



Original article

Determination of synergistic effects of antibiotics and ZnO NPs against isolated *E. Coli* and *A. Baumannii* bacterial strains from clinical samples



Alshareef O. Fadwa^{a,*}, Ahmed M. Albarag^b, Dena K. Alkoblan^a, Ayesha Mateen^a

^a Department of Clinical Laboratory Science, College of Applied Medical Science, King Saud University, Riyadh, Saudi Arabia

^b Department of Pathology, College of Medicine, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:

Received 6 December 2020

Revised 21 May 2021

Accepted 23 May 2021

Available online 3 June 2021

Keywords:

Multidrug resistance

A. baumannii

E.coli

ZnO NPs

Carbapenem antibiotics

MIC

FICI

Time kill curve

ABSTRACT

The mortality rates has been increased globally due to multidrug resistant (MDR) *E.coli* and *A.baumannii* bacterial strains and also there is an emerging resistance of the Enterobacteriaceae family of bacteria to Carbapenem antibiotics (CRE) in Saudi Arabia. The main aim of our research study is to isolate *E.coli* and *A. baumannii* bacterial species from various collected clinical samples and to evaluate the MIC and FICI of Colistin, Ciprofloxacin, Meropenem and ZnO NPs and in combination of Colistin, Ciprofloxacin, Meropenem with ZnO NPs.

The clinical isolated strains of *A. baumannii* (MRO-17-13) and *A. baumannii* (MRO-17-25) was found to be sensitive towards colistin with 0.5 µg/mL concentration, whereas, all the isolated *A. baumannii* strains showed similar MIC value 2 mg/mL when tested with ZnO NPs, the MIC value for the ZnO NPs was found to be similar for all the *E.coli* strains 0.25 mg/mL. The effects of all Ciprofloxacin concentrations used in the study were bacteriostatic against *E. coli* (01UR19006568-01) strain but 1 mg/mL concentration of ZnO NPs alone is showed bactericidal activity, ZnO NPs effect was found to be concentration dependent, as highest concentration of ZnO NPs showed strongest antibacterial effect. In conclusion, more investigation is required to evaluate the acceptable concentration of ZnO NPs and antibiotics selected to avoid toxicity and must be tested against more clinically isolated gram-negative bacterial strains.

© 2021 Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

As the significant rate of mortality and morbidity caused by multidrug-resistant (MDR) has been increased in intensive care units has resulted an enormous challenge globally to public health

[Clark et al., 2016]. Many research studies reported, that there is an emerging resistance of the Enterobacteriaceae family of bacteria to Carbapenem antibiotics (CRE) in Saudi Arabia province (Kader and Kumar, 2004), as we have selected Meropenem broad-spectrum antibiotic belongs to the Carbapenem class and moreover a recent, study in Saudi Arabia on Carbapenem-resistant Enterobacteriaceae concluded that most reports related to CRE

came from the central part of Saudi Arabia (Riyadh) (Alotaibi, 2019), not only the Carbapenem resistance, other types of antibiotic also requires global attention to limit the spread of infections (Zowawi et al., 2013).

ZnO NPs is listed as generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) (Xie et al., 2011), as reported in the previous studies the toxic effect of ZnO NPs against Gram-negative and Gram-positive bacteria as well as against human T lymphocytes, nanoparticles have selective toxicity towards bacteria while having negligible toxicity towards human cells (Kumar et al., 2017, Reddy et al., 2007).

Colistin antibiotics are used as a last resort for the treatment of most of the infections globally, resistance to Colistin by Gram-negative bacteria has already been reported in the later studies (Zowawi, 2016, Loho and Dharmayanti, 2015). As reported in the previous case study, Colistin-resistant *E. coli* carrying the *mcr-1* gene on conjugative plasmids from a blood sample were first reported in Saudi Arabia a case of plasmid mediated Colistin-resistance in the Arabian Peninsula (Sonnevend et al., 2016). It has been updated in the recent review that, the colistin mechanisms of action and various mechanism to develop resistance

* Corresponding author at: College of applied medical science, Department of Clinical laboratory science, Saudi Arabia.

E-mail address: fadoalshareef@ksu.edu.sa (A.O. Fadwa).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

against colistin in the past two decades has been discussed (El-Sayed Ahmed et al., 2020).

Another study conducted on *A. baumannii* isolates obtained from a Saudi tertiary care hospital in 2015 concluded that the rate of susceptibility of these isolates to Colistin was 95.6%, while 50% of the isolates were susceptible to Meropenem (Elabd et al., 2015). Moreover, A recent report from the Al-Ahsa region identified 11% of *A. baumannii* ICU isolates to be resistant to Colistin (Badger-Emeka et al., 2018). Taken together, these surveillance reports suggest that bacterial Colistin resistance is an increasing problem in Saudi Arabia. In the search for ways to tackle this problem, combination therapy offers one promising approach (Petrosillo et al., 2008).

The checkerboard method is a traditional method that tests synergy between two or more drug combinations. It reflects the bacterial growth inhibition based on MIC results, by using an equation to calculate the fractional inhibitory concentration index (FICI). The resulting index is interpreted as synergy, additive, indifference, or antagonism (Martinez-Irujo et al., 1996, Cappelletty and Rybak, 1996). Time-kill curve is another traditional method to test synergy between drug combinations. Synergy, additive, or antagonism results are determined by comparing the difference in colony counts of an organism over time intervals after exposure to the drug alone and after exposure to the combination therapy of two or more drugs (Cappelletty and Rybak, 1996).

The purpose of this study is to isolate *E.coli* and *A. baumannii* bacterial species from various clinical samples, to evaluate the MIC and FICI of Colistin, Ciprofloxacin, Meropenem and ZnO NPs and in combination of Colistin, Ciprofloxacin, Meropenem with ZnO NPs.

2. Methodology

2.1. Clinical samples collection and isolation of *E.coli* and *A.baumannii* strains

All the clinical samples have been collected from KKHU(King Khalid University hospital), King Saud university, Riyadh. The different clinical samples as Respiratory/Sputum, blood, urine, abdominal drainage and body fluid have been collected in sterile bottles and shifted to the laboratory for the isolation of *E.coli* and *A.baumannii* bacterial strains. Reference standard bacterial strain *E. coli* ATCC 25,922 were used for quality control in all procedures and assays of the study, ensuring that the MIC values of reference strain were in the acceptable range according to Clinical Laboratory Standards Institute guidelines (Wayne, 2018).

The clinical samples were collected between 2016 and 2018 from different body sites of patients. The clinical isolates that we were able to identify their sites are shown in (Table 1). They were routinely cultured and identified by conventional laboratory techniques based on colony morphology and lactose fermentation on

Table 1
The Isolates Codes and their Isolation Sites.

Site of Isolates	Number of Isolates	Isolate Serial Number
Urine	2	<i>E. coli</i> (01-UR19006568) <i>E. coli</i> (01-UR19000135)
N/A	2	<i>E. coli</i> (KPC-16-7) <i>E. coli</i> (KPC-18-19)
Respiratory/ Sputum	2	<i>A. baumannii</i> (MRO-17-13) <i>A. baumannii</i> (MRO-17-26)
Blood	1	<i>A. baumannii</i> (MRO-17-25)
Wound	1	<i>A. baumannii</i> (MRO-16-8)
N/A	1	<i>A. baumannii</i> (CRA-16-8)

(N/A) not been identified.

MacConkey agar plates, and by the Micro Scan Walk-Away[®] system (Beckman Coulter Inc.) by KKHU. Isolates that showed resistance or elevated MIC were kept for further investigation, stored at – 80 °C in skim milk media with glycerol, which was purchased ready-made (Oxoid Ltd, Hampshire, UK).

2.2. Antibacterial agent and ZnO NPs stock solution preparation

Pure powdered antibiotics, namely Meropenem trihydrate ≥ 98.0% (HPLC) (Sigma–Aldrich, St. Louis, USA), Ciprofloxacin ≥ 98.0% (HPLC) (Sigma–Aldrich, St. Louis, USA), Colistin sulfate salt ≥ 15000 U/mg (Sigma–Aldrich, St. Louis, USA), and ZnO NPs (50 nm diameter) (Sigma–Aldrich, St. Louis, USA), were all used as antibacterial agents.

Antibiotic stock solution was prepared for Meropenem trihydrate at a concentration of 1.6 mg/mL in Dimethyl sulfoxide (DMSO) as a solvent, Ciprofloxacin stock solution was prepared at a concentration of 1.6 mg/mL in 0.1 N HCL as an aqueous acidic solvent and the Colistin stock solution was prepared at a concentration of 3.2 mg/ml in sterile distilled water.

The ZnO NPs suspension was prepared at a concentration of 16 mg/ml in sterile distilled water.

2.3. Minimum inhibitory concentration (MIC)

The broth microdilution method was followed to determine the MIC of all the isolated bacterial strains and standard strain, Costar 96-well Polystyrene Cell Culture Cluster with flat bottom plate (Corning Inc., Corning, N.Y.) were used.

To prepare the broth microdilution method, 100 µL MHB was added to the microtiter plate, from column two to column twelve and first column was left empty, the antibiotic stock solution, previously prepared in Section 2.2, was taken out of the –20 °C storage, and thawed. After thawing, this stock solution was diluted 1/100 in a separate tube containing MHB, with 20 µL of the stock solution added to 1980 µL MHB to get a concentration of 16 µg/mL. Then, 200 µL of this dilution was added to all the wells of the first column in the microtiter plate, and double diluted by taking 100 µL from the first column to the second one using the multichannel pipette, using new tips for each dilution step until reaching the tenth column. The mixing for each step was done separately using the multichannel pipette. Finally, 10 µL of the bacterial suspension, prepared was as described in Section 2.1, was added to the whole plate except column number twelve. Column twelve was the negative control, and contained only 100 µL MHB. Column eleven was used as a positive control; it contained MHB and the bacterial suspension. MIC was determined by comparing the growth of test bacteria in the wells with the positive and negative controls.

2.4. Checkerboard assay

The checkerboard assay was performed for the combinations of colistin and ZnO NPs, Ciprofloxacin and ZnO NPs and Meropenem and ZnO NPs against Isolated *E.coli* and *A.baumannii* strains using doubling dilutions.

To prepare the checkerboard plate, first, the antibiotic stock solution, previously prepared as described in Section 3.2, was taken out of the –20 °C storage, and thawed. After thawing, this stock solution was diluted 1/100 in a separate tube containing MHB, with 40 µL of the stock solution added to 3960 µL MHB to get a concentration of 16 µg/mL. Then, eight tubes were arranged in a rack and each was filled with 2 mL MHB. The first prepared antibiotic concentration (16 µg/mL) was double diluted by placing 2 mL of it in one of the tubes containing 2 mL MHB, making a concentration of 8 µg/mL, and this process was repeated for each

further tube until reaching a final concentration of 0.06 µg/mL. Next, 100 µL of the first concentration (16 µg/mL) was added to all the wells in column two of a microtiter plate, except the well in the top row (row A). This process was repeated for the rest of the dilutions, moving across the columns from the highest to the lowest concentration, until column number ten. Then, the wells in row A, which had been left empty, were filled with 200 µL of the antibiotic dilutions, starting from the 2A well containing the first concentration (16 µg/mL), until column number ten, with well 1A remaining empty. Columns eleven and twelve were used as positive and negative controls respectively. The positive control contained 200 µL MHB and the bacterial suspension, while the negative control contained only 200 µL liquid media (MHB).

Fractional inhibitory concentration index (FICI) values were calculated as

$$FICI = \frac{FIC A (MIC \text{ antibiotic A in combination})}{(MIC \text{ antibiotic A}) + \frac{FIC B (MIC \text{ antibiotic B in combination})}{(MIC \text{ antibiotic B})}}$$

2.5. Time-Kill method

The time-kill method is used to test the bactericidal activity of selected antibiotic and ZnO NPs alone and in combination. The Microtiter plate was prepared for the time-kill method contained the antibiotic alone in one row (row A), the ZnO NPs suspension alone in another row (row C), and the combination of these two agents in a separate row as well (row B). To prepare the microtiter plate, first, the antibiotic MIC for the tested strain was determined as described in Section 2.3. Second, the stock solution of the antibiotic of choice, prepared as described in Section 2.2, was diluted to a concentration equal to four times the MIC ($4 \times MIC$) for the tested bacterial strain. Third, serial double dilutions were done to get concentrations equal to double the MIC ($2 \times MIC$) and the MIC concentration of the antibiotic for the tested strain. Next, three wells from the first row (row A) of the 96-well microtiter plate were each filled with 200 µL of the decreasing concentrations of the antibiotic, starting with ($4 \times MIC$), then ($2 \times MIC$), and finally (MIC) concentration. In the same way, three wells from the second row (row B) were also filled with 100 µL of antibiotic concentration ($4 \times MIC$, $2 \times MIC$, and MIC). In some cases, half the MIC and quarter the MIC ($0.5 \times MIC$, $0.25 \times MIC$) were also added. Like in the case of highly resistant isolates that had a very high MIC value, using $4 \times MIC$, and $2 \times MIC$ was not possible because of the possibility of a toxic effect to occur. Also, in the case where the MIC value was equal to the MBC value, half the MIC and quarter the MIC ($0.5 \times MIC$, $0.25 \times MIC$) were also added.

For the ZnO NP suspension, the following steps were carried out: First, 1 mL of the stock ZnO/water suspension was prepared as described in Section 3.2., with a concentration equal to ($8 \times MIC$) for the tested organism. Then, serial double dilution was done in separate tubes. To do this, four tubes were arranged in a rack and each filled with 0.5 mL sterile water; the first concentration ($8 \times MIC$) was double diluted by taking 0.5 mL from it and adding this to the next tube, with this process being repeated until reaching a concentration of (MIC). Then, 0.5 mL MHB was added to each of these tubes to support the bacterial growth. This gave us four tubes containing 1 mL of decreasing concentrations of ZnO NP suspension in MHB, starting with a concentration equal to four times the MIC ($4 \times MIC$), then double the MIC ($2 \times MIC$), MIC concentration (MIC), and half the MIC ($0.5 \times MIC$) for each tested strain. Next, using the same microtiter plate as for the antibiotic solution, three wells of the second row (row B), which already contained 100 µL antibiotic solution, were filled with 100 µL of ($0.5 \times MIC$) ZnO NP suspension prepared. Then three wells of the

third row (row C) were each filled with 200 µL of decreasing concentrations of the ZnO NPs suspension prepared. This resulted in the second row (row B) having different combinations of antibiotic and ($0.5 \times MIC$) of nanoparticle suspension, with a final volume of 200 µL. Additionally, one well was used as a positive control, with 200 µL of the MHB and the bacterial suspension, while another well was used as a negative control, with 200 µL of the MHB only. All the wells except the negative control were inoculated with 20 µL of the prepared bacterial suspension as described in Section 3.1. Finally, the 96-well plate was incubated at 37 °C in a 5% CO₂ incubator.

Bacteriostatic and bactericidal activities are defined as $< 3 \log_{10}$ and $\geq 3 \log_{10}$ reductions in CFU/mL at 24-hour, respectively, relative to the starting inoculum. Synergy is defined as a $\geq 2 \log_{10}$ decrease in CFU/mL for the combination in comparison to its most active agent after 24 h. Indifference is defined as $\leq 1 \log_{10}$ CFU/mL decrease compared to the most active agent alone at 24 h. Additive is $< 2 \log_{10}$ CFU/mL decrease after 24 h.

3. Results

To analyse the effect of combination of ZnO NPs with antibiotics meropenem, ciprofloxacin and colistin, the minimum inhibitory concentration and fractional Inhibitory Concentration Index is calculated to see the effectiveness of these antibiotics and ZnO NPs in controlling the *E.coli* and *A. baumannii* infections. *A. baumannii* (MRO-17-13) and *A. baumannii* (MRO-17-25) was found to be sensitive towards colistin with 0.5 µg/mL concentration, where as all the isolated *A. baumannii* strains showed similar MIC value 2 mg/mL when tested with ZnO NPs. The standard *E.coli* (ATCC 25922) strain showed sensitivity to Ciprofloxacin 0.008 µg/mL, more over other two isolated strains *E. coli* KPC-18-19 and *E. coli* 01-UR19006568 was resistant with MIC values 16 and 32 µg/mL, *E. coli* (01-UR19000135) was sensitive with MIC value 0.0625 µg/mL, whereas, the MIC value for the ZnO NPs was found to be similar for all the *E.coli* strains 0.25 mg/mL (Table 2).

The Fractional inhibitory concentration index of ZnO NPs and in combination with Meropenem, Ciprofloxacin and Colistin has been done but we did not get the synergistic effects for all the combination, *A. baumannii* (MRO-17-25), *A. baumannii* (MRO-16-8) and *A. baumannii* (MRO-17-26) has shown additive effect to combination of colistin + ZnO NPs with FICI values 0.58, 0.53 and 0.55, whereas, the all *E.coli* strains were not been tested for this combination. Where as indifference results were shown for Meropenem and ZnO NPs combination against *E. coli* (KPC-18-19) and *A. baumannii* (MRO-17-13) strains with FICI values 1.25 and 2, in contrast Ciprofloxacin and ZnO NPs combination also showed indifference effects on some of *E.coli* and *A. baumannii* strains (Table 3).

3.1. Time-kill curve of *E. Coli* (ATCC 25922)

The ATCC 25,922 *E. coli* strain was found to be sensitive to Ciprofloxacin, after 4-hour incubation at a concentration of 0.032 µg/mL showed no growth, whereas, when treated with 0.016 µg/mL showed no growth even after 8-hour incubation (Fig. 1). When treated with ZnO NPs (0.25, 0.5, 1) mg/mL concentrations showed bactericidal effect after 24-hour incubation and when treated with 0.125 mg/mL ZnO NPs showed bacteriostatic effect, which indicates that effect of ZnO NPs is concentration dependent.

An additive effect has been showed for the combination of 0.008 µg/mL Ciprofloxacin and 0.125 mg/mL ZnO NP in contrast an indifference result was seen for the combination of 0.125 mg/mL ZnO NP and 0.004 µg/mL Ciprofloxacin.

Table 2
Minimum Inhibitory Concentration (MIC) of Antibiotics and ZnO NPs of isolated *E.coli* and *A.baumannii* strains.

Organism	Antibiotic $\mu\text{g/mL}$			ZnO NPs (mg/mL)
	Meropenem	Ciprofloxacin	Colistin	
<i>E. coli</i> ATCC 25,922	N/T	0.008(S)	N/T	0.25
<i>E. coli</i> KPC-16–7	16 (R)	N/T	N/T	0.25
<i>E. coli</i> KPC-18–19	N/T	16 (R)	N/T	0.25
<i>E. coli</i> 01-UR19006568	N/T	32 (R)	N/T	0.25
<i>E. coli</i> 01-UR19000135	N/T	0.0625 (S)	N/T	0.25
<i>A. baumannii</i> MRO-17–13	4 (I) ± 0	0.125 (S)	0.5 (S)	2
<i>A. baumannii</i> MRO-17–25	N/T	8 (R)	0.5 (S)	2
<i>A. baumannii</i> MRO-16–8	N/T	N/T	2 (S)	2
<i>A. baumannii</i> MRO-17–26	N/T	N/T	2 (S)	2
<i>A. baumannii</i> CRA-16–8	N/T	N/T	4 (R)	2

(R) Resistant, (S) Sensitive, (I) Intermediate, (N/T) not been tested.

*MIC value breakpoints: Meropenem ($S \leq 2$, $I = 4$, $R \geq 8$), Ciprofloxacin ($S \leq 1$, $I = 2$, $R \geq 4$), and Colistin ($S \leq 2$, $R \geq 4$).

Table 3
Fractional Inhibitory Concentration Index (FICI) for the combination of ZnO NPs with Meropenem, Ciprofloxacin, and Colistin.

Organism	FICI Values		
	Meropenem	Ciprofloxacin	Colistin
<i>E. coli</i> ATCC 25,922	N/T	0.82 (A)	N/T
<i>E. coli</i> KPC-16–7	1.25 (I)	N/T	N/T
<i>E. coli</i> KPC-18–19	N/T	1.03 (I)	N/T
<i>E. coli</i> 01-UR19006568	N/T	1.0009 (I)	N/T
<i>E. coli</i> 01-UR19000135	N/T	1.22 (I)	N/T
<i>A. baumannii</i> MRO-17–13	2 (I)	1.35 (I)	1.18 (I)
<i>A. baumannii</i> MRO-17–25	N/T	0.59 (A)	0.58 (A)
<i>A. baumannii</i> MRO-16–8	N/T	N/T	0.53 (A)
<i>A. baumannii</i> MRO-17–26	N/T	N/T	0.55 (A)
<i>A. baumannii</i> CRA-16–8	N/T	N/T	1.01 (I)

(S) Synergy, (A) Additive, (I) Indifference, (N/T) not been tested.

*FICI value interpretation: Synergy ≤ 0.5 , additive > 0.5 and ≤ 1.0 , Indifference > 1 and ≤ 4.0 and Antagonism > 4.0 .

3.2. Time-kill curve of *E. Coli* (01UR19006568-01)

The effects of all Ciprofloxacin concentrations used in the study were bacteriostatic against *E. coli* (01UR19006568-01) strain, whereas, 1 mg/mL concentration of ZnO NPs alone is showed bactericidal effect, but the rest of ZnO NPs concentrations were bacteriostatic. Moreover, combination of 0.125 mg/mL ZnO NPs and (32, 16) $\mu\text{g/mL}$ Ciprofloxacin was found to be bactericidal, and the effect of these combinations is considered to be additive (Fig. 2) (see Fig. 3).

3.3. Time-Kill curve of *E. Coli* (KPC-18–24)

The *E. coli* (KPC-18–24) strain was found to be resistant to Ciprofloxacin, with MIC $> 32 \mu\text{g/mL}$ and no antibacterial effect

was seen, whereas, increase in the log₁₀ CFU/mL was observed for all concentrations. The effect of ZnO NPs was concentration dependent, as highest concentration of ZnO NPs showed strongest antibacterial effect and bacteriostatic effect of ZnO NPs for this resistant isolate was detected. The largest decline in viable CFU/mL was achieved by the effect of all the combinations of ZnO NPs with Ciprofloxacin, synergy was found (Figures).

4. Discussion

Infections caused by Multi drug resistant bacteria pose a major threat to global public health, application of nanotechnology in combination with antibiotics are being explore to counter this threat is becoming particular interest of choice. This study has explored the effect of using ZnO NPs in combination with Meropenem, Ciprofloxacin, and Colistin, against Gram-negative resistant and sensitive bacteria. Following CLSI guidelines, microdilution susceptibility testing, checkerboard, and time-kill methods were used to test the effect of the combination of the antibacterial agents (Wayne, 2012, Wikler, 2006).

It has been reported in previous studies that shape of the nanoparticles plays an important role which may influence their effect, however research data on the influence of shape on the effect of nanoparticles is conflicting but, a study carried out on different shapes of particles concluded that rods show the highest uptake by cells, followed by spheres, cylinders, and cubes (Gratton et al., 2008), in our present study nanoparticle of choice is present as nano-powder and shown by TEM to have irregular shaped nanoparticles and some rod-shaped particles with smooth surfaces (Liu et al., 2017). Another study investigated the cytotoxic and inflammatory potential of different shapes of ZnO NPs, and found that there was no significant effect of shape on cytotoxicity (Heng et al., 2011).

In our study, *A. baumannii* (MRO-17–25), *A. baumannii* (MRO-16–8) and *A. baumannii* (MRO-17–26) has shown additive effect to combination of colistin and ZnO NPs with FICI values 0.58, 0.53 and 0.55 in contrast to the other studies synergetic effect of Colistin with combination of conventional and unconventional drugs against MDR bacteria (Vidaillac et al., 2012), as the ability of Colistin to disturb outer membrane permeability facilitates the

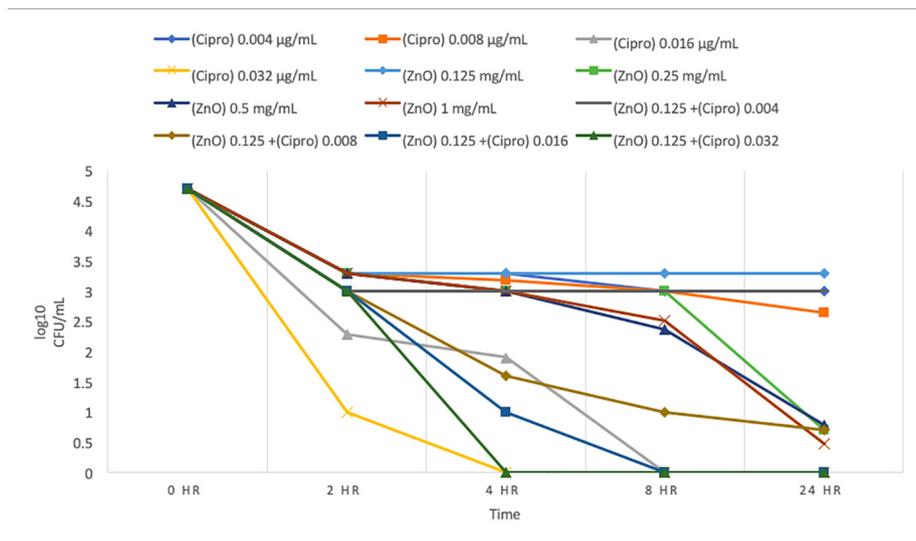


Fig. 1. Time-Kill Curve for *Escherichia coli* (ATCC 25922). This Figure shows the growth curves against Ciprofloxacin alone, ZnO alone, and the combination of different concentrations of Ciprofloxacin (0.004, 0.008, 0.016, 0.032) µg/mL with 0.125 mg/mL ZnO NPs at different time intervals. (Cipro) Ciprofloxacin, (ZnO) ZnO NPs suspension.

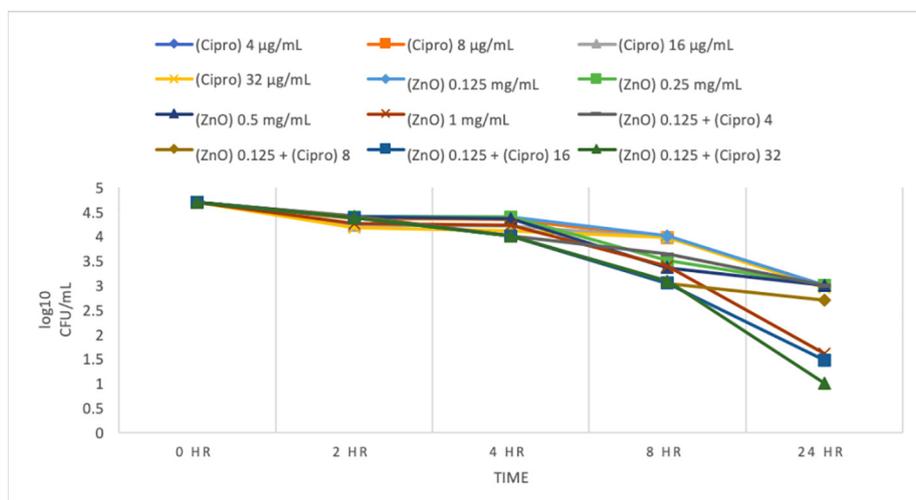


Fig. 2. Time-Kill Curve for *Escherichia coli* (01UR19006568-01), shows growth curves against Ciprofloxacin alone, ZnO alone, and the combination of different concentrations of Ciprofloxacin (4,8,16,32) µg/mL with 0.125 mg/mL ZnO NPs at different time intervals. (Cipro) Ciprofloxacin, (ZnO) ZnO NPs suspension.

accumulation of the other drug inside the bacterial cell (Gordon et al., 2010) and it has been observed that polymyxins disrupt the efflux pumps which leads to increased intracellular concentration of ZnO NPs which obtains a synergistic interaction (Bowers et al., 2015), moreover, positively charged colistin binds to negatively charged phosphate groups of lipid A of lipopolysaccharide (LPS) in Gram-negative bacterial cell and leads to inhibition of NADH-quinone oxidoreductase activity (Deris et al., 2014). As due to limited data availability for the combination of Colistin with nanoparticles, our data suggest that Colistin may improve the effect of the ZnO NPs on bacteria and its ability to disturb outer membrane permeability may have led to the synergetic effect with ZnO NPs. In contrast, Ciprofloxacin targets an inner part of the bacteria, the DNA gyrase (Campoli-Richards et al., 1988), which does not help the ZnO NPs to accumulate inside the bacterial cells. Consequently, there might be better interaction between Colistin and the nanoparticles.

In the present study, we found no correlation between the site of the infection and the results of the different MIC values, we cannot confirm until it is done on a large-scale sample size, as our tested isolates were limited in number.

5. Conclusion

As Combination therapy is being used in the treatment of infectious diseases, in our study we explore the synergistic and additive effects in combination of ZnO NPs and selected antibiotics, present research work might be useful as combination therapy in the treatment of *E.coli* and *A. baumannii* bacterial strains infections, moreover further investigation is required to evaluate the acceptable concentration of ZnO NPs and antibiotics selected to avoid toxicity and must be tested against more clinically isolated gram negative bacterial strains.

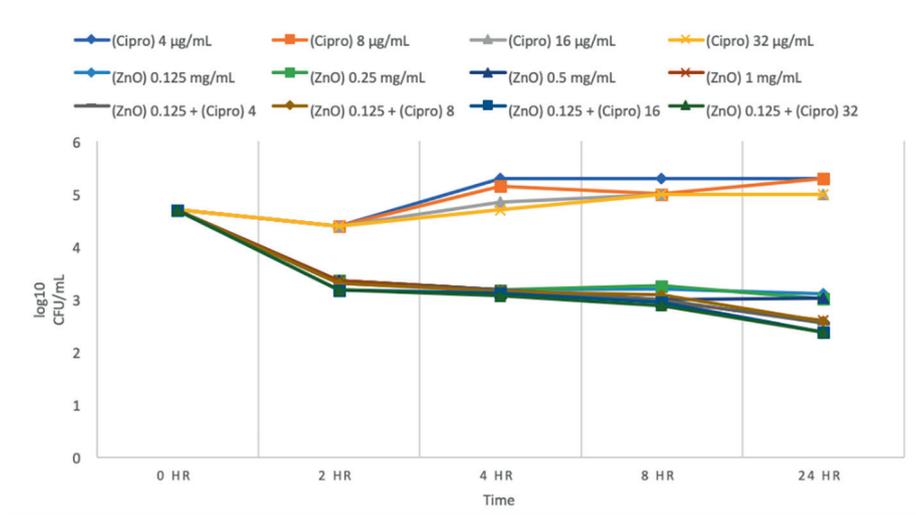


Fig. 3. Time-Kill Curve for *Escherichia coli* (KPC-18–24). This Figure shows growth curves against Ciprofloxacin alone, ZnO alone, and the combination of different concentrations of Ciprofloxacin (4,8,16,32) µg/mL with 0.125 mg/mL ZnO NPs at different time intervals. (Cipro) Ciprofloxacin, (ZnO) ZnO NPs suspension.

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.

Acknowledgement

The authors acknowledge the support of grant from the “Research Center of the Female Scientific and Medical Colleges”, Deanship of Scientific Research, King Saud University for supporting this research project, and also special thanks to all the authors for their constant support and sincere efforts to carry out the present research work.

References

Alotaibi, F., 2019. Carbapenem-resistant Enterobacteriaceae: an update narrative review from Saudi Arabia. *J. Infect. Public Health* 12 (4), 465–471.

Bowers, D.R., Cao, H., Zhou, J., Ledesma, K.R., Sun, D., Lomovskaya, O., Tam, V.H., 2015. Assessment of minocycline and polymyxin B combination against *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 59 (5), 2720–2725.

Badger-Emeka, L., Al-Sultan, A.A., Alrashed, A.S., Alhaddad, M.S., Al-Barjas, A.K., 2018. Antimicrobial susceptibility pattern of Gram negative bacteria isolated from intensive care units in Al-Ahsa, Kingdom of Saudi Arabia. *African J. Microbiol. Res.* 12 (31), 747–753.

Cappelletty, D.M., Rybak, M.J., 1996. Comparison of methodologies for synergism testing of drug combinations against resistant strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 40 (3), 677–683.

Clark, N.M., Zhan, G.G., Lynch III, J.P., 2016. Emergence of antimicrobial resistance among *Acinetobacter* species: a global threat. *Current Opin. Crit. Care* 22 (5), 491–499.

Campoli-Richards, D.M., Monk, J.P., Price, A., Benfield, P., Todd, P.A., Ward, A., 1988. Ciprofloxacin. *Drugs* 35 (4), 373–447.

El-Sayed Ahmed, M.A.E.G., Zhong, L.L., Shen, C., Yang, Y., Doi, Y., Tian, G.B., 2020. Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerg. Microbes Infect.* 9 (1), 868–885.

Deris, Z.Z., Akter, J., Sivanesan, S., Roberts, K.D., Thompson, P.E., Nation, R.L., Li, J., Velkov, T., 2014. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. *J. Antibiot.* 67 (2), 147–151.

Elabd, F.M., Al-Ayed, M.S., Asaad, A.M., Alsareii, S.A., Qureshi, M.A., Musa, H.A.A., 2015. Molecular characterization of oxacillinases among carbapenem-resistant *Acinetobacter baumannii* nosocomial isolates in a Saudi hospital. *J. Infect. Public Health* 8 (3), 242–247.

Gratton, S.E., Ropp, P.A., Pohlhaus, P.D., Luft, J.C., Madden, V.J., Napier, M.E., DeSimone, J.M., 2008. The effect of particle design on cellular internalization pathways. *Proc. Natl. Acad. Sci.* 105 (33), 11613–11618.

Gordon, N.C., Png, K., Wareham, D.W., 2010. Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multidrug-resistant strains of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54 (12), 5316–5322.

Heng, B.C., Zhao, X., Tan, E.C., Khamis, N., Assodani, A., Xiong, S., Ruedl, C., Ng, K.W., Loo, J.S.C., 2011. Evaluation of the cytotoxic and inflammatory potential of differentially shaped zinc oxide nanoparticles. *Arch. Toxicol.* 85 (12), 1517–1528.

Kader, A.A., Kumar, A.K., 2004. Prevalence of extended spectrum beta-lactamase among multidrug resistant gram-negative isolates from a general hospital in Saudi Arabia. *Saudi Med. J.* 25 (5), 570–574.

Kumar, R., Umar, A., Kumar, G., Nalwa, H.S., 2017. Antimicrobial properties of ZnO nanomaterials: A review. *Ceram. Int.* 43 (5), 3940–3961.

Loho, T., Dharmayanti, A., 2015. Colistin: an antibiotic and its role in multiresistant Gram-negative infections. *Acta Medica Indonesiana.* 47 (2).

Liu, J., Kang, Y., Yin, S., Song, B., Wei, L., Chen, L., Shao, L., 2017. Zinc oxide nanoparticles induce toxic responses in human neuroblastoma SHSY5Y cells in a size-dependent manner. *Int. J. Nanomed.* 12, 8085.

Martinez-Irujo, J.J., Villahermosa, M.L., Alberdi, E., Santiago, E., 1996. A checkerboard method to evaluate interactions between drugs. *Biochem. Pharmacol.* 51 (5), 635–644.

Petrosillo, N., Ioannidou, E., Falagas, M.E., 2008. Colistin monotherapy vs. combination therapy: evidence from microbiological, animal and clinical studies. *Clin. Microbiol. Infect.* 14 (9), 816–827.

Reddy, K.M., Feris, K., Bell, J., Wingett, D.G., Hanley, C., Punnoose, A., 2007. Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. *Appl. Phys. Lett.* 90, (21) 213902.

Sonnevend, A., Ghazawi, A., Alqahtani, M., Shibl, A., Jamal, W., Hashmey, R., Pal, T., 2016. Plasmid-mediated colistin resistance in *Escherichia coli* from the Arabian Peninsula. *Int. J. Infect. Dis.* 50, 85–90.

Vidaillac, C., Benichou, L., Duval, R.E., 2012. In vitro synergy of colistin combinations against colistin-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* isolates. *Antimicrob. Agents Chemother.* 56(9), 4856–4861.

Wikler, M.A., 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. CLSI (NCCLS), 26, M7-A7.

Wayne, P.A., 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. In: *Clinical and Laboratory Standards Institute Approved Standard*, ninth ed., pp. M07–M9.

Wayne, P.A., 2018. *Clinical and Laboratory Standards Institute Performance standards for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 11th ed CLSI standard M07 Clinical and Laboratory Standards Institute.

Xie, Y., He, Y., Irwin, P.L., Jin, T., Shi, X., 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 77 (7), 2325–2331.

Zowawi, H.M., Balkhy, H.H., Walsh, T.R., Paterson, D.L., 2013. β-Lactamase production in key gram-negative pathogen isolates from the Arabian Peninsula. *Clin. Microbiol. Rev.* 26 (3), 361–380.

Zowawi, H.M., 2016. Antimicrobial resistance in Saudi Arabia: An urgent call for an immediate action. *Saudi Med. J.* 37 (9), 935.