Research Article

Kinetics activity of *Yersinia Intermedia* Against ZnO Nanoparticles Either Synergism Antibiotics by Double-Disc Synergy Test Method

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Received: March 16, 2015; **Revised:** July 11, 2015; **Accepted:** December 27, 2015

Background: Bacterial resistance to the commonly used antibacterial agents is an increasing challenge in the medicine, and a major problem for the health care systems; the control of their spread is a constant challenge for the hospitals.
Objectives: In this study, we have investigated the antimicrobial activity of the Zinc Oxide nanoparticles against clinical sample; *Yersinia intermedia* bacteria.
Materials and Methods: Nanoparticle susceptibility constants and death kinetic were used to evaluate the antimicrobial characteristics of the Zinc Oxide (ZnO) against the bacteria. Antimicrobial tests were performed with 10^8 cfu.mL^{-1} at baseline. At first, Minimum Inhibitory Concentration (MIC) of ZnO was determined and then nanoparticle suspension at one and two times of the MIC was used for death kinetic and susceptibility constant assay at 0 to 360 min treatment time.
Results: ZnO nanoparticles with size ranging from 10 to 30 nm showed the highest susceptibility reaction against *Y. intermedia* (Z=39.06 mL.μg^{-1}). The process of *Y. intermedia* death in ZnO suspension was assumed to follow the first-order kinetics and the survival ratio of bacteria decreased with the increasing treatment time. An increased concentration of the nanoparticle was seen to enhance the bactericidal action of the nanoparticle. Then we performed the best ratio of the nanoparticles on semi-sensitive and resistance antibiotic for the bacteria. However, based on experimental results, synergy of ZnO nanoparticles and Oxacilin was determined and *Y. intermedia* showed a higher sensitivity compared to the ZnO nanoparticles alone.
Conclusions: The results of the present study illustrates that ZnO has a strong antimicrobial effect and could potentially be employed to aid the bacterial control. It could also improve- antibacterial effects in combination with the antibiotics.

Keywords: Kinetic activity of nanoparticles; *Yersinia intermedia*; ZnO

1. Background

Aquatic environments have an important role in the spread of the diseases. *Yersinia* species have an immense ability to adapt to the aquatic environments as they need minimum requirements for life, therefore they can live for a long time in the cold waters; hence surface waters and sewage could be good habitats for spreading of the diseases: Yersiniosis (1). In addition, the bacteria belonging to the *Yersinia intermedia* species could be seen in different animal kingdom (e.g. fish, oyster, shrimp, spiel, and domestic animals), as well as foods (milk, cream, flesh), and sometimes in ill or even healthy individuals especially in their stool (2).

It does not seem that *Yersinia intermedia* to have intestinal pathogenic genes. As well, it is not clearly defined whether they are pathogenic for human, as they are usually non-pathogenic unless they become clinically consistent with the human body. Nevertheless, according to the report by Agbonlahor the species causes four kinds of acute diarrhea (3, 4).

The species *Yersinia intermedia* is a member of the genus *Yersinia* that was identified as a distinct species of the *Y. enterocolitica*. This distinction was done based on the acid production when microbe is exposed to D-melibiose, D-raffinose, α-methyl-diglucose, L-rhamnose. Applying citrate distinguishes *Y. intermedia* from the other *Yersinia* species (5) and has introduced as a new species in 1980s by Brenner and colleagues. This species was found to belong to family of *Entrobacteriaceae* (2). They are Gram- negative, poly

type bacilli that show bipolar staining. Also, they are oxidase negative and catalase positive.

Y. intermedia was defined as a new species distinct from *Y. enterocolitica* in 1980 based on DNA-DNA hybridizations and biochemical characteristics (6). The lipopolysaccharides of *Y. intermedia* is similar to those of *Y. enterocolitica*, and its carbohydrate utilization profile is similar to that of *Y. enterocolitica* as well as *Y. pseudotuberculosis* (6).

Y. intermedia has been mostly isolated from aquatic environments, such as freshwater, sewage, as well as invertebrate hosts such as fish, oysters, shrimps, snails, which are living in the aquatic environments. Association of the microbe with the mammals is evident as it could be isolated from milk, cream, and meat. It has also been reported that the bacterium could be isolated from the human urine, stool specimens, as well as from wound infections (7, 8), however it is rarely associated with the disease in human (9).

The bacteria are resistant to oxacillin, penicillin G, amoxicillin, and are sensitive to cefotaxime, ceftazidime, chloramphenicol, ceftriaxone and sulfonamides. As well, they are sensitive to a lower extent to the nalidixic acid, ampicillin, and amikacin (1, 2, 10, 11). With regard to the tetracycline antibiotic, there were reports indicating diverse results through undertaking a set of different essays so far. In one case it was found that the bacteria are resistant, while in the other investigations it was found that they are susceptible to the Tetracycline (2, 11).

There is a large interest in the application of the inorganic antimicrobial agents as they have shown their advantages over the organic antimicrobial agents.

This interest, in part, is because of an improved safety and stability. Zinc oxide (ZnO) is currently being investigated as an antimicrobial agent both in the microscale as well as nanoscale formulations. The obtained results so far have indicated that ZnO nanoparticles show an apparently superior antibacterial activity than ZnO microparticles (12, 13). The precise mechanism underlying the antibacterial action of the ZnO nanoparticles is not entirely well elucidated as yet. Different assumptions have so far been made, among which, the influence that reactive oxygen species (ROS) might have (14), the effect of the released Zinc ion, as well as the mechanical damage of the cell wall through adhesion to the cell membrane which might result and have an influence on the reaction system pH value . At higher concentration, small particles with a larger surface area ensure a more efficient antibacterial behavior, while, the crystalline structure, and particle shape probably have less influence (15).

Nowadays, researchers are trying to find a more pertinent method for treating this disease. Thus, nanomaterial that increasingly have received an expanded applications, have found their ways to our lives, and introduction of a new solution for treatment of the diseases. The various products of the nanotechnology have taken their applications in biology and medicine through many functions attributed to them. These materials are moving rapidly towards improving a new generation of medicines.

2. Objectives

The aim of the present study was to focus and find a solution for lifting drug resistance, in addition to using a lower dosage of the antibiotics for treating infections caused by the bacteria.

3. Materials and Methods

3.1. Preparation of the Culture Medium

Y. intermedia bacteria were obtained from positive culture of the clinical samples taken from patients with Yersiniosis and sent to Tehran University, Medical Sciences for culturing, using CIN Agar (Cefsulodin-Irgasan-Novobiocin, Merck, Germany) (16, 17).

3.2. Sensitivity Test using the Kirby-Bauer Method

In order to test the resistance of the *Y. intermedia* to the antibiotics, positive culture of the clinical samples of the *Y. intermedia* were cultured in Muller Hinton Agar medium (Merck, Germany), and Kirby - Bauer disc diffusion method. The discs used in the present study were cefotaxime, tetracycline, nalidixic acid, oxacillin, amoxicillin, ampicillin, chloramphenicol, ceftazidime according to the following concentrations in micrograms: 30, 30, 30, 30, 30, 10, 25, 10 µg (Mast group Ltd. UK) respectively. The incubation was performed for 24 h at 3ºC supplemented with %5 CO_2 (semi-aerobic condition). After reading the zone of growth inhibition, a clinical specimen that was resistant to Oxacillin was used for further evaluation.

3.3. Nanoparticle Suspensions Preparation

ZnO nanoparticles (purity more than 99.7%) with a size ranged from 10-30 nm was purchased from US NANO. To prepare a stock solution of nanoparticles, 10 g of the nanoparticles was turned into the suspension in one litter of the sterile medium. As well, for a proper dispensing of the particles, Electrosonic System

(Bandelier Sonorex RK 31H) was used for 35 min. In order to prevent errors, the preparation of the nanoparticle suspension and the microbial tests were performed simultaneously.

3.4. Preparation of the Microbial Suspensions

In order to prepare microbial suspension, first bacterial cells were picked from CIN agar medium by using a sterile loop and mixed in 10 mL of the sterile PBS (Buffered- phosphate) so that samples were prepared to a half McFarland turbidity (Colony Forming Unit (CFU) 1 to 1.5×10^8). The absorbance was measured by applying a UV/visble spectrophotometer (UNICO-2100, US) at 620 nm and absorption rate was adjusted in a range of 0.08-0.1 (18).

3.5. Minimum Inhibitory Concentration (MIC) Test

Using the standard method suggested by the national committee of the clinical laboratories standards (CLSI), MIC was calculated for the samples in contact with the nanoparticle suspension (16). MIC is defined as the minimum concentration of nanoparticles that prevent organisms' growth in the medium. Tubes containing the medium as batch systems were prepared with the different concentrations of the silver nanoparticle. In this method 12 tubes was used, each of which containing 2 mL of the sterile Triton Soya Broth (TSB) medium. In the first tube only 4 mL of the suspension was added and was mixed. Then 2 mL of this mixture was added to the second tube, and from this tube it was added to the next tube respectively. This process was done up to the 12^{th} tube (the resulting concentrations from the first to the twelfth tube declined accordingly from 10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, 19.53, 9.765 to a final of 4.883 $\mu g.mL^{-1}$), respectively. Then the tubes containing nanoparticles and bacteria were incubated at 37ºC for 24 h in a shaker incubator at a rate of shaking of 60 rpm.

3.6. Bacterial Killing and Kinetics Activity

The study of the bacterial kill-kinetics reveals the degree of the bacterial death and kinetics of which in contact with the nanoparticle suspension. The bacterial suspension was added to the medium in the tube containing nanoparticles with the MIC and then it was incubated in the shaker incubators (600 rpm, for maximum 24 h at 37ºC), considering the duration (from Zero to 360 min). Samples were taken from the nanoparticle-bacterial suspension and were cultured on the plates containing Brain Heart Infusion (BHI) medium. The formed colonies at each time, bacteria and concentration were counted and were recorded. Survival rates (N/N_o) was calculated through dividing the number of colonies at the time of the sampling (N) by the number of colonies at the time when they had no contact with the silver nanoparticle suspension (N_o). In order to study the kill-kinetics of the bacteria, the first order kill-kinetics of the bacteria was used. The general formula for this kinetics is:

$$dN/dt = -kN_o$$

In this equation k is the death rate constant, N_o, the number of initial bacterial colonies, N, the number of bacterial colonies at the time t (19-21).

3.7. Calculating Sensitivity Coefficients to the Nanoparticle Suspensions

Here, the sensitivity coefficient of the nanoparticles is calculated through application of the following equation ($\mu g.mL^{-1}$), suggested by Yoon *et al*. (22).

$$Z = \frac{Ln\ (N/N_o)}{c}$$

3.8. Preparation of the Nanoparticles Impregnated Discs

Sterile blank discs were saturated with the desired concentration of the nanoparticle suspension and incubated at room temperature for 24 h.

3.9. DDST Susceptibility Test

Candidate antibiotics to which the *Y. intermedia* were resistant *i.e*. Penicillin, Oxacillin and semi- sensitive, *i.e*. Amoxicillin and Ampicillin, were assessed for DDST. Also the sensitive antibiotic were studied to investigate whether ZnO resonate the inhibitory effect or not. Hinton Moular agar medium were prepared, and 10 µg amikacin, 10 µg penicillin, 30 µg amoxicillin and 10 µg ampicillin discs were placed at a distance of 10 mm from center to center. The culture mediums were incubated at 37ºC for 18 h, then zone of inhibition for each disc was recorded.

Table 1. Sensitivity of the *Y. intermedia* to the applied antibiotics

Antibiotic	mg	Zone Diameter (mm)
Nalidixic acid	30	35
Tetracycline	30	34
Cefotaxime	30	36
Ceftazidime	10	25
Chloramphenicol	25	25
Ampicillin	10	13
Amoxycilin	30	8
Oxacilin	30	0

4. Results

4.1. Kirby- Bauer Susceptibility Test

(Table 1) summarizes the bacterial resistance to the antibiotics as were assessed through application of the impregnated discs in comparison to the standard samples.

4.2. Preparation of the Nanoparticle Suspensions

Figure 1A shows the X-ray powder diffraction (XRD) patterns of the commercial ZnO powder. There was not any diffraction pattern detection originating from impurity. Figure 1B shows the morphology and the corresponding particle size distributions over the volume of the prepared ZnO powders as were examined using TEM.

4.3. Bacteria Killing and Kinetics Activity

According to the results obtained through experiments for revealing antibacterial activity of the ZnO nanoparticle suspension against *Y. intermedia*, the MIC value for the bacteria in ZnO nanoparticles was found to be 39.06 $\mu g.mL^{-1}$.

The sensitivity coefficient of the *Y. intermadia* to the nanoparticle at different sampling periods was calculated. The result of such mean of sensitivity coefficient are shown in (Figure 2).

4.4. DDST Susceptibility Test

The results accumulated in the (Table 2) show significant DDST differences. It seems that there is a loss of sensitivity in synergy with the applied ZnO nanoparticles with Amoxcycilin and Ampicilin, as the zone became vanished, however, it also shows a higher sensitivity for Oxacilin due to visibility of the zone.

5. Discussion

The results obtained through application of the ZnO nanoparticle XRD, the purity of such nanoparticles were validated, as there is no impurity except oxygen. In addition, XRD images obtained for the nanoparticle show peaks which indicate the precise crystal structure of the nanoparticles (23, 24). The Transmission Electron Microscopy (TEM) pictures show the shape and size range of the nanoparticles. According to the image, ZnO nanoparticles' have relatively regular spherical shape, as well as, a size in the nanometer range. Reducing the nanoparticles size causes both structural and physico-chemical features to change, as a result, providing the organisms to have access for them, and, accordingly an increased toxic property (25). The reduction in the size, increase in the specific surface area, along with the rising of the reactive groups' number on nanoparticles surface have been already reported. The increase in the surface active groups may become as active sites for the formation of the reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydrocele radical that lead to the oxidative stress. Regarding to (Figure 2), due to increased nanoparticles concentration, the inhibitory property of the nanoparticles against *Y. intermadia* have increased. Increasing nanoparticles concentration to achieve the MIC for that strain, it was observed a slight growth of the bacteria. In the other words, ZnO nanoparticles did not have the ability to kill the bacteria in the tested strain. However, results achieved in the present study have also indicated that for some synergies, an inhibitory effect could be observed as shown in (Tables 1 and 2).

Numerous studies regarding the antibacterial activity of the ZnO nanoparticles have been carried out,

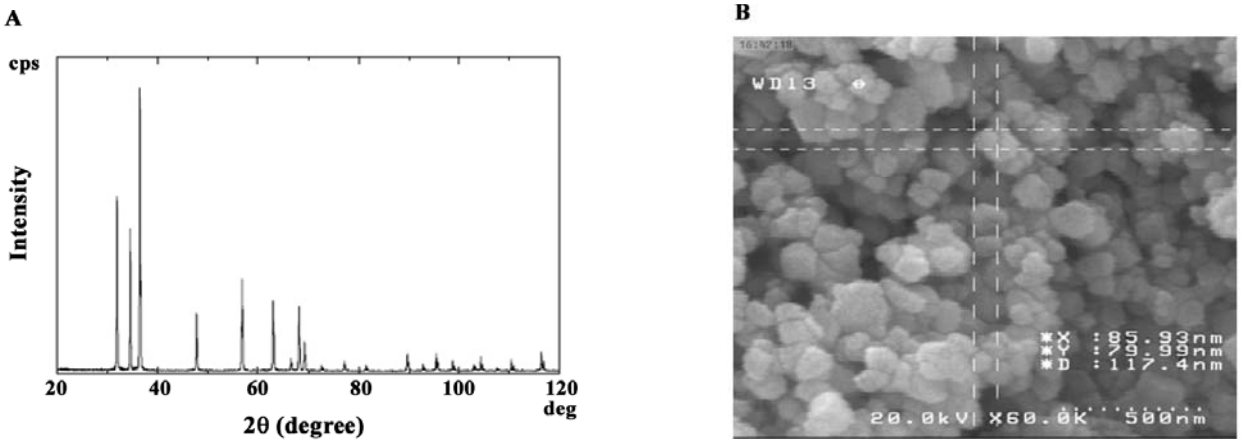

Figure 1. A: XRD of ZnO nanoparticles, B: TEM of the ZnO nanoparticles

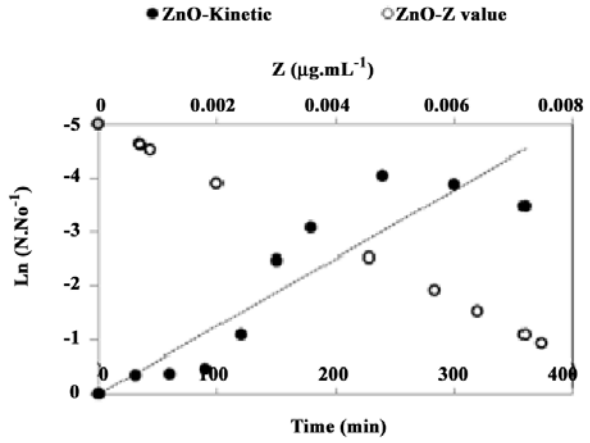

Figure 2. Coefficient sensitivity of *Y. Intermedias* population with respect to the time of investigation

however, most of which were focused on *E. coli* and *S. aureus*, and their synergy with a variety of antibiotics. M. Banoee *et al*. in 2009 have reported that the antibacterial activity of ciprofloxacin against the two clinical test strains: *S. aureus* and *E. coli* could be improved in the presence of ZnO nanoparticles (26). In another study, Reddy KM. *et al*. in 2007 have shown that the introduction of ~13 nm ZnO nanoparticle kills Gram-negative *E. coli* at concentration of ≥3.4 mM, whereas, growth of the Gram-positive *S. aureus* was prevented at much lower concentrations (≥1 mM) (13). Seil *et al*. in 2012 have reported that the small-diameter ZnO nanoparticles exhibit strong antibacterial properties against *S. aureus* that can also be additionally enhanced in the presence of ultrasound. Ahmad A. Tayel *et al*. (2011) have studied ZnO antibacterial activity on foodborne bacteria. Their results indicate that the exposure of *Salmonella typhimurium* and *Staphylococcus aureus* to their relevant minimal inhibitory concentrations of the ZnO nanoparticle

Table 2. Sensitivity of *Y. intermedia* in synergy with ZnO nanoparticles

Antibiotic	mg	Zone Diameter (mm)
Nalidixic acid	30	35
Tetracycline	30	34
Cefotaxime	30	36
Ceftazidime	10	25
Chloramphenicol	25	25
Ampicillin	10	0
Amoxycilin	30	0
Oxacilin	30	16

reduced the cell number to zero within 8 and 4 h, respectively.

Researches have stated that the dominant nanoparticles surface charge is a function of the pH in the culture medium. In a case when pH of the culture medium is lower than nanoparticles' pH, nanoparticles surface area will have positive charge. In addition, in a case when the nanoparticles' surface pH is higher than medium, nanoparticles surface area will have negative charge. Demetri (26) has theoretically calculated the pH of the many syntheses such as ZnO nanoparticles. According to his studies, pH of ZnO nanoparticles is 10. The studies for determining pH of the bacteria indicate that pH range for growth of microorganisms is between 2 and 4 (27). Gram-negative bacteria have a layer of lipopolysaccharide outside of their cell wall, under this cell wall there is a thin layer (7-8 nm) of peptidoglycan. Lipopolysaccharides are not as hard as peptidoglycans, because, there is an equipotential relation between lipid and polysaccharide. Lipopolysaccharides have negative charge, so they are attracted by nanoparticles with positive charge. Then as stated above, it can be concluded that the bacterial surface charge reduces as a result of repulsive forces (27). Hence, in order to overcome this inhibiting force, there must be a higher concentration of the nanoparticles in the culture medium, and perhaps a reason for the less sensitivity of the bacteria in the lower concentrations might be due electrostatic repulsive force.

Additionally, investigators have also suggested that nanoparticles may specifically become bound to the cell membrane outer space, and likely, they penetrate into the cells at higher concentrations (28).

6. Conclusions

According to the results presented in this study it could be concluded that ZnO nanoparticles at different concentrations show a growth inhibitory effect on bacteria causing clinical infections, and at concentrations higher than MIC, can kill bacteria effectively.

ZnO nanoparticle suspensions has inhibiting effects on the bacterial activity kinetics. In addition to the inhibitory effects, such nanoparticles, along with antibiotics could be a right choice for treating the bacteria causing clinical infections. However, this issue demands further studies in order to obtain a conclusive understanding of the nanoparticle action.

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