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Synthesis, Characterization and Antimicrobial Activity of Zinc Oxide Nanoparticles against Drug-Resistant *Escherichia coli* Isolated from Potable Water

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ABSTRACT

Nanoparticles have outstanding properties due to their small size. Their high surface-to-volume ratio facilitate them a better interaction with biomolecules. Metal oxide nanomaterials like CuO, Ag and ZnO have been in frequent use for several purposes. Their inherent ability and surface chemistry make them suitable for the antimicrobial agent against pathogens. In this regard, the present study focuses on the synthesis and antibacterial activity of ZnO-NPs against Enterotoxigenic *Escherichia coli* (ETEC). Synthesis of ZnO-NPs was achieved by chemical method followed by capping of citrate. This allowed the particles to be separate and monodispersed. After characterization, the synthesized ZnO-NPs were tested as antibacterial agent against ETEC. The time dependent growth inhibition was also performed. The synthesized ZnO-NPs were highly effective and showed bactericidal activity after 16 hours. The present study offers ZnO-NPs as a potential antimicrobial agent which can be used against ETEC.

KEY WORDS: ZnO-NPs, ETEC and Antibacterial activity.

1. INTRODUCTION

Prevalence of ETEC in potable water is one of the alarming situations for both rural and urban world. This leads to diarrhea and causes mortality and morbidity in children (below age five). Globally, Over 6.5 million children died under age of five (Mattioli, 2014). Basically, *Escherichia coli* reside in the intestines of human and other animals too and is transmitted by food or water (Walker, 2013). Most types of *E. coli* causes no harm but some of them are pathogenic and can cause disease. ETEC secretes heat-labile (LT) and heat-stable toxin (ST) toxins which allow them to rouse the intestines for further secretion of fluid, followed by abdominal cramping and watery diarrhea. Majority of ETEC strains exhibit antibiotic resistance (Agarwal, 2014; Tomar, 2015). Antimicrobial agents play crucial role in the control of these infectious diseases and spread of these pathogens. The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have stated on the serious intimidation due to antimicrobial-resistance in pathogenic bacteria (Wang, 2004). The exhaustive use of antimicrobial agents has generated the selective pressure to encourage the gradually increasing rates for antimicrobial resistance (Levy, 2004). Hence there is no single antimicrobial agent available for human and animal use that has not demonstrated resistance against microorganisms (Raffi, 2010). There has been much recent interest in using silver, zinc and copper nanoparticles in new technologies with respect to their improved properties such as high and reactive surface area of nano size materials (Lee, 2013). Larger the surface area ensures an increased range of interaction with bio-organic material present on the viable cell surface. These synthesized nanomaterials are designed and engineered for incorporation into consumer products (Diaz, 2012; Kahrilas, 2014). The antimicrobial activities of synthesized nanoparticles for example CuO, ZnO, TiO_2, SiO_2 etc. and their selective bactericidal effect suggests their potential application in various fields such as diagnostic, therapeutic and nano medicine based antimicrobial agent (Auffan, 2009). Zinc and Copper slowly release their cation in small amount which are toxic to bacteria (Rizzello, 2014). This leads to necessity for the development of potential new alternative materials in order to combat this problem (Karvani, 2011; Wakshlak, 2015). The present study aims on the synthesis and antimicrobial activity of ZnO-NPs against ETEC isolated from potable water of Gwalior.

2. MATERIALS & METHODS

Sample collection: Water samples (2L) were collected for the isolation of Enterotoxigenic *Escherichia coli* from six different sites of Gwalior city (Agarwal, 2015). All samples were collected and brought to laboratory for further processing on the same day. Standard and modified protocols were followed to collect, concentrate and clean-up the sample.

Isolation and detection of ETEC: Water samples collected from each site were concentrated using centrifugation followed by filtration (cellulose nitrate filter of 0.22 µm pore size; Millipore, USA). Membrane filters, immobilized with ETEC and other microflora were placed on culture media containing EMB (Hi-media) under aseptic conditions and incubated for 37 ± 1°C for 18-24 h. Colonies with greenish metallic sheen (presumptive ETEC) were selected and streaked onto EMB plates and incubated overnight at 37 ± 1°C. The confirmed isolates were preserved at -70°C glycerol stocks.

Molecular Characterization of ETEC: Isolates of *E. coli* were incubated at 37^0C overnight. One ml grown culture was centrifuged at 6500 x g for 2 min. Supernatant was removed and the pellet was resuspended in 200 µl of sterile nuclease-free double distilled water. Suspended cells were lysed by boiling them for 30 min followed by centrifugation (7000 rpm, 2 min). Approx 250 µl of the supernatant, containing the DNA was carefully collected and precipitated using sodium acetate (0.3 M, pH 5.2) and ethanol. The precipitated DNA was washed twice by 70% ethanol and resuspended in 100 µl TE (pH 8.0). All isolated DNA samples were visualized on agarose gel electrophoresis. Well characterised DNA of isolated strains were amplified for detection using the signature virulent gene *LT-1* (heat labile enterotoxin). PCR primers were adopted from published work (Ram, 2008). *E. coli* MTCC 723 were used as positive control for *LT1* gene. Amplicons were analyzed on 1.8% agarose gel, visualized and recorded.

Antibiotics susceptibility test: ETEC isolates were subjected to the antibiotic-susceptibility testing. Briefly, individual isolates were spread on Muller-Hinton agar plates followed by placement of antibiotic discs on them as per CLSI guidelines.

Synthesis of Zinc nanoparticles: Zinc acetate dihydrate (Zn $(CH_3COO)_2 \cdot 2H_2O$) (Fisher Scientific 99.0%); Sodium Hydroxide (NaOH) (Fisher Scientific,99.0%); Methanol (Fisher Scientific, 99.0%); Acetone, (Fisher Scientific, 99.0%); Isopropyl alcohol (Fisher Scientific, 99.0%) were used for the synthesis. ZnO-NPs were prepared by co-precipitation method. Briefly, 0.02 M aqueous solution of zinc acetate dihydrate was put into 50 ml of HPLC Grade de-ionized water under vigorous stirring. Sodium Hydroxide aqueous solution (2.0 M) was mixed in the above aqueous solution. This resulted in the change in colour from colorless to white. The colour change was observed. The precipitate was then taken out and washed again and again with HPLC Grade de-ionized water followed by ethanol and acetone to remove the impurities.

Characterization of Synthesized Nanoparticles:

a) UV-Visible Spectroscopy: UV-Visible spectra was taken in an optical quality quartz cuvette with a 1 cm path length, require 2.5 ml of solution to fill past the light path of the instrument. Spectra were seen at room temperature, while Zinc Acetate solution was used as a blank. Solution was diluted immediately before analysis. Spectrum was seen from range 200-600 nm.

b) Transmission Electron Microscopy: ZnO-Nps samples were concentrated via centrifugation prior to use for it. Transmission electron micrographs were obtained.

Antimicrobial Assays: The antimicrobial activity of ZnO-NPs was tested against ETEC (Environmental isolates) and reference strain of ETEC (MTCC 723). The assays were set by preparing the ZnO-NPs at different concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL, which were suspended in Nutrient Broth. Each concentration of NPs was added to 10^8 CFU/mL of bacterial suspension and incubated at 37^0C for 18 h and 150 rpm. During the incubation the growth of bacteria was monitored by measuring the optical density (OD) at 600 nm at regular interval of two hours. Further the bacterial growth was determined by on plating the treated culture and control upon EMB agar.

3. RESULTS AND DISCUSSION

- Occurrence of virulence determinants in ETEC isolates: Samples collected from six different sites, three sites were positive for the ETEC. The 150 bp amplicon were observed in these samples on gel electrophoresis after PCR. Results showed the 50 % occurrence of ETEC strains isolated from the potable water exhibiting virulent gene. Remaining isolates were negative for the presence of *LT1* gene. Our interpretation indicated that the drinking water is contaminated by ETEC exhibiting virulent *LT1* gene. Contaminated water and poor hygiene are the reasons for diarrheal diseases throughout the world. In present study, we found potable water samples contaminated with strains of ETEC. The presence of ETEC in potable water suggests the possibility of contamination of water supplies. Water channels flowing in the old city and other areas are often leaky and unmanaged. As a result they are often in contact with the sewage pipelines. Most of the strains were in culturable state, which shows the presence of high organic matter in potable water. Still some of the strains may have undergone in viable but not culturable (VBNC) state which could not be cultured easily (Jyoti, 2015; Tomar, 2016)
- Determination of antimicrobials susceptibility: ETEC isolated from contamination point were screened for susceptibility for existing antimicrobials by disc diffusion method as described by Clinical and Laboratory Standards Institute. All of the potential ETEC isolates in the present study were resistant to at least one of the antimicrobial. We found that some of isolates were resistant to more than two antimicrobials. Out of the isolates we recovered, some exhibited resistance to the β-lactam class of antimicrobials. It was found that site 3 showed high resistance pattern (45.87%) as compared to site 1 (30.22%) and site 2 (28.09%). Site 1 has high intermediate pattern (47.12%) as compared to other sites.
- Synthesis and Characterization of Zinc nanoparticles: Synthesis of ZnO-NPs was achieved by reducing an aqueous solution of zinc ions with an *in-situ* capping of metallic nanoparticles with citrate ions. This allowed to control the particle size and the stability was also enhanced. The ZnO nanoparticles were synthesized and capped by

using citrate. Citrate is a good stabilizing agent. The ZnO-NPs exhibit the Surface Plasmon Resonance (SPR) related spectra in the UV-visible range at 330 nm. The size and shape of the synthesized Zn-NPs were determined by transmission electron microscopy (TEM) (Figure.1). TEM image of Zn-NPs synthesized by using Chemical means is predominates with spherical oval, morphologies ranging from 25 to 30 nm (approx). The nanoparticles were mono dispersed and no aggregates were found, indicating stabilization of the nanoparticles by capping agent.

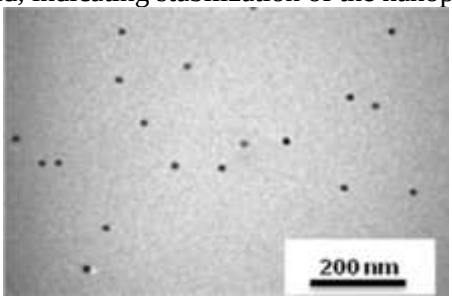

Figure.1. Transmission Electron Micrograph of ZnO-NPs

- Antimicrobial Assay: The antimicrobial activity of Zn-NPs against ETEC environmental strains was performed. It evident that there was an increase in the inhibitory activity as we gradually increases the concentration of nanoparticles. Growth inhibition of ETEC was examined both on EMB agar plates and also in Nutrient broth containing a range of concentration (0, as a control, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) of Zn-NPs. ETEC at stationary phase were inoculated into media (Nutrient Broth) 10^8 CFU/ml. The growth of bacteria was evaluated by measuring the optical density (OD) at 600 nm at different time intervals. It was found that at growth of bacteria was inhibited at concentration 0.5 mg/ml as it reaches time 2 h. On the plate spread with 10^8 ETEC CFU/Plates and in broth inoculated in equivalent number of cells, bacterial growth was inhibited at 0.5 mg/ml of ZnO-NPs. ZnO-NPs has a modest effect on cell growth, which result in the recovery of few viable cells (Figure.2). The ZnO-NPs was found to exhibit the lesser antibacterial property in Nutrient broth which might be due to either prevention of interaction of zinc nanoparticle via component of Nutrient broth.

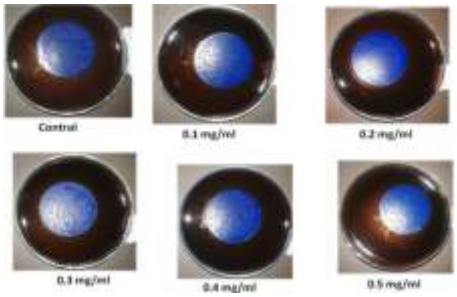

Figure.2. Growth inhibitory effects of ZnO-NPs on ETEC

Table.1. Primer sequence of *LT1* gene of ETEC

Gene	Primer Sequence (5'- 3')	Tm (^{0}C)	Amplicon
LT1	GGCAGGCAAAAGAGAAATGG	54.5	150 bp
	TTGGTCTCGGTCAGATATGTG	54.4	

4. CONCLUSION

The presence of pathogenic strains of ETEC in Gwalior city is alarming which may be the major roadblocks for the management of waterborne outbreaks. Therefore, the presence of ETEC in potable water of Gwalior city requires increased examination for risk assessment and prevention strategies for the protection of public health. Zn-NP was synthesized by chemical reduction methods with citrate as capping agent. In view of these, the Zn-NPs show, in fact, enhanced antibacterial properties, and their synthesis procedures are quite easy and cheeper. We can visualize that this study offers novel insights into antimicrobial actions of Zn-NPs and also demonstrates Zn-NPs as a novel class of contemporary antimicrobial agent for the treatment of water borne infectious diseases.

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