

Zinc Oxide Nanoparticles: A Promising Solution for Controlling the Growth of Gentamicin-Resistant Uropathogenic *Escherichia coli*

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ABSTRACT

Backgrounds: Uropathogenic *Escherichia coli* is one of the most important etiological agents of UTI. The aim of this study was to investigate the antibacterial effects of zinc oxide nanoparticles (ZnONPs) on aminoglycoside-resistant *E. coli* isolates from patients with UTI.

Materials & Methods: After identifying *E. coli* strains in 100 out of 250 urine samples, antibiotic susceptibility was evaluated against six antibiotic classes (with emphasis on aminoglycosides) by disk diffusion method according to CLSI-2020 guidelines. The presence of aac(6')-Ie-aph(2'') gene in isolates was investigated by PCR. Antibacterial properties and minimum inhibitory concentration (MIC) of zinc oxide nanoparticles were evaluated by agar well diffusion and broth microdilution assays, respectively.

Findings: Among 100 *E. coli* isolates, the highest and lowest antibiotic resistance rates were observed against tetracycline (70%) and ofloxacin (10%), respectively. Of 30 gentamicin-resistant *E. coli* isolates, 17 (56.5%) isolates harbored the aac(6')-Ie-aph(2'') gene. In agar well diffusion assay, 22 (74%) gentamicin-resistant isolates were eliminated by zinc oxide nanoparticles at a concentration of 150 mg/L, while ZnONPs at 300 mg/L could eliminate all gentamicin-resistant isolates. Furthermore, ZnONPs could inhibit all bacteria at a concentration of 200 µg/mL (MIC₉₀ ≥ 100).

Conclusions: Spread of the aac(6')-Ie-aph(2'') gene could increase gentamicin resistance among *E. coli* strains causing UTI. Given the favorable antibacterial effects of zinc oxide nanoparticles *in vitro*, the clinical application of these nanoparticles in the treatment of UTIs caused by multidrug-resistant *E. coli* could be investigated in future studies.

Keywords: Aminoglycoside resistance, Uropathogenic *Escherichia coli*, Zinc oxide

CITATION LINKS

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Introduction

Urinary tract infections (UTIs) are one of the most common infectious diseases and one of the most important causes of community-acquired infections. Most UTIs are usually harmless and not serious; however, misdiagnosis or delay in treatment could lead to serious complications, such as urinary tract disorders, hypertension, uremia, preterm delivery, or even miscarriage in pregnant women. Studies in different communities have shown that Gram-negative bacilli are the most common etiological causes of UTIs, among which *Escherichia coli* accounts for more than 80% of UTIs [1,2]. Proper treatment of UTIs is based on the selection of a suitable and highly effective antibiotic. Unfortunately, inappropriate and excessive use of antibiotics has led to the emergence of resistant bacteria. Among antibiotic-resistant *E. coli* strains, the prevalence of aminoglycoside-resistant strains is high [3-5]. Gentamicin is an aminoglycoside antibiotic that inhibits bacterial protein synthesis by binding to 30S ribosomal component, thereby inhibiting the growth of dividing bacteria. Resistance to this antibiotic occurs in three ways: change in drug target, change in drug transport, and enzymatic inactivation of drug by producing aminoglycoside-modifying enzymes. In recent years, the role of several genes in the development of antibiotic resistance has been confirmed, including the *aac(6')-Ie-aph(2'')-Ia* gene which is transmitted through transposons and plasmids [6,7]. Given the increasing prevalence of antibiotic resistance, it seems necessary to find more effective antibiotics and novel antimicrobial compounds. Metal nanoparticles are of great importance in microbiology and nanotechnology due to their interesting chemical and antimicrobial properties. Zinc oxide nanoparticles (ZnONPs) with strong antimicrobial properties have been

widely used in industry and medicine. These nanoparticles have also been widely used as one of the safest nanoparticles to combat Gram-positive and Gram-negative bacteria [8,9].

Objectives: The aim of this study was to determine the pattern of antibiotic resistance of uropathogenic *E. coli* (UPEC) isolates to selective aminoglycosides and to evaluate the antibacterial activity of zinc oxide nanoparticles against these isolates.

Materials and Methods

Study population: In this descriptive cross-sectional study, 250 urine samples were collected from all symptomatic and asymptomatic patients with UTIs in hospitals in Gorgan (northeastern Iran) in 2020. The inclusion criterion was positive urine culture (i.e., the presence of 10^5 colony forming units (CFU)/mL in asymptomatic patients and 10^4 CFU/mL in symptomatic patients) [10].

First, morning urine samples were collected from urine drainage bags. Blood agar, eosin methylene blue, and MacConkey agar were used to culture the urine samples. The cultured samples were incubated at 37 °C for 18-24 hours. Next, *E. coli* strains were identified based on conventional microbiological and biochemical tests such as Gram staining, glucose and lactose fermentation, gas production, indole production, Voges-Proskauer (VP) test on triple sugar iron (TSI) agar, sulfide-indole-motility, and methyl red-VP. Antibiotic susceptibility was evaluated by Kirby-Bauer method (disk diffusion) using the following antibiotic disks: tetracycline (30 µg), gentamicin (10 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), and fosfomycin (200 µg containing 50 µg glucose-6-phosphate). All antibiotic disks except fosfomycin (CONDA, Spain) were purchased from PadtanTeb Co., Iran. The

results were interpreted according to CLSI-2020 guidelines [11].

Investigation of molecular pattern of gentamicin- resistance by polymerase chain reaction (PCR): Genomic DNA was extracted by boiling method. After assessing the purity of the extracted DNA samples by spectrophotometry at 280 nm, they were stored at -20 °C until analysis. PCR reaction was performed using specific primers [12] (forward: 5' CTT GGG AAG ATG AAG 3' and reverse: 5' CCT CGT GTA ATT CAT GTT CTG GC 3'). The reaction was performed in a final volume of 20 µL, containing 1 µL of DNA sample, 0.5 µL of each primer, 0.5 µL of dNTP, 2 µL of PCR buffer, 1 µL of MgCl₂, 0.2 µL of Taq DNA polymerase (5 U/µL), and distilled water (14.8 µL). Cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Finally, PCR products were electrophoresed on 1% agarose gel for the presence of *aac* (6')-Ie-aph (2'') gene.

Investigation of antibacterial properties of zinc oxide nanoparticles: The antibacterial properties of zinc oxide nanoparticles were investigated by agar well diffusion method. For this purpose, ZnONPs powder (Nanopioneer, Iran) with a diameter of 20 nm was inoculated in a tube containing sterile distilled water and 20% dimethyl sulfoxide to obtain a final concentration of 300 mg/L. After shaking for 30 min, the solution was subjected to sonication, and serial dilutions (37.5, 75, 150, and 300 mg/L) were prepared in sterile distilled water. In order to evaluate the antimicrobial effects of different concentrations of ZnONPs, bacterial suspensions were prepared from all gentamicin-resistant isolates (with a density equivalent to half the McFarland standard)

and cultured uniformly on Mueller-Hinton agar. Then wells with a diameter of 7 mm were made on the culture medium, and 100 µL of each concentration of zinc oxide nanoparticles was poured into each well. A well containing distilled water was used as a negative control. After incubating the plate at 37 °C for 24 hours, the diameter of the growth inhibition zone around each well was measured and recorded in millimeters. Bacteria with a growth inhibition zone diameter of ≤ 10 mm were considered as nanoparticle-resistant [9].

Determination of minimum inhibitory concentration (MIC) of ZnONPs by broth microdilution method: In order to prepare an initial stock solution of ZnONPs, 1 g of nanoparticle powder with a concentration of 3 mg/mL was first added to a tube containing 5 mL of sterile distilled water. The tube was placed on a shaking incubator for 4 hours. The solution was centrifuged at 3800 rpm, and the resulting precipitate was mixed with distilled water. Absorbance of the resulting solution was determined at 600-700 nm by absorption spectroscopy. To determine the MIC, 100 µL of the initial stock solution of ZnONPs was inoculated into wells of a microplate containing Mueller-Hinton broth (Merck, Germany). Simultaneously with inoculation of bacterial suspension (3×10⁸ CFU/mL), dilutions of 0.39-200 µg/mL were prepared. Wells containing only bacterial suspension and nanoparticles were considered as positive and negative controls, respectively. After 24 hours of incubation at 37°C, MIC values were determined by measuring optical density at 595 nm. The strain *E. coli* ATCC 25922 was used as a control strain.

Data were analyzed with SPSS software (Version 18) and Microsoft Excel 2007 using one-way ANOVA and Chi-square tests. A *p*-value of less than 0.05 was considered statistically significant.

Table 1) Demographic characteristics of hospitalized patients with UTI

Variables	All Isolates		Gentamicin-Resistant Isolates		P-Value	
	Number	%	Number	%		
Gender	Male	35	35	11	36.67	.67
	Female	65	65	19	63.33	
Age (years)	<15	7	7	1	3.33	.31
	16-30	19	19	8	26.67	
	30-45	23	23	8	26.67	
	>45	51	51	13	43.33	
Hospital ward	Intensive care unit	53	53	8	26.67	.02*
	Operating room	9	9	7	23.33	
	Infectious disease	17	17	7	23.33	
	Internal medicine	21	21	8	26.67	

*Significant difference between the study groups based on the Chi-square test

Findings

Among 250 urine samples, *E. coli* was detected in 100 (40%) samples, most of which were taken from female patients (65%), patients over 45 years (51%), and patients in the intensive care unit (ICU) (53%). The frequency of UPEC and gentamicin-resistant isolates was significantly different in different hospital wards ($p=.02$) (Table 1). Based on the analysis of antibiotic susceptibility pattern, the highest and lowest rates of antibiotic resistance among *E. coli* isolates were observed against tetracycline (70%) and ofloxacin (10%), respectively (Table 2).

PCR-based detection of antibiotic resistance genes: The detection of 500 bp fragments confirmed the presence of the *aac(6')-Ie-aph(2'')-Ia* gene encoding gentamicin resistance. Of 30 gentamicin-resistant *E. coli* isolates identified in the phenotypic test, 17 (56.5%) isolates harbored the gentamicin resistance gene.

Results of determining the antimicrobial effect of ZnONPs by agar well diffusion method: Out of 30 gentamicin-resistant *E. coli* isolates, 22 isolates (74%) were eliminated by ZnONPs at a concentration of 150 mg/L. In addition, nanoparticles at

a concentration of 300 mg/L were able to eliminate all gentamicin-resistant *E. coli* isolates. The bactericidal properties of nanoparticles increased in a dose-dependent manner (Figure 1).

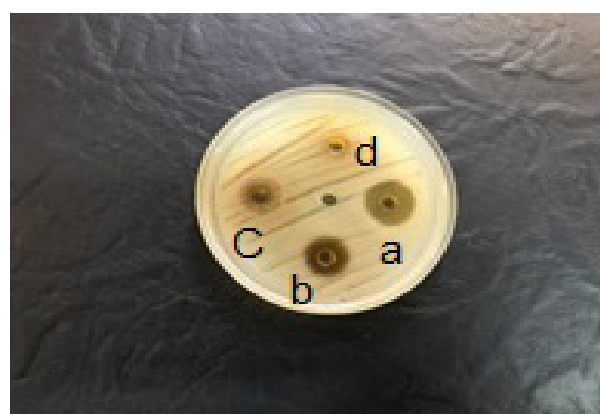


Figure 1) Antibacterial effects of ZnO nanoparticles at different concentrations: a: 300 mg/L, b: 150 mg/L, c: 75 mg/L, d: 37.5 mg/L

Determination of MIC of ZnONPs: The results of antimicrobial effects of ZnONPs on the growth of gentamicin-resistant *E. coli* isolates showed that 90% of the isolates could not grow at 100 $\mu\text{g/mL}$ ($\text{MIC}_{90} \geq 100$), and their bacteriocidal property increased upon the increase of the concentration (Table3).

Table 2) Pattern of antibiotic resistance of UPEC isolates

Drug Class	Antibiotics	Number (%)	Number (%)	Number (%)
		Resistant	Intermediate	Susceptible
Cyclines	Tetracycline	70(70)	12(12)	18 (18)
Cephems	Ceftazidime	18(35)	20(20)	62(62)
Phosphonic acids	Fosfomycin	11(11)	28(28)	61(61)
Phenicols	Chloramphenicol	24(24)	25(25)	51(51)
Aminoglycosides	Gentamicin	30(30)	12(12)	58(58)
Quinolones	Ciprofloxacin	20(20)	15(15)	65(65)

Table 3) MIC of ZnONPs against gentamicin-resistant UPEC isolates

Gentamicin-Resistant <i>E. coli</i> Isolates	MIC ($\mu\text{g/mL}$)									
	200	100	50	25	12.50	6.25	3.12	1.56	0.78	0.39
No. 1	-	-	-	✓	-	-	-	-	-	-
No. 2	-	-	✓	-	-	-	-	-	-	-
No. 3	-	-	-	-	✓	-	-	-	-	-
No. 4	-	-	-	✓	-	-	-	-	-	-
No. 5	✓	-	-	-	-	-	-	-	-	-
No. 6	-	-	✓	-	-	-	-	-	-	-
No. 7	-	-	-	-	✓	-	-	-	-	-
No. 8	-	-	✓	-	-	-	-	-	-	-
No. 9	-	-	-	✓	-	-	-	-	-	-
No. 10	-	-	✓	-	-	-	-	-	-	-
No. 11	-	-	-	✓	-	-	-	-	-	-
No. 12	-	-	✓	-	-	-	-	-	-	-
No. 13	-	-	✓	-	-	-	-	-	-	-
No. 14	-	-	✓	-	-	-	-	-	-	-
No. 15	✓	-	-	-	-	-	-	-	-	-
No. 16	-	-	-	✓	-	-	-	-	-	-
No. 17	-	-	✓	-	-	-	-	-	-	-
No. 18	-	-	✓	-	-	-	-	-	-	-
No. 19	-	-	-	-	✓	-	-	-	-	-
No. 20	-	-	-	-	✓	-	-	-	-	-
No. 21	✓	-	-	-	-	-	-	-	-	-
No. 22	-	-	✓	-	-	-	-	-	-	-
No. 23	-	-	✓	-	-	-	-	-	-	-
No. 24	-	-	✓	-	-	-	-	-	-	-
No. 25	-	-	✓	-	-	-	-	-	-	-
No. 26	-	-	✓	-	-	-	-	-	-	-
No. 27	-	✓	-	-	-	-	-	-	-	-
No. 28	-	✓	-	-	-	-	-	-	-	-
No. 29	-	✓	-	-	-	-	-	-	-	-
No. 30	-	✓	-	-	-	-	-	-	-	-

Discussion

Urinary tract infection is one of the most common bacterial infections in humans. Approximately 80-90% of all community-acquired UTIs and 30-50% of nosocomial UTIs are caused by *E. coli*. In addition, UTI is one of the main reasons for hospitalization and imposes a high burden on healthcare systems [13, 14].

In the present study, the frequency of UPEC isolates was 40%. Previous studies in Iran in 2010 [15] and 2012 [16] have reported prevalence rates of 60.87 and 38%, respectively. In this study, the frequency of UTI was higher in women than in men, which is in line with findings of previous studies [16, 17]. In addition, most *E. coli* isolates (53%) were isolated from ICU patients, which is consistent with the results of a study conducted by Neamati et al. (2014) [18].

Prolonged hospitalization, short urethral length in women, and the use of invasive devices such as urinary catheters might be related to the high prevalence of UTI in ICU patients, especially women. Depending on the type and method of antibiotic prescription in each country, resistance patterns of UTI-causing *E. coli* isolates to antibiotics are significantly different [19]. In the present study, the frequency of gentamicin resistance in *E. coli* isolates was 30%. In previous studies, the frequency of gentamicin resistance has been reported to be 56.82% [20] and 13.07% [17]. This difference in the rate of gentamicin resistance could be due to differences in time of study, geographical location, and patient's conditions. The most common mechanism of resistance to aminoglycosides in *E. coli* is enzymatic modification and inactivation of aminoglycosides, which leads to inhibition of antibiotic binding to ribosome [21]. Also, the *aac(6')-Ie-aph(2'')-Ia* gene that encodes aminoglycoside-modifying enzymes (acetyltransferase and phosphotransferase) confers resistance to

all clinically used aminoglycosides except streptomycin [22].

Based on the results of PCR-based detection of antibiotic resistance genes, the frequency of the *aac(6')-Ie-aph(2'')-Ia* gene in *E. coli* isolates was 56.5%. This gene is located on a transposon or plasmid that allows it to be easily transferred to other bacteria and induce resistance to most aminoglycoside antibiotics. The crucial role of this gene in inducing gentamicin resistance among members of *Enterobacteriaceae* has been well demonstrated [23]. Therefore, finding novel and effective antibacterial agents to control drug-resistant pathogenic bacteria seems necessary. In recent years, nanoparticles have been proposed as suitable alternatives to common antibiotics. Protein degradation, DNA damage, and cell wall disruption have been suggested as the main mechanisms through which zinc oxide nanoparticles exert their inhibitory effects on bacteria [24, 25].

According to the findings, increasing the concentration of ZnONPs to 300 mg/mL could enhance their bactericidal properties. Similarly, a previous study in Iran reported that zinc oxide nanoparticles were highly effective against *E. coli*, and this inhibitory effect increased in a dose-dependent manner [26]. In another study, sub-MIC concentrations of ZnONPs inhibited biofilm formation in *Staphylococcus aureus* and *E. coli* [27]. A study in 2016 on extended-spectrum beta-lactamase-producing *E. coli* and *Klebsiella pneumoniae* strains showed that neodymium-doped zinc oxide nanoparticles had stronger antibacterial effect than ZnONPs alone [28]. According to various studies results, the antibacterial effect of ZnONPs is correlated to their size and shape so that smaller nanoparticles exert greater antimicrobial activity. In previous studies, ZnONPs with a diameter of 100 nm have been shown to exert the

highest antimicrobial effects against *E. coli* and *S. aureus* [29, 30].

Conclusion

The relatively high abundance of the *aac(6)-Ie-aph(2'')*-Ia gene among *E. coli* strains isolated from patients with UTI indicates the importance of this gene in inducing gentamicin resistance. The spread of this gene could further increase resistance in *E. coli* strains causing UTI. The present study findings indicated the favorable efficacy of zinc oxide nanoparticles in inhibiting the growth of gentamicin-resistant *E. coli* isolates *in vitro*. Further studies are required to evaluate the efficacy of these nanoparticles against resistant *E. coli in vivo*.

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Authors' Contribution: Conceptualization: FL and MS, data curation: FL, MS and NGH, formal analysis: FL and MS, Investigation: FL, methodology: FL, MS and NGH, project administration: FL, supervision: FL, writing of original draft: FL, MS and NGH, writing-review and editing: FL and MS.

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