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SYNERGISTIC ANTIBACTERIAL EFFECT OF ZINC OXIDE NANOPARTICLES AND CIPROFLOXACIN AGAINST KLEBSIELLA PNEUMONIAE ISOLATED FROM URINARY TRACT INFECTIONS IN SALAH AL-DIN GOVERNORATE, IRAQ

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ABSTRACT

Klebsiella pneumoniae is one of the most common causes of urinary tract infections (UTIs), and is becoming increasingly resistant to antibiotics. The zinc oxide nanoparticles (ZnO-NPs) may be a potential alternative approach to combat resistant bacteria. We wanted to know the prevalence of uropathogenic *K.pneumoniae*, its antibiotic resistance profile and the ability of ZnO-NPs to inhibit the growth of uropathogenic *K.pneumoniae*, either alone or in combination with ciprofloxacin. This is a cross sectional study which was conducted in Tikrit Teaching Hospital in Iraq from September 2023 to June 2024. 250 patients with UTIs were screened and *K. pneumoniae* isolates were tested for their antibiotic resistance and multidrug resistance (MDR). We subsequently investigated the effect of ZnO-NPs on 24 selected isolates by using the MIC, MBC and checkerboard assays. 58/250 (23.2%) patients were positive for *K. pneumoniae*. The majority (70.7%) were MDR and 15.5% were reduced-susceptible to carbapenems. All 24 isolates tested were inhibited by ZnO-NPs, the MIC values ranged between 32 to 256 µg/mL. ZnO-NPs were synergistic with ciprofloxacin in 58.3% of the ciprofloxacin-resistant isolates, reducing the ciprofloxacin MIC by a median of four-fold. ZnO-NPs could be effective against uropathogenic *K. pneumoniae* and enhance the activity of Ciprofloxacin in some of the resistant isolates in laboratory setting. They need further research to be established on their safety and clinical potential.

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Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, affecting an estimated 150 million people each year worldwide [1,2]. They cause painful symptoms, repeated visits to hospital, and, when the infection reaches the kidneys or blood, serious illness. Women, older adults, diabetic patients and people with urinary catheters are at higher risk.

Klebsiella pneumoniae is one of the main causes of UTI after *Escherichia coli*, and it is especially common in hospital-acquired and catheter-associated infections [3,4]. It was among the six leading pathogens for deaths associated with antimicrobial resistance worldwide in 2019 [5]. Population genomic studies show that it carries many resistance genes [6], and in hospital patients it acts mainly as an opportunistic pathogen, with infections increasingly complicated by ESBL production and carbapenem resistance [7]. Some strains are also resistant to carbapenems. The World Health Organization places carbapenem-resistant and third-generation cephalosporin-resistant Enterobacterales, which include *K. pneumoniae*, in its critical priority group for new antibacterial development [8].

K. pneumoniae also forms biofilms on the bladder wall and on catheter surfaces. Bacteria in a biofilm survive antibiotic doses that kill free-living cells, which helps explain recurrent and persistent UTI [9,10]. In vitro tests of 120 clinical strains show that biofilm formation on urinary catheters varies widely between strains [11]. Because choices for oral treatment are shrinking, new agents and ways of improving existing antibiotics are needed.

Zinc oxide nanoparticles, or ZnO-NPs, are extensively researched antimicrobial agents. Reactive oxygen species production, zinc ion release, and cell envelope destruction are just a few of the multiple ways they function simultaneously [12,13]. Activity against both Gram-positive and Gram-negative bacteria is described in reviews [14, 15]. They may assist an antibiotic reach its target and remain active against bacteria that are resistant to antibiotics because they reach several targets. Zinc oxide is already used in medical and skin products, which makes its use in coatings or topical and bladder-irrigation preparations plausible. ZnO-containing coatings have reduced bacterial growth and biofilm on catheters in laboratory and animal studies [16,17], and sub-MIC ZnO-NPs reduced biofilm formation in uropathogenic *E. coli* [18].

In Iraq, local data on the resistance of *K. pneumoniae* from UTI are limited, the available studies report resistance among urinary isolates, including ESBL production in *K. pneumoniae* [19,20]. The aim of this study is to evaluate the antibacterial and antibiofilm activity of ZnO-NPs, alone and combined with ciprofloxacin, against *K. pneumoniae* isolated from UTI patients in Salah al-Din Governorate.

Materials and Methods

Study design, setting and ethics

This cross-sectional laboratory study was carried out at Tikrit Teaching Hospital in Salah al-Din Governorate from September 2023 to June 2024, using patients from the outpatient clinics and the urology and medical wards. Informed consent was obtained from adult patients and from parents or guardians of children.

Inclusion criteria included symptoms of UTI (for example dysuria, frequency, suprapubic or flank pain, fever) with significant bacteriuria (at least 10^5 CFU/mL of a single organism) excluding asymptomatic bacteriuria, mixed growth of three or more organisms (probable contamination), and antibiotic use in the previous 7 days.

Sample collection

Clean-catch midstream urine was collected in a sterile container after instruction on cleaning the genital area. In catheterized patients, urine was drawn aseptically from the catheter sampling port. Samples reached the laboratory within 2 hours, or were kept at 4 °C. Age, sex, diabetes,

catheter use, previous hospital stay and antibiotic use in the past 12 months were recorded on a data sheet.

Isolation and identification of *K. pneumoniae*

Urine was cultured with a calibrated loop (0.001 mL) on blood agar, MacConkey agar and CLED agar at 37 °C for 24 hours. Colony count was expressed as CFU/mL. Mucoid, lactose-fermenting colonies were identified as *K. pneumoniae* by Gram stain (Gram-negative rods), the biochemical profile (indole negative, methyl red negative, Voges-Proskauer positive, citrate positive, urease positive, non-motile, triple sugar iron acid/acid with gas and no H₂S), and confirmed by [VITEK-2 GN card / API 20E]. The hypermucoviscous phenotype was screened by the string test (a string longer than 5 mm when a colony is stretched with a loop, Russo and Marr, 2019). Isolates were stored in brain heart infusion broth with 20% glycerol at -20 °C.

Antibiotic susceptibility testing and ESBL detection

Susceptibility was tested by disk diffusion on Mueller-Hinton agar and interpreted according to CLSI M100 ([edition, e.g. 2024]) [21]. The antibiotics tested were ceftriaxone, ceftazidime, cefepime, piperacillin-tazobactam, imipenem, meropenem, ciprofloxacin, gentamicin, amikacin, trimethoprim-sulfamethoxazole and nitrofurantoin. An isolate was defined as multidrug-resistant (MDR) if it was non-susceptible to at least one agent in three or more antimicrobial categories [22]. Carbapenem non-susceptibility was defined as an intermediate or resistant result to imipenem or meropenem.

Zinc oxide nanoparticles

ZnO-NPs were purchased commercially (XFNANO, INC, China). The manufacturer's data sheet states a particle size of 30-80 nm and purity of 99%. A stock suspension of 5 mg/mL was prepared in sterile deionized water, sonicated for 10-15 minutes in a water-bath sonicator immediately before each experiment, and diluted in Mueller-Hinton broth. Particle size was taken from the supplier's certificate.

MIC and MBC of ZnO-NPs

MIC was determined by broth microdilution in 96-well plates [23]. Two-fold dilutions of ZnO-NPs from 512 to 8 µg/mL were prepared in cation-adjusted Mueller-Hinton broth and inoculated with 5×10^5 CFU/mL. Plates were incubated at 37 °C for 18 to 20 hours. Because nanoparticle suspensions are turbid, growth was read by adding (0.015% resazurin) for 2 hours [24] (blue = no growth, pink = growth) or by comparing optical density at 600 nm with ZnO-NP-only control wells. The MIC was the lowest concentration without growth. For MBC, 10 µL from wells at and above the MIC was spotted on nutrient agar, and the MBC was the lowest concentration giving at least 99.9% killing. Each test was repeated three times on separate days. Controls were growth control, sterility control, and ZnO-NP-only control.

Isolates tested

A total of 24 isolates chosen to include MDR and non-MDR strains.

Statistical analysis

Data were analyzed with SPSS 24. Categorical data are given as number and percentage (with 95% Wilson confidence interval for prevalence), continuous data as mean ± SD, or median and range when not normally distributed. Proportions were compared with the chi-square. Two groups (MDR and non-MDR isolates) were compared with the T-test, and more than two groups with ANOVA. A p value below 0.05 was considered significant.

Results

A total of 250 patients with culture-confirmed UTI were enrolled (90 males and 160 females, age range 3 to 82 years). *K. pneumoniae* was isolated from 58 patients (23.2%, 95% CI 18.4-28.8%). Isolation was significantly higher in catheterized patients and in patients who had taken antibiotics in the previous 12 months (Table 1).

Table 1. Patient characteristics and *K. pneumoniae* isolation.

Characteristic	Patients (n)	<i>K. pneumoniae</i> positive, n (%)	p value
Male	90	17 (18.9)	0.22
Female	160	41 (25.6)	
Age under 18 years	40	8 (20.0)	0.82
Age 18 to 60 years	140	32 (22.9)	
Age over 60 years	70	18 (25.7)	
Urinary catheter	50	21 (42.0)	0.001
No catheter	200	37 (18.5)	
Diabetes	60	19 (31.7)	0.07
No diabetes	190	39 (20.5)	
Antibiotic use in past 12 months	120	38 (31.7)	0.002
No antibiotic use	130	20 (15.4)	
Total	250	58 (23.2)	

Resistance was highest to trimethoprim-sulfamethoxazole (69.0%) and ceftriaxone (65.5%), and lowest to meropenem (10.3%) and imipenem (12.1%) (Table 2).

Table 2. Antibiotic susceptibility of *K. pneumoniae* isolates, n = 58.

Antibiotic	Class	Resistant, n (%)	Intermediate, n (%)	Susceptible, n (%)
Ceftriaxone	3rd-generation cephalosporin	38 (65.5)	2 (3.4)	18 (31.0)
Ceftazidime	3rd-generation cephalosporin	36 (62.1)	3 (5.2)	19 (32.8)
Cefepime	4th-generation cephalosporin	31 (53.4)	4 (6.9)	23 (39.7)
Piperacillin-tazobactam	Penicillin + inhibitor	24 (41.4)	5 (8.6)	29 (50.0)
Imipenem	Carbapenem	7 (12.1)	2 (3.4)	49 (84.5)
Meropenem	Carbapenem	6 (10.3)	2 (3.4)	50 (86.2)
Ciprofloxacin	Fluoroquinolone	33 (56.9)	5 (8.6)	20 (34.5)
Gentamicin	Aminoglycoside	26 (44.8)	3 (5.2)	29 (50.0)
Amikacin	Aminoglycoside	12 (20.7)	4 (6.9)	42 (72.4)
Trimethoprim-sulfamethoxazole	Folate pathway inhibitor	40 (69.0)	0 (0.0)	18 (31.0)
Nitrofurantoin	Nitrofurantoin	27 (46.6)	14 (24.1)	17 (29.3)

ZnO-NPs inhibited all 24 tested isolates. MIC values ranged from 32 to 256 µg/mL (median 128, MIC50 128, MIC90 256) and MBC values from 64 to 512 µg/mL. The MBC/MIC ratio was 4 or lower in 22 of 24 isolates (91.7%), indicating mainly bactericidal activity. The median MIC was higher in MDR than in non-MDR isolates (128 vs 64 µg/mL, p = 0.04) (Table 3).

Table 3. MIC and MBC of ZnO-NPs, µg/mL.

Isolate group	n	MIC range	MIC median	MIC50 / MIC90	MBC range	MBC/MIC ratio, median (range)
All isolates	24	32-256	128	128 / 256	64-512	2 (1-8)
MDR	16	64-256	128	128 / 256	128-512	2 (1-8)
Non-MDR	8	32-128	64	64 / 128	64-256	2 (1-4)

In 12 ciprofloxacin-resistant isolates, the combination was synergistic in 7 (58.3%, mean FICI of synergistic isolates 0.40), showed no interaction in 5 (41.7%), and was antagonistic in none (Table 4). In combination, the ciprofloxacin MIC fell by a median of 4-fold (range 2- to 16-fold).

Table 4. Checkerboard results, MICs in µg/mL.

Isolate	Cipro MIC alone	ZnO-NP MIC alone	Cipro MIC combined	ZnO-NP MIC combined	FIC I	Interpretation
K1	16	128	2	32	0.38	Synergy
K2	8	128	1	32	0.38	Synergy
K3	32	256	4	64	0.38	Synergy
K4	16	128	4	16	0.38	Synergy
K5	4	64	1	16	0.50	Synergy
K6	8	128	2	32	0.50	Synergy
K7	32	128	2	32	0.31	Synergy
K8	16	256	8	64	0.75	No interaction
K9	8	128	4	64	1.00	No interaction
K10	32	128	16	64	1.00	No interaction
K11	16	64	8	32	1.00	No interaction
K12	4	256	2	128	1.00	No interaction

Discussion

The present study recovered *Klebsiella pneumoniae* from 58 of 250 patients with culture-confirmed urinary tract infection, giving a prevalence of 23.2% (95% CI 18.4–28.8%). This indicates that *K. pneumoniae* was an important uropathogen in the studied population in Salah al-Din Governorate. The frequency is clinically relevant because *K. pneumoniae* can cause complicated and healthcare-associated urinary infections, particularly in patients with urinary devices or prior exposure to healthcare and antibiotics [3,4].

K. pneumoniae was isolated significantly more often from catheterized patients than from those without a catheter (42.0% vs. 18.5%, $p = 0.001$). This is not surprising, since catheters give bacteria an easy route into the urinary tract and a surface to attach to and persist on, which makes preventing catheter-associated infections an important clinical priority. Isolation was also higher among patients who had taken antibiotics in the previous 12 months (31.7% vs. 15.4%, $p = 0.002$). Earlier antibiotic use may favor resistant organisms and disrupt the normal urinary microbiota, although a cross-sectional design like ours cannot establish that one causes the other. Diabetes showed a similar trend (31.7% vs. 20.5%), but the difference did not reach statistical significance ($p = 0.07$). Neither sex nor age group was significantly associated with *K. pneumoniae* isolation in this dataset.

The resistance profile is worrisome, since 70.7% of the *K. pneumoniae* isolates were MDR. Trimethoprim–sulfamethoxazole (69.0%), ceftriaxone (65.5%), ceftazidime (62.1%), and cefepime (53.4%) had the highest resistance rates. The results indicate that a number of antimicrobial agents used in this population are not reliable empiric drugs and highlight the importance of local, periodically updated antibiograms and culture-directed antimicrobial therapy. The carbapenems resistance was less than the cephalosporins resistance with 9/58 (15.5%) isolates reported as non-susceptible to the carbapenems. This is clinically relevant as ESBL-producing Enterobacterales are frequently a cause of serious infections that require carbapenems as treatment [25,26]. Our results are consistent with the general issue of AMR in urinary *K. pneumoniae*. When comparing them directly with other Iraqi studies, then there should be a degree of caution because the patient population, dates of study and the susceptibility-testing procedure vary from study to study [19,20].

All 24 *K. pneumoniae* isolates tested were inhibited by ZnO-NPs with MICs ranging from 32 to 256 µg/mL and a median and MIC₅₀ value of 128 µg/mL and a MIC₉₀ of 256 µg/mL. The

MBCs were between 64 and 512 µg/mL. The ratio of MBC/MIC for all isolates was found to be 4 or less and this indicated that the nanoparticles had exhibited the bactericidal effect predominantly under the assay conditions. It is believed that ZnO-NPs exert their activity in a number of ways such as the generation of zinc ions, induction of oxidative stress, and disruption of the bacterial envelope. These mechanisms could account for their activity in isolates of varying susceptibility [12-14]

In the checkerboard assay, the FICI values of the synergistic isolates ranged from 0.40 to 0.50, with a mean of 0.40, when ZnO-NPs were used with ciprofloxacin against 7 of the 12 isolates (58.3%) that were ciprofloxacin-resistant. The ciprofloxacin MIC decreased by a median of fourfold (range of two- to 16-fold) and no antagonism was observed in any isolate. The results indicate that this combination could increase the ciprofloxacin activity against some resistant isolates in vitro. There have also been other studies reporting synergy between ZnO-NPs and other antibiotics against clinical isolates, thus aiding in further research on combinations of nanoparticles and antibiotics [26]. However, this effect was not consistent, with five isolates not showing an interaction. Should not be presumed to aid all isolates and checkerboard data cannot demonstrate clinical efficacy.

The strength of this study is that the clinical isolates were detected locally, the ZnO-NPs were quantitatively evaluated through MIC/MBC and the interactions of ZnO-NPs with ciprofloxacin were assessed by checkerboard assay. However, there are some restrictions. First, the experiments conducted on the nanoparticles were done with 24 selected isolates of *K. pneumoniae* and not the full 58 isolates, which decreases precision and generalizability. Secondly, this study was conducted in one governorate only and thirdly, the cross-sectional design cannot allow it to make causal inferences between patient characteristics and the detection of the pathogen.

Conclusion

Klebsiella pneumoniae was a significant UTI etiology in our study population, and resistance rates were high, especially for trimethoprim-sulfamethoxazole and third generation cephalosporins. Urinary catheterization and previous antibiotic use were both significantly associated with isolation of the organism. In vitro activity of zinc oxide nanoparticles (ZnO-NPs) against all 24 isolates studied were mostly bactericidal. In combination with ciprofloxacin, ZnO-NPs showed synergistic effects in 58.3% of the ciprofloxacin-resistant isolates and no antagonism was detected. Based on these results, it can be inferred that ZnO-NPs may be used as an adjunct to conventional antibiotics. However, more isolate collections, toxicity testing and in vivo studies are required before it could be considered for clinical use.

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