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Original Research Article

Deterrence in metabolic and biofilms forming activity of *Candida* species by mycogenic silver nanoparticles

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ABSTRACT

Candida, a commensal and opportunistic fungal pathogen has been typically known for its biofilm forming ability and device-associated hospital acquired infections in human. The study aimed at exploring the *in-vitro* anti-biofilm and anti-metabolic activity of AgNPs against *C. albicans* ($n=2$), *C. tropicalis* ($n=2$) and *C. parapsilosis* ($n=2$) isolated from urine samples. Broth dilution method revealed greater than 50% inhibition at 100 ppm against Ag NPs in 24 h. An overall reduction of 55–86% in biomass (crystal violet staining assay) and 20–73% in metabolic activity (XTT assay) was observed in 24 h old biofilms. However, *C. albicans* proved to be more susceptible to AgNPs compared to *C. tropicalis* and *C. parapsilosis*. Scanning Electron Microscopy revealed patchy growth and deterrence in biofilm biomass when Ag NPs were coated on urinary catheter. Furthermore, viable cell counts of *Candida* were significantly reduced on AgNPs coated catheter compared to control.

Introduction

An about 60–80% of human infections occur as a consequence of biofilms formation by pathogenic microorganisms (Harriott et al., 2010; Seneviratne et al., 2008). *C. albicans* belongs to the group of commensal fungi that are generally present asymptomatically on skin, oral cavity, vaginal and gastrointestinal tracts (Brown et al., 2012). Strains of *Candida* become pathogen due to evolving resistance to antimicrobials and changing micro-environmental conditions. Pathogenic *Candida* is associated with mucosal to invasive systemic life threatening infections such as *Candida* septicemia (Nobile and Johnson, 2015). *Candida* spp. are characterized based on their virulence factors with diverse attributes. The most prominent virulence factors are the cell surface adhesins and invasions. Additionally, the phenotypic switching, biofilm forming ability along with production of different hydrolytic enzymes are involved in candidiasis (Deepa et al., 2015). The highly structured biofilms of *Candida* spp. are composed of multiple cell types enclosed in exo-polymeric substances. Attachment and colonization to the biotic surfaces such as mucosal linings as well as the abiotic medical implants provide the basis for the development of complex multicellular biofilm (Uppuluri et al., 2010).

Infections caused by biofilm forming *Candida* spp. are difficult to treat due to greater (up to 1000 fold) resistance to drugs compared to planktonic cells (Mah and O'Toole, 2001). Most of the proteins identified in the *Candida* biofilm matrix are suggested to be the hydrolyzing enzymes (Zarnowski et al., 2014). Among these, extracellularly secreted enzymes, aspartyl proteinases are of vital importance. S-aspartyl proteases activity has been directly related to the number of SAP genes in pathogenic species of *Candida* (Staniszewska et al., 2012). The enzyme not only involves in acquisition of essential nitrogen for growth, but it also provides means for enhanced fungal colonization, and penetration through impaired host barriers. Besides, the fungal proteinases may help evading host defenses through direct degradation of host molecules associated with an intracellular lysosomal enzyme and activation of complement system (Naglik et al., 2003).

Candida biofilms have been primarily studied on abiotic surfaces of medical devices such as stents, shunts, oral dentures and implants, pacemakers, endotracheal tubes and other indwelling catheters (Harriott et al., 2010; Seneviratne et al., 2008). Recent reports have even indicated catheter associated biofilm infections that are >50% (approx.) due to different bacterial and fungi strains (Nobile and Johnson, 2015; Mermel et al., 2009). Current remedial measures including different anti-fungal physical and chemicals

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methods proved to be having limited efficacy due to developing resistance among species of *Candida*. Besides, use of high dose of antifungal agents proved to be damaging vital human organs like kidney, liver etc. Medical implants associated with *Candida* infections have been reported to cause mortality rates of up to 30%. Moreover, the biofilm associated with medical devices not only complicate the treatment and removal but it also make them costly and hazardous (Nobile and Johnson, 2015; Andes et al., 2012; Peres-Bota et al., 2004). Thus, for the deterrence of *Candida* biofilm infections, it is essentially important to device cheap and sustainable procedures and practices.

Recent studies have investigated different physical or chemical methods to control *Candida* biofilms. In this context, the role of silver nanoparticles (AgNPs) has been gaining much importance due to their vital role in controlling bacterial and fungal infestation (Lateef et al., 2016; Li et al., 2014; Monteiro et al., 2011; Kim et al., 2009 and Kim et al., 2008). Still, these studies were mostly conducted at initial levels for biofilm deterrence during different stages of biofilm growth (Monteiro et al., 2015; Li et al., 2014). In the recent past, the AgNPs anti-biofilm effect was determined but limited data is available regarding the mechanism of action thus needs further evaluation. A detailed mechanistic approach has to be elucidated related to the anti-biofilm properties of AgNPs against test fungal strains. Previously, antifungal action by AgNPs was proposed to be linked with cell membrane disruption (Lara et al., 2015 and Kim et al., 2009). However, details on the said process are vague and need further experimental evidences.

Cotemporary growing methods to control pathogenic microbes include the application of metal nanoparticles. Comparatively, the biological synthesized metal nanoparticles are gaining more importance over other physical and chemical due their low cost and eco-friendly nature (Lateef et al., 2016; Naqvi et al., 2014). Immense data has been generated that emphasized the importance of microbes and related bioprocess for development of nanoparticle. Among microbes, filamentous fungi have gained prime importance for metal nanoparticles synthesis because of their certain morphological and physiological features. They are easy to grow and manage thus favoring significant amount of extracellular enzymes production. Large fungal biomass production also neutralize the toxigenic effects of metals and biotransform and bio-accumulate them. The extracellular synthesis of metal nanoparticles by fungi has been reported efficient and require minimum of efforts during downstream processing (Naqvi et al., 2014; Zhang et al., 2011; Das and Thiagarajan, 2012 and Rai et al., 2009).

The research work investigated the biofilm deterring ability of biologically synthesized AgNPs against different pathogenic species of *Candida* (*C. albicans*, *C. tropicalis* and *C. parapsilosis*). Similarly, anti-biofilm activity of AgNPs was studied when they were coated on urinary catheter surface.

Materials and methods

Fungal cultures and growth conditions

Clinical isolates ($n = 6$) of *C. albicans* (CA46, CA72), *C. tropicalis* (CP17, CP65) and *C. parapsilosis* (CT28, CT92) were provided by Medical Microbiology and Immunology lab. They were previously collected from different urine samples and used in the study. American Type Culture Collection (ATCC) culture of *C. albicans* (ATCC 24433) was used as a control for comparative biofilm studies. The isolates used in the study showed resistance against fluconazole, amphotericin B and voriconazole. The cultures were refreshed on Sabouraud dextrose agar medium (SDA, Oxoid) at 37 °C for 24–48 h and maintained at 4 °C during storage.

Mycogenic silver nanoparticles synthesis

Mycogenic AgNPs were provided by Microbiology Research Lab, synthesized previously using *Aspergillus flavus*. Most of the nanoparticles were found to be spherical in shape and size ranged between 3 and 80 nm. During nanoparticles synthesis, the color of reaction mixture was changed from light yellow to dark brown which indicated the surface plasmon excitation of AgNPs. Also, the UV–vis spectra of reaction mixture showed a strong surface plasmon response at 400–500 nm, with an increase in intensity with reaction time thus color change was easily investigated by UV-spectrophotometry (Naqvi et al., 2014).

Minimum inhibitory concentrations (MICs) of biogenic Ag NPs against *Candida* spp. biofilms

The Clinical and Laboratory Standards Institute (CLSI, 2013) broth microdilution method was used to determine the *in vitro* minimum inhibitory concentrations (MIC) of AgNPs against *C. albican, C. tropicalis* and *C. parapsilosis*. Different concentrations of AgNPs including 25, 50, 75 and 100 ppm were prepared in deionized water. After incubation, inhibition was evaluated based on lowest concentration causing 50% reduction in turbidity related to the control growth (Jebali et al., 2014).

***Candida* biofilm development and inhibition by Ag NPs treatment**

C. albicans, C. tropicalis and *C. parapsilosis* biofilms were developed in 96 well microtiter plates. Then, 200 μl of *Candida* cell suspension (1.0×10^7 cells/ml) was prepared in Sabouraud dextrose broth (SDB, Oxoid) and supplemented with 10% glucose. For the development of biofilms at intermediate stage, plates containing cell suspension were grown for 24 h at 37 °C and 120 rpm. After 10–15 h, the wells were replaced with fresh medium. After 24 h, wells were washed with 400 μl of PBS (pH 7.2-7.4) to remove non-adherent cells. In the next, step 200 μl solution of AgNPs was prepared in deionized water was added to wells along with 200 μl of fresh medium. The wells were incubated for 24 h after treatment with AgNPs under shaking at 120 rpm at 37 °C. Wells with cell suspension but without AgNPs were designated as growth controls and for sterility purpose wells with sterile media (without cell suspension and AgNPs) were used (Monteiro et al., 2015). The OD was determined at the wavelength of 492 nm using microtiter plate reader (Platos, R492).

$$\%\text{age inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

Effect of AgNPs on total biofilm biomass

Total biofilm biomass of *C. albicans, C. tropicalis* and *C. parapsilosis* were evaluated after treatment with AgNPs by biomass reduction assay using crystal violet (CV) for staining. The biofilms were developed using 1.0×10^7 cells/ml of cultures as inoculums. After biofilm development and treatment with AgNPs, medium was discarded. Subsequently wells were washed with 400 μl of PBS to remove any non-adherent cells. Fixation of adhered *Candida* cells were achieved by 200 μl of 95% ethanol incubated for 15 min. After 15 min ethanol was removed and wells containing different *Candida* spp. biofilms were dried at room temperature. Consequently 400 μl of 1% w/v CV was applied for 5 mins. Excess strain was removed by washing with deionized water. Crystal violet bound to adhered cells was solubilized by using 33% acetic acid. Afterwards the absorbance was measured using microtiter plate reader at wavelength of 492 nm. The test was performed in duplicate and independently for each sample under investigation (Dhanasekaran et al., 2014).

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