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## ORIGINAL ARTICLE

# Assessment of Zinc Nanoparticle Effect and Expression of Zinc Uptake Gene in Drug Resistance *Acinetobacter baumannii* Strain FMHLN5

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### SUMMARY

***Background:*** **Drug resistance bacteria pose an increasing threat to public health. Antimicrobial agents often lack efficacy toward recently developed drug resistance bacteria. Nanoparticles are one of the most effective treatment agents. The aim of this study was to evaluate the expression of zinc uptake regulator gene with zinc nanoparticle stress in drug resistant *Acinetobacter baumannii* strain FMHLN5.**
***Methods:*** **The antimicrobial susceptibility technique was evaluated through disk diffusion methods. ZnO nanoparticles (ZnO-NPs) were synthesized using an acetate precursor-based sol-gel route. *In vitro*, the FMHLN5 strain susceptibility to ZnO-NPs has been tested by the agar wells diffusion method, using varying sizes (20 to 520 nm) of ZnO NPs. The purities and sizes of Nano-ZnO was determined by X-ray diffraction (XRD) and scanning electron microscope (SEM). The expression of the zinc-uptake gene was investigated through the qRT-PCR technique.**
***Results:*** **The results revealed that the ZnO NPs against clinical FMHLN5 strain were useful at different sizes. The expression of the zinc-uptake gene was observed.**
***Conclusions:*** **The effect of ZnO NPs was strong, as reflected by inhibition zones at different sizes. Thus, ZnO NPs potentially induced bactericidal effect for fighting FMHLN5 strain.**
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**KEY WORDS**

antibacterial, nano-zinc oxide, qRT-PCR, strain, Zur gene

### LIST OF ABBREVIATIONS

PCR - Polymerase Chain Reaction
rRNA - Ribosomal RNA
qRT-PCR - Quantitative real time PCR
BLAST - Basic Local Alignment Search Tool
NCBI - National Center for Biotechnology Information
MDR - Multi-drug resistance
DR-AB - Drug resistant *Acinetobacter baumannii*
ZnO-NPs - ZnO nanoparticles

Zur gene - zinc uptake regulator gene
MeO-NPs - Metal oxide nanoparticles
XRD - X-ray diffraction

## INTRODUCTION

In the 21st century, the phenomenon of multidrug resistance (MDR) has emerged as a significant public health issue, causing many healthcare-associated infections which are difficult to manage with current antibiotics [1]. Typically, this is due to the weak health system and excess and irrational use of antibiotics [2]. However, safe and alternate regulation of the emergence of MDR pathogens is a critical issue for public health. Serious MDR bacteria have been included in the "ESKAPE" acronym for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. [3]. MDR *A. baumannii* is a significant pathogen associated with hospital-acquired infections [4]. In the 1970s, *A. baumannii* was considered sensitive to most antibiotics and today tends to be highly resistant to most antibiotics [5]. Therefore, the development of new medicines is required; natural agents may be obtained from biological sources, such as bacteriocins, or synthesized organic/ inorganic compounds [6] and use of nanoparticles to provide a potential strategy for the management of MDR bacteria. The antibacterial effect of NPs may target various biomolecules and have the ability to reduce or eliminate the evolution of MDR bacteria [7]. Metal oxide nanoparticles (MeO-NPs) showed an attractive alternative source for combating microbes which are highly resistant to various antibiotic classes. Besides showing antimicrobial properties, MeO-NPs often act as drug carriers, thereby barely providing any chance for developing resistance [8], such as ZnO, TiO2, and CuO [9]. Zinc oxide nanoparticles (ZnO-NPs) stand out as antimicrobial agents [10]. ZnO is a bio-safe substance that exhibits photo-oxidizing and photo-catalysis impacts on microorganisms [11]. Herein, the current study aimed to evaluate the bactericidal effect of ZnO-NPs against drug resistant *Acinetobacter baumannii* strain FMHLN5 and to analyze the expression of the zinc-uptake gene.

## MATERIALS AND METHODS

### Ethical approval

Ethical approval was not applicable, because this article does not contain any studies with human or animal subjects.

### Bacterial sources and identification

A total of 60 *A. baumannii* isolates were collected from Baghdad hospitals. The isolate sources were wound infections (n: 24), burn infections (n: 12), urinary tract infections (n: 16), and blood (n: 8). The VITEK 2 system was used to identify the isolates (Version 5.01 BioMérieux).

### Antimicrobial susceptibility testing

The susceptibility of *A. baumannii* was analyzed using the Kirby-Bauer disk diffusion technique according to CLSI 2019 guidelines [12]. Duplicate antibiotic solutions were made. Muller-Hinton agar free of antibiotic was used as a positive control, and the standard strain *E. coli* ATCC 25922 as a negative control.

### ZnO synthesis and characterization

ZnO nanoparticles were synthesized using the acetate precursor-based sol-gel route [13]. Purity was determined using XRD methods and SEM.

### Antibacterial activity of ZnO nanoparticles

Agar wells diffusion method was performed for determination of antibacterial activity [14]. Using sterile cotton swabs, 100 μL *A. baumannii* isolates were swabbed homogeneously on the plates and left to dry. Using gel puncture, 10 mm diameter wells were made on Mueller-Hinton agar plates. Then, 50 μL of different sizes of ZnO nanoparticle (20 - 520 nm) was added to each well. Plates were incubated 24 hours at 37°C. Three replicates of experiments were carried out.

### PCR amplification and DNA sequencing

First, DNA was extracted under normal conditions by DNA extraction kit (G-spin™ Total DNA Extraction Mini Kit, iNtRON Biotechnology). To detect the 16s rRNA and Zur genes, PCR reactions were performed on a thermal cycler (Agilent (8800)/USA), Table 1, 2. The primers were designed by Integrated DNA Technologies/USA. Then, the PCR products were purified from agarose gel. They were then sequenced by a national instrumentation center for environmental management, Korea (NICEM). Sequenced alignments of the 16s rRNA gene region data were analyzed by the Basic Local Alignment Search Tool (BLAST) program and submitted to National Center Biotechnology Information (NCBI).

### Evaluation of Zur Gene Activity by qRT-PCR

RNA was extracted using an RNA extraction kit (ZR Fungal/Bacterial RNA MiniPrep™, Zymo/USA). In the next step, the cDNA was synthesized using KAPA SYBR® FAST One-Step qRT-PCR Kit, Kappa/USA. Then, qRT-PCR was done by thermal cycler (Bio-Rad) Table 3. The 16S rRNA, was used as a reference gene in *A. baumannii* strain FMHLN5 to compare gene expression. The mRNA amount was measured in each sample then compared with the amount of mRNA of the 16S rRNA gene as a reference. The relative mRNA level was determined by calculating the threshold cycles of specific genes (ΔCt), using the classic ΔCt method, on the basis of the standard curves of 16S rRNA expression.

**Table 1. Specific primers and molecular size of PCR products.**

| Target gene | Primer sequence | Fragment size (bp) | References |
|---|---|---|---|
| 16S rRNA | F: 5'- AGAGTTTGATCCTGGCTCAG- 3'<br>R: 5'- GGTTACCTTGTTACGACTT- 3' | 1,250 bp | Current study |
| Zur | F: 5'- ACTTTATGTACTGCGGTCGG - 3'<br>R: 5'- ATAAACAGTAGGAGGGGCGG - 3' | 130 bp | |

**Table 2. PCR Programs of Genes.**

| Gene | Stage | Temperature (time) | |
|---|---|---|---|
| 16S rRNA and Zur | Initial denaturation | 94°C (3 minutes) | |
| | Denaturation | 95°C (1 minute ) | 40 cycles |
| 16S rRNA | Annealing | 52°C (45 seconds) | |
| Zur | Annealing | 55.0°C (45 seconds) | |
| 16S rRNA and Zur | Extension | 72°C (1 minute ) | |
| | Final extension | 72°C (10 minutes) | |

**Table 3. The amplification program used in the qRT-PCR experiment.**

| Step | Temp. (°C) | Time | Cycle |
|---|---|---|---|
| Reverse transcription | 42°C | 10 minutes | hold |
| Enzyme activation | 95°C | 3 minutes | hold |
| Denaturation | 95°C | 15 seconds | 40 |
| Annealing/Extension | 55°C | 15 seconds | |

**Table 4. Ct, ΔCt, ΔΔCt values and fold ratio.**

| | No. of isolates | Ct | ΔCt | ΔΔCt | Fold ratio | Average |
|---|---|---|---|---|---|---|
| Zur gene | AB1 | 21.0 | 3.9 | -13.4 | 10,809.41 | 12,301.88 |
| | AB2 | 21.2 | 3.4 | -13.9 | 15,286.81 | |
| | AB3 | 20.9 | 3.9 | -13.4 | 10,809.41 | |

## RESULTS

### PCR results

The 16s rRNA and Zur genes have been identified via PCR and the findings have been positive. Sequenced alignments of the 16s rRNA genes of *A. baumannii* FMHLN5 strain which is available online at (https://www.ncbi.nlm.nih.gov/nuccore/1409899189). The partial sequence was deposited in GenBank under accession number MH542624.1.

### ZnO-NPs characterization

ZnO nanoparticle was successfully synthesized (Figure 1). SEM analyses provided confirmation that Zn aggregates were made up of nanoparticle oxides, with varying sizes of 20 - 520 nm. XRD pattern of ZnO film refers to the polycrystalline of the film. Diffraction peaks were connected to the ZnO wurtzite structure (JCPDS

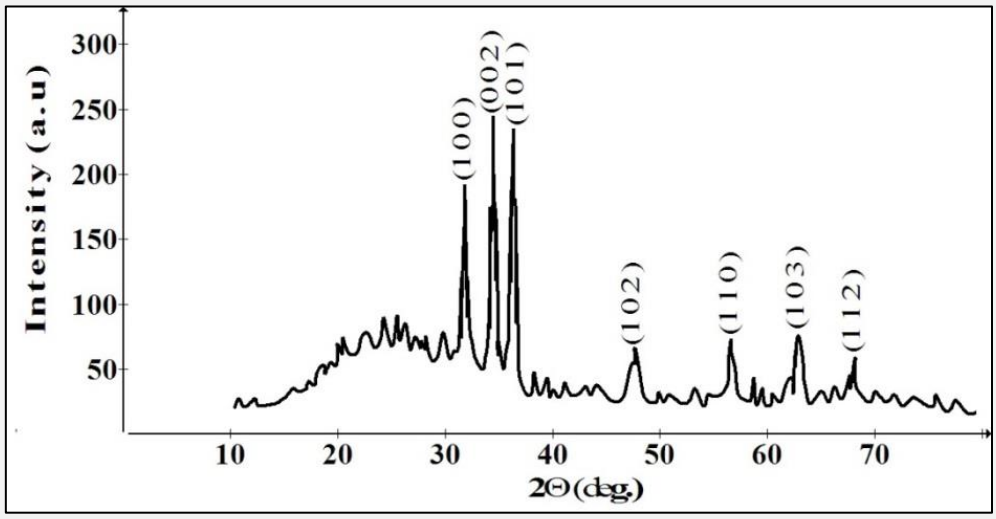


**Figure 1. XRD of Zn oxide nanoparticles film on a glass substrate.**

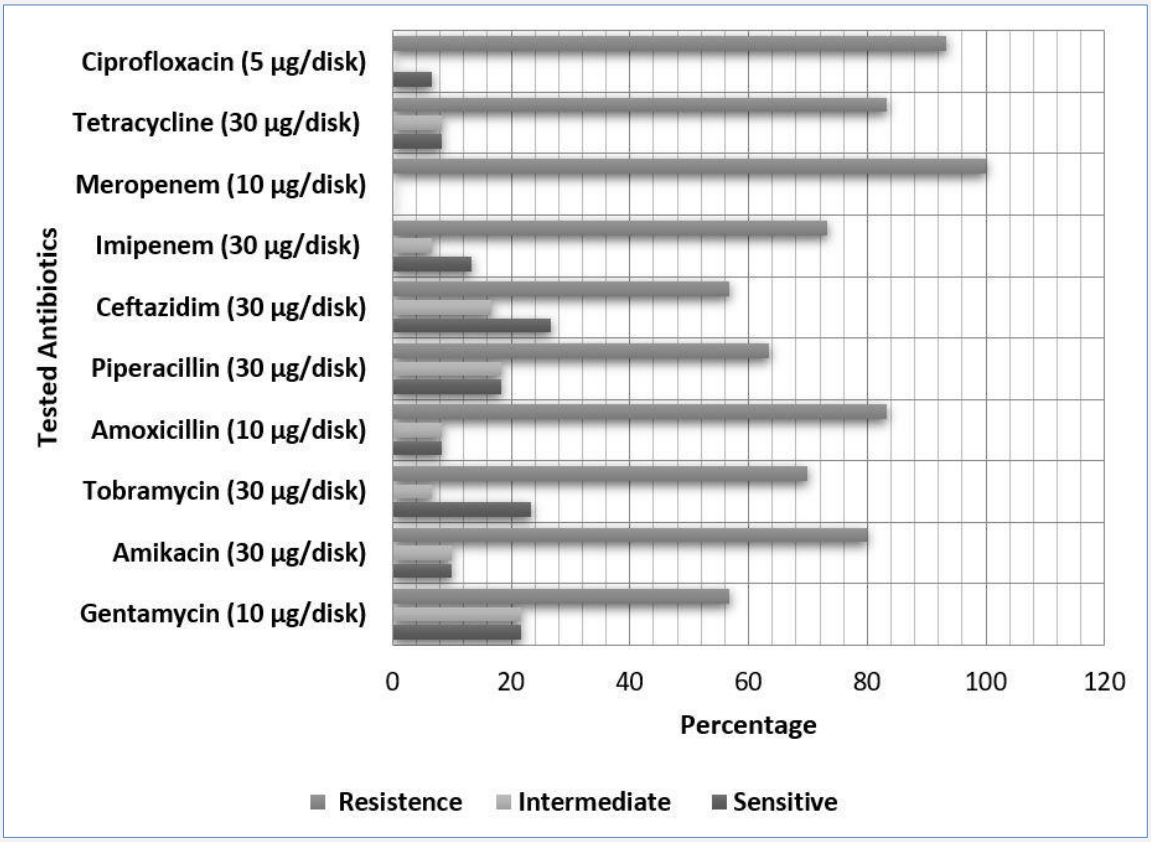


**Figure 2. Antimicrobial susceptibility results of *A. baumannii* isolates.**

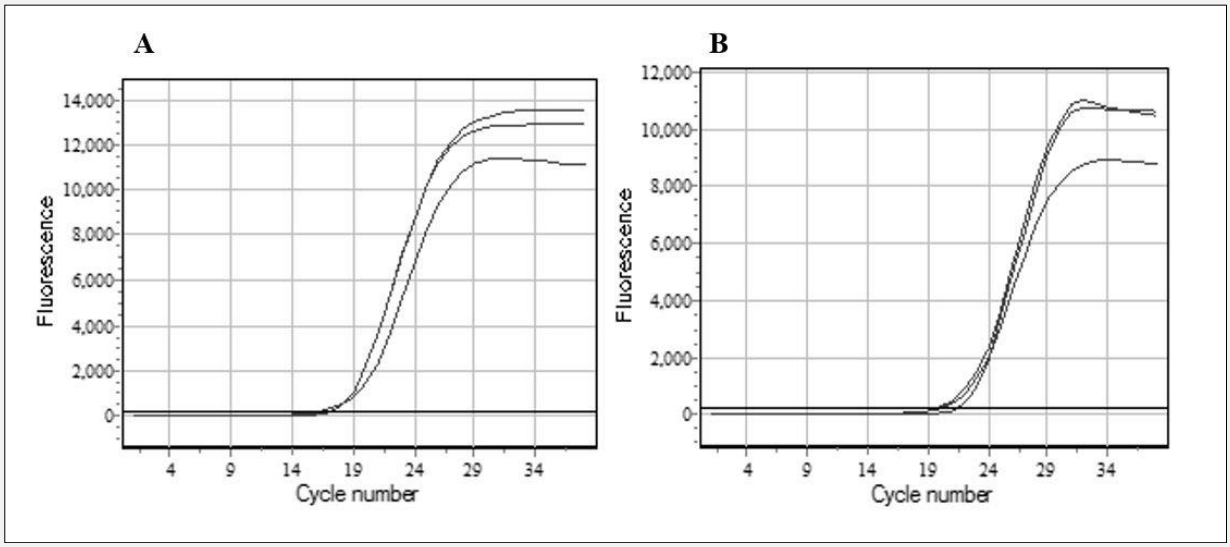


**Figure 3. Ct values A: Zur gene, B:16sr RNA gene.**

Card, No. 36-1451).

**Antibacterial activity**

**Antimicrobial susceptibility testing**

Current findings showed that the *A. baumannii* strain FMHLN5 was resistant to the antibiotics used as shown in Figure 2. Strain was highly resistant to Meropenem, Ciprofloxacin, Amoxicillin and Amikacin antibiotics. Overall, we noticed that the FMHLN5 strain showed resistance to various classes of antibiotics.

**Agar well diffusion method results**

After 24 hours, the inhibition zones of *A. baumannii* strain FMHLN5 were detected at different sizes. It indicates that ZnO-NPs can induce broad-spectrum bactericidal effects against *A. baumannii* strain FMHLN5 with different sizes.

**Zur gene expression**

To assess the Zur gene expression with ZnO NP stress, the *A. baumannii* strain FMHLN5 was subjected to evaluation. The results nicely demonstrated that Zur gene is expressed (Figure 3). The results of Ct values of Zur gene are shown in Table 4, Ct values for bacterial strains: AB1 = 21.0, AB2 = 21.2, and AB3 = 20.9 which indicted high copy number of RNA and, therefore, high copy number of cDNA as compare with 16sr RNA Ct values: AB1 = 17.1, AB2 = 17.8, and AB3 = 17.0.

## DISCUSSION

Regretfully, due to develop a variety of antibiotic resistance types, resistance gene acquisition, transposons, and integrons among *A. baumannii* isolates, as well as the ever-increasing inability to treat patients is a clinical challenge in treatment today. So, we have decided to evaluate the effect of Nano-ZnO as a substitute for antibiotics and the expression level of Zinc uptake gene in *A. baumannii.*

In this study, agar media have been chosen rather than broth because a proportion of ZnO NP can precipitate in broth, which makes it difficult to estimate the actual ZnO NP exposure levels [15]. However, ZnO-NP size is a significant antimicrobial factor [16,17]. Studies have shown that the smaller NP size is more toxic to microorganisms [18,19]. Overall, ZnO-NPs used in the current study were presented between 20 to 520 nm and were effective at their different sizes. This can be explained by the disruptive effects of ZnO NPs on the pathogenic bacteria, with increased production of active oxygen such as hydrogen peroxide, which ultimately caused cell death [20]. ZnO NPs exhibit bactericidal activity, photographic degradation, and drug delivery applications [21,22]. Besides, ZnO-NP has been widely evaluated against G+ve and G-ve bacteria, such as *E. coli* and *S. aureus* which have shown sensitivity to ZnO-NPs [20,23]. Effects of ZnO-NPs on bacterial cells involve a reduction in cell viability, generation of adenosine triphosphate, and reactive oxygen species, subsequently, the membrane leaks reducing sugars, proteins, and DNA [24,25].

Metals such as iron, zinc, copper, and manganese are key cell components but are toxic when excessive [26]. In bacteria, their free intracellular levels are kept within a restricted range [27]. Zinc is an essential nutrient that pathogenic bacteria acquire from their hosts, including *A. baumannii*, and they have catalytic, structural, redox, and regulatory roles [28]. Zinc homeostasis is maintained by controlling the transcription of regulatory genes for the acquisition, utilization, trafficking, and export of zinc by specific metal-sensing metalloregulatory proteins and zinc uptake regulator (Zur) [29].

Zur are intracellular proteins that mediate the regulation of *znuABC* genes required to adapt to avoid Zn toxicity and limitation. Zn proteins bound to Zur recognize a conserved Zur box sequence when Zn is available, thus inhibiting transcription. Under Zn-deplete conditions, Zur is no longer bound by the Zur box, and the target genes are expressed. The oversensitivity of Zur to Zn levels reflects the importance of proper metal homeostasis in the bacterial cell [30]. Collectively, current findings define the Zur gene within the *A. baumannii* strain FMHLN5 and suggest Zn levels may affect the expression of the Zur gene. Finally, the 16S rRNA sequence of the *A. baumannii* strain FMHLN5 was recorded.

## CONCLUSION

In summary, inappropriate and irrational use of antibiotics accelerates development and spread of Superbugs bacteria. Importantly, ZnO-NP may be an appropriate candidate as an alternative to antibiotics against drug resistant *A. baumannii*. There has been a rise in antibiotic resistance in Iraq hospitals, and the current study recommends using the antibiotic footprint as an effective tool to aid in reducing the risk of antibiotics resistant bacteria.

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**Disclosure:**
The authors report no conflicts of interest in this work.

**Declaration of Interest:**
None of the authors have any challenging conflicts of interests. The paper was read and approved by all the authors.

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