

## Research Article

### Studies on isolation of *Proteus mirabilis* from urinary catheter biofilm

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#### Abstract

#### Keywords

*Proteus mirabilis*,  
Congo red stain,  
TCP,  
Silver nanoparticles.

In the present study *P. mirabilis* was reported as a major biofilm forming dangerous bacterial cell that supplemented LB medium was sufficient to sustain cell growth. Biofilm formation was determined by Congo red agar method and tissue culture plate by using microplate reader (model 680, Bio-rad) at 595nm. The OD values were considered as an index of bacteria adhering to surface and forming biofilms. Biofilm formation is detected in many organisms synthesizing exopolysaccharides. *Proteus mirabilis* was grown to form biofilm for 24hrs in microtiter plate wells and then treated with varying concentrations of silver nanoparticles. Treatment for 2hrs with concentration of 50nM of silver nanoparticles resulted in a decrease of 95% and 98% of the biofilm formed and 10nM resulted in a 50% reduction in biofilm.

#### Introduction

Surface associated communities of bacteria (biofilm) are prevalent in environmental and clinical settings. Biofilm bacteria are less susceptible to antimicrobial agents and are protected from the host immune response, giving rise to chronic infections that are notoriously difficult to eradicate (Lewis, 1988). The extracellular polymeric substance (EPS) is thought to maintain the biofilm architecture and function as a matrix, or glue, holding biofilm cells together (Schwank, 1998).

A thorough understanding of the biofilm formed by a bacterial group and its colonisation predominant in majority of the catheters screened has to be studied in detail. Infections of the urinary tract with strains of *P. mirabilis* have a special predilection for the upper urinary tract where they cause much greater kidney damage (Burke, 1983). *P. mirabilis* produce urease which causes damage and death to the renal epithelium, inactivation of complement and conditions favouring the development of renal stones. *P. mirabilis* associated with urinary catheter also invade the blood stream and give rise to septicaemia (Burke, 1983). Hence *P. mirabilis* isolated from the urinary catheter was studied in detail.

#### Materials and Methods

##### Biofilm quantifications

*P. mirabilis* isolated from urinary tract catheter was stored in Luria-Bertani medium (LB medium). The catheter was revived one ml of overnight bacterial culture were grown in 10 ml of fresh liquid LB medium to reach a mid- log phase recognised by OD 570 value of 0.16 which is equivalent to a cell density of  $10^8$  CFU/ ml<sup>-1</sup>.

The biofilm formation assay was performed in microtiter plates using a modification of previously reported protocols (O'Toole *et al.*, 2000). Samples of *Proteus mirabilis* were prepared in 10-fold diluted LB medium ( $10^8$  CFU/ml<sup>-1</sup>). The experiments showed that the supplemented LB medium was sufficient to sustain cell growth. A 200µl aliquot of each sample was placed in each well of the 96 well plate in 6-8 replicates, and the plates were incubated at 37°C without shaking for different times (12-160 hrs). After a predefined incubation time, the supernatant was gently removed and the wells were rinsed with phosphate buffer (PBS), leaving only adherent bacteria in the wells. For fixation of the biofilm, 99% ethanol was added and the plates were allowed to air dry.

Then, the plates were stained and incubated at room temperature with 0.4% (v/v) Crystal violet solution.

The unbound Crystal violet was removed by rinsing the plates under running tap water. Crystal violet stained biofilm was extracted with 1% (v/v) SDS and incubated at room temperature for 15min to allow all of the biofilm-associated dye to re elute. Finally, 100µl from each well was transferred to a new polystyrene plate, and absorbance was determined with a plate reader at 595nm. Baseline biofilm formation (termed relative biomass) was defined as that for the untreated sample that contained  $10^8$  CFUml<sup>-1</sup> of *Proteus mirabilis* (without silver nanoparticles) and was set as 100% the relative percentage of biomass. All samples were incubated together in the same plate and under the same conditions.

#### Determination of biofilm formation by Congo red agar method (CRA)

The determination of biofilm formation was carried out by the method described by Freeman *et al.*, (1989). This method utilized a specially prepared solid medium brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red for screening the formation of biofilm by *Proteus mirabilis*. The medium composes of BHI (37 g/L), sucrose (50 g/L), agar No.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared in the form of concentrated aqueous solution and it was autoclaved at 121°C for 15min, separately from other medium constituents. Following autoclave, the concentrated solution was added to agar which was previously cooled to 55°C. Plates were inoculated and incubated aerobically for 24-48 hrs at 37°C.

#### Tissue culture plate method (TCP) – *in vitro* biofilm formation assay

To determine the efficacy of silver nanoparticles in elimination of formed biofilm, TCP method was carried out with suitable modifications (Christensen *et al.*, 1985). Individual wells of sterile, polystyrene, 96 well flat bottom tissue culture plates were filled with 180µl of BHI broth and inoculated with 10µl of overnight culture. To the mixture 10µl of silver nanoparticles were added from the stock so that final concentration was made between 10nM and 100nM. The tissue culture plates were incubated for 24hrs at 37°C. After incubation, content of each well was gently removed. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS pH 7.2) to remove free floating 'Planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by thorough washing with deionised water and plates were kept for drying. After drying, 95% ethanol was added to the wells and the optical densities (OD) of stained adherent bacteria were determined with a microplate reader (model 680, Bio-rad) at 595nm (OD595nm). These OD values were considered as an index of bacteria adhering to surface

and forming biofilms. Experiment was performed in triplicate, the data was then averaged and the standard deviation was calculated.

## Results and Discussion

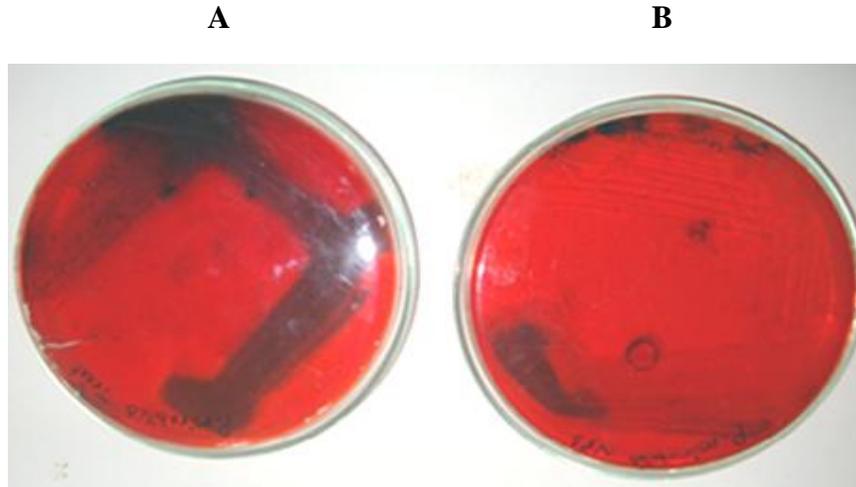
Biofilm formation is detected in many organisms synthesizing exopolysaccharides. The biofilm is made up of microorganisms adhering to the surface coated with slime the exopolysaccharide matrix which protects the microbes from the unfavourable environmental factors (Christensen *et al.*, 1985). Biofilm formation by *Proteus mirabilis* were tested by growing the organism in Brain heart infusion agar supplemented with Congo red (BHIC) with and without silver nanoparticles. When the colonies were grown without silver nanoparticles in the medium, the organisms appeared as dry crystalline black colonies, indicating the production of exopolysaccharides, which is the prerequisite for the formation of biofilm (Fig 1 A, B). Whereas when the organisms were grown on BHIC with silver nanoparticles, the organisms did not survive. During the treatment with reduced concentrations of silver nanoparticles (10nM), the organisms continued to grow, but silver nanoparticles treatment has inhibited the synthesis of exopolysaccharides, indicated by the absence of dry crystalline black colonies. The presence of nanoparticles at a certain level inhibited bacterial growth by more than 90%. When the exopolysaccharide synthesis is arrested, the organism cannot form biofilm. Therefore, 50nM of silver nanoparticles significantly arrested biofilm formation without affecting viability, whereas 100nM inhibited the growth of the organism itself.

The microbial biofilm formation of *Proteus mirabilis* was investigated *in vitro* by monitoring the binding of the dye crystal violet to adherent cells which directly revealed their effective ability in formation of biofilm (Judith *et al.*, 2005).

*Proteus mirabilis* was grown to form biofilm for 24hrs in microtiter plate wells and then treated with varying concentrations of silver nanoparticles. Treatment for 2hrs with concentration of 50nM of silver nanoparticles resulted in a decrease of 95% and 98% of the biofilm formed and 10nM resulted in a 50% reduction in biofilm. These data demonstrate that silver nanoparticles induced detachment of *Proteus mirabilis*.

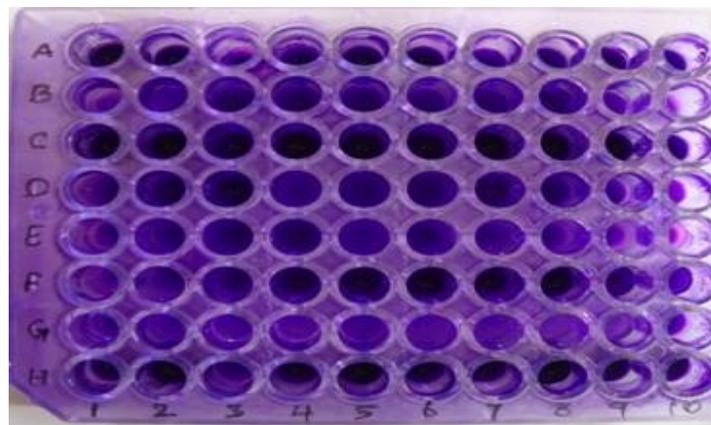
Therefore the ability of the nanoparticles to inhibit the formation of the biofilms and formed biofilms were checked against *Proteus mirabilis*. The exopolysaccharides synthesized by the bacteria not only protects the bacteria from the host defence mechanism but also mediates the adhesion of the organism between the lens and the corneal epithelium (Stepanovic *et al.*, 2007). Moreover the ability of the organisms to form biofilm was confirmed by the formation of dry crystalline colonies on BHIC agar plates. Here the ability of the silver nanoparticles to inhibit the growth of the organisms under consideration was tested by well diffusion

Figure 1: Detection of biofilm production by Congo red Agar medium



A- Colonies of biofilm on Congo red Agar medium by *Proteus mirabilis* and  
B- Silver nanoparticles inhibited colonies of *Proteus mirabilis*

Figure 2: Quantitative assay for biofilm formation on microtitre plate,



Rows A, C, F - Clinical isolates of *P. mirabilis* isolates tested for biofilm,  
Row E - Negative Control, Row G - Positive control *P. mirabilis*

method of antibiotic assay. The organism's growth was effectively impeded by the silver nanoparticles at the concentration of 100nm. The concentration of silver nanoparticles was lesser than the previous reports for the toxic concentration of the silver nanoparticles (Kalishwaralal *et al.*, 2009). The recurrence of biofilms in spite of treatment with various anti-microbial agents was attributed to the impedance created by the biofilm matrix (Mohammed and Fattani, 2006). Although water channels are present in the biofilms, the deep lying organisms escape the treatment as the matrix hinders the diffusion of the drug. Therefore, inhibition of biofilm formation is very much essential in the case of prevention of microbial invasion on catheters and various other disorders, as the exopolysaccharide slime formed reduces the susceptibility

of the organism to the administered drug. Antibiotic treatments effectively kill the bacteria which remain individual, but the efficiency is very much reduced when the organism forms slime. This makes the organisms to revert the disease when the antibiotic treatment is finished because of the healing of symptoms. This is the reason behind the biofilm infections showing recurring symptoms even after cycles of antibiotic therapy until the mass is surgically removed. Therefore in this report we investigated whether silver nanoparticles has anti-microbial activity and also anti-biofilm function by using BHIC. Here, silver nanoparticles not only inhibited the growth but also inhibited the ability of the organism to synthesize the exopolysaccharide.

This shows that silver nanoparticles have the ability to block the exopolysaccharide synthesis of the organism otherwise the biofilm. Another method was found to be useful in determining the ability of the moiety to disrupt the biofilm formation is the crystal violet method on TCP. This assay is most widely used and was considered as standard test for detection of biofilm formation (Christensen *et al.*, 1985). The ability of the organism to form biofilm and the effect of various concentrations of silver nanoparticles in inhibiting the formation was checked on a 96 well plate, where an increase of silver nanoparticles concentration negatively regulated the biofilm formation (Fig 2).

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