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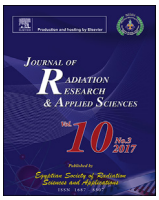

# Control of imipenem resistant-*Klebsiella pneumoniae* pulmonary infection by oral treatment using a combination of mycosynthesized Ag-nanoparticles and imipenem

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ABSTRACT

*Klebsiella pneumoniae* (Kp) a common cause of pneumonia leads to intense lung injury and mortality that are correlated with infective exacerbations. A significant increase in the prevalence of imipenem resistant *K. pneumoniae* (IRKP) has been observed in common human pathogens. The rapid emergence of IRKP has limited the availability of anti-bacterial treatment options. Silver nano-particles (AgNPs) are one of the well-known antibacterial substances showing such multimode antibacterial action. Therefore, AgNPs are suitable candidates for use in combinations with imipenem in order to improve its antibacterial action. Fifteen fungal species were screened for mycosynthesis of silver nanoparticles (AgNPs), only eight fungal species were found to reduce the silver salt into silver nanoparticles which was characterized by UV−visible spectrophotometric analysis, Energy Dispersive Analysis of X-ray (EDX) and transmission electron microscopy. Consistent with characterized silver nanoparticles, mycosynthesized AgNPs by *Verticillium albo-atrum* (RCMB 039001) was found the higher in concentration (as detected by UV−visible spectrophotometric analysis) and the least in size (as detected by TEM analysis) so, it was chosen for further studies such as *in vitro* antibacterial activity against Imipenem resistant *K. pneumonia* (IRKP), MIC, FIC measurements and in *vivo* study. In this work a strong synergistic antibacterial effect between AgNPs and imipenem was detected *in vitro* (with FIC Index 0.07) and *in vivo* against IRKP strain. These results suggested that sliver nanoparticles have an effective antibacterial action on bacterial count, histopathology as well as protective immune response in an IRKP rat model.


## 1. Introduction

*Klebsiella pneumoniae* (Kp) is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium of worldwide importance causing pneumonia associated with high morbidity and mortality (Armstrong, Conn, & Pinner, 1999; Mizgerd, 2006). Pneumonia caused by Kp is characterized by an exacerbated inflammatory response, associated with excessive neutrophil and macrophage infiltration, high production of pro-inflammatory cytokines and severe lung injury (Soares et al., 2006; Zhang, Summer, Bagby, & Nelson, 2000). Although local inflammation is beneficial following pathogen infection by preventing pathogen dissemination, non-resolving hyper-inflammation is accompanied by mortality and chronic inflammatory disorders (Medzhitov, 2008). Kp infection is being recognized as a major health threat due to the increasing antibiotic-resistance therefore limiting efficient therapies. Alternatively, strategies to reprogram lung defences and improve immune response to clear bacteria could be effective against pulmonary Kp infection. In this context, probiotics have emerged as a strong potential candidate. (Reid, Jass, Sebulsky, & McCormick, 2003).

*Klebsiella pneumoniae* is one of the pathogens responsible for the majority of hospital infections in the United States (Boucher et al., 2009). Resistance of *K. pneumoniae* to carbapenem antibiotics has spread to all regions of the world and in some countries carbapenem resistance is present in more than half of the patients treated for *K. pneumoniae* infections (WHO, 2014). *K. pneumoniae* is also emerging as an agent of severe community-acquired infection including bacteraemia pneumonia (Lin, Jeng, Chen, & Fung, 2010).

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Imipenem is recommended as a first-line therapy for multi-drug resistant Gram-negative bacteria; however, resistance to imipenem has emerged (Ardanuy et al., 1998; Cao et al., 2000; Gülmez et al., 2008; Normann, Cuzon, & Naas, 2009). The mechanism of imipenem resistance involves the production of specific carbapenemases as well as the loss of porin proteins (Doménech-Snchez, Hernndez-Allés, Martnez-Martnez, Bened, & Albert, 1999; Hesna et al., 2002; Yang, Guo, & Zhang, 2009). Imipenem resistance caused by the alteration in lipopolysaccharide (LPS) levels has also been reported in *Enterobacter aerogenes* (Bornet, Davin-Regli, Bosi, Pages, & Bollet, 2000; Leying, Cullmann, & Dick, 1991).

In recent years, the application of bio nanotechnology has been investigated as an alternative to chemical and physical ones. Research in bio-nanotechnology has shown to provide reliable, eco-friendly processes for synthesis of noble nanomaterials. Biological synthesis of nanoparticles using various biological systems such as yeast, bacteria, fungi, algae and plant extract have been reported (Yen & Mashitah, 2012).

A large number of fungal strains are capable to synthesize silver nanoparticles (AgNPs) extracellularly, among which *Fusarium oxysporum* (Ahmad et al., 2003), *Aspergillus fumigatus* (Bhainsa & D'Souza, 2006), *Aspergillus niger* (Gade et al., 2008), *Fusarium semitectum* (Basavaraja, Balaji, Lagashetty, Rajasab, & Venkataraman, 2008), *Penicillium brevicompactum* (Shaligram et al., 2009), *Cladosporium cladosporioides* (Balaji et al., 2009), and *Aspergillus clavatus* (Verma, Kharwar, & Gange, 2010) have been previously described.

Recently, several studies have indicated that AgNPs may strengthen the antibacterial effects of antibiotics against both susceptible and resistant bacteria, either additively or synergistically. The additive effect was shown in antibiotics of different mode of action against various bacterial strains (Birla et al., 2009; Fayaz et al., 2010; Ghosh et al., 2012; Naqvi et al., 2013; Muhsin & Hachim, 2014).

In the present study, we attempted to evaluate antibacterial activity of characterized mycosynthesized AgNPs singly and in combination with imipenem against imipenem resistant -*Klebsiella pneumonia*.

## 2. Materials and methods

### 2.1. Fungal species

*Alternaria tenuissima* (RCMB009006), *Aspergillus clavatus* (RCMB0020161), *Aspergillus oryzae* (RCMB002015), *Cladosporium herbarum* (RCMB027002), *Emericella nidulans* (RCMB004002), *Eurotium repens* (RCMB003003), *Fusarium equiseti* (RCMB008004), *Fusarium oxysporum* (RCMB008006), *Kluyveromyces marxianus* (RCMB007004), *Penicillium echinulatum* (RCMB0010042), *Penicillium hordei* (RCMB0110056), *Rhizoctonia solani* (RCMB031004), *Rhizopus microsporus* (RCMB014004), *Trichoderma longibrachiatum* (RCMB017007) and *Verticillium albo-atrum* (RCMB039001) were kindly provided from the Regional Centre for Mycology and Biotechnology.

### 2.2. Fungal biomass preparation

All fungi under investigation were grown on malt extract broth at 28 °C on a rotary shaker (120 rpm) for 96 h. The biomasses were harvested by filtration using Whatman filter paper No. 1, followed by washing with distilled water to remove any components of the medium. The biomass (25 gm) wet weight was placed in individual flasks containing 100 ml of deionized water and incubated as described above for 24 h. The biomass was filtered, and the cell filtrate was collected and used for biosynthesis of AgNPs according to Kathiresan, Manivannan, and Nabeel (2009).

### 2.3. Biosynthesis of AgNPs

For biosynthesis of AgNPs, 50-ml of cell filtrate was mixed with 10-ml $AgNO_3$ solution (1 mM) and reaction mixture without $AgNO_3$ was used as control. The prepared solutions were incubated at 28 °C for 72 h. All solutions were kept in dark to avoid any photochemical reactions during the experiment. The AgNPs were collected by centrifugation at 10,000 rpm for 10 min twice, and collected for further characterization according to Kathiresan et al. (2009).

### 2.4. Characterization of AgNPs

After 24 h of synthesis, Repeated rinses were performed to remove impurities by centrifugation at 14,000 rpm for 30 min at room temperature. The pellet of AgNPs was re-suspended in 1 ml sterile water. The production of AgNPs in aqueous solution was monitored at the Regional Centre for Mycology and Biotechnology (RCMB) using.

i. **UV–visible Spectroscopy Analysis:** Change in colour of the cell free filtrate incubated with silver nitrate solution was visually observed over a period of time. Absorption measurements were carried out using UV–visible spectrophotometer (Milton-Roy Spectronic 1201). UV–Visible analysis of several weeks old samples was also carried out to check the stability of synthesized AgNPs.
ii. **Transmission Electron Microscopy (TEM):** For TEM analysis, a drop of the solution was placed on the carbon coated copper grids and dried by allowing water to evaporate at room temperature. Electron micrographs were obtained using GEOL GEM-1010 transmission electron microscope at 70 kV.
iii. **Energy Dispersive Analysis of X-ray (EDX):** The presence of elemental silver was confirmed through EDX. The EDX microanalysis was carried out by X-ray micro-analyzer (Oxford 6587 INCA) attached to JEOL JSM-5500 LV scanning electron microscope at 20 kV. The EDX spectrum recorded in the spot profile mode from one of the densely populated silver nanoparticles region on the surface of the film. The nano crystallites were analyzed using Quanta 200 FEG.

### 2.5. Bacterial strains

Imipenem resistant *K. pneumoniae* clinical strain was kindly provided from culture and collection unit of the Regional Centre for Mycology and Biotechnology with code (RCMB 5621).

### 2.6. Antibiotic

Imipenem was purchased from SIGMA. Imipenem was dissolved in 10 mM phosphate buffer (pH 7.0).

### 2.7. Determination of minimum inhibitory concentration (MIC) and fractional inhibitory concentrations (FIC) of imipenem and Ag-nanoparticles

The MICs of imipenem, AgNPs and combined imipenem–AgNPs for imipenem *resistant K. pneumoniae* RCMB5621 determined by the agar dilution method using MH agar. MIC was defined as the lowest drug concentration that inhibited bacterial growth. To measure the synergistic effect of AgNPs and imipenem against IRKP

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