthesized silver nanoparticles on the expression of the CTX-*M*-15 (a type of extended-spectrum β-lactamase (ESBL)) gene in *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus* isolates. The results revealed that biosynthesized silver nanoparticles suppressed the expression of the CTX-*M*-15. Alinaghiyan et al. [38] investigated the effects of chitosan nanoparticles on fos A (fosfomycin resistance) gene expression in 14 isolates of *Proteus mirabilis* isolated from urine samples. The results exhibited that chitosan nanoparticles decreased the expression of the fos A gene by 1.78 times compared to the control group with a $P < 0.001$.

4. Conclusion

Multidrug-resistant *K. pneumoniae* (MDR-KP) is significantly increasing worldwide and has limited the use of treatment options, especially carbapenem antibiotics. The results of this study showed that the biosynthesized Bi NPs have a spherical shape and a size of about 22.36 nm, which can reduce the expression of the NDM gene in multi-drug resistant *K. pneumoniae* strains by 1.6 times. It seems that the effect of Bi NPs on the NDM gene in *K. pneumoniae* isolates was reported for the first time in this study. Considering the high potential that these nanoparticles show, we can hope that the use of metal nanoparticles is an option to deal with antibiotic resistance.

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Data availability statement

All the data generated in this research work has been included in the manuscript.

Ethical approval

This study was reviewed and approved by the Ethics Committee of Kerman University of Medical Sciences, Iran with the approval number: IR.KMU.REC.1399.416, dated 5/10/2020.

CRediT authorship contribution statement

Amin Sadeghi Dousari: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Mojtaba Shakibaie:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Hossein Hosseini-Nave:** Writing – review & editing, Writing – original draft. **Hamid Forootanfar:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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