

Original Research Article

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## Rabbit Serum Raised against *P. aeruginosa* Biofilm Exhibit Significant Biofilm Reduction and Removal Activity

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### ABSTRACT

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*P. aeruginosa* biofilms can offer resistance to antibiotics, and have evolved mechanisms to evade innate and acquired immune systems. Biofilms infection causes stimulation of innate and acquired immune systems. However, their persistent activation may result in more immune-mediated tissue damage with limited success in the removal of the biofilm pathogen. In this study we evaluated the potential of the serum raised against *P. aeruginosa* biofilm antigen in reducing and removing *P. aeruginosa* biofilms. We raised polyclonal sera in a Rabbit against *P. aeruginosa* biofilm antigens. The efficacy of the antigen was tested using slide agglutination test. We finally tested the efficacy of polyclonal sera to reduce and remove biofilm formation. We found that the anti-sera from rabbit showed strong immune response and disinfection activity against *P. aeruginosa* biofilm antigens. Passive immunotherapies can be important in attenuating acute *P. aeruginosa* infection and interfering with chronic infection.

### Introduction

*P. aeruginosa* is known to cause catheter-associated urinary tract infections through biofilm formation on the surface of indwelling catheters (Cole *et al.*, 2014; Mulcahy *et al.*, 2013). These biofilms can offer resistance to antibiotics, and have evolved mechanisms to evade innate and acquired immune systems (Drenkard *et al.*, 2002; Anderson *et al.*, 2008).

Biofilms infection cause stimulation of innate and acquired immune systems

(Anderson *et al.*, 2010). However, their persistent activation may result in more immune-mediated tissue damage with limited success in the removal of the biofilm pathogen (Kimbrell *et al.*, 2001). The antibodies produced against *P. aeruginosa* virulence factors may also contribute to the host tissue damage.

Studies have documented the direct killing of gram-negative bacteria by serum, mediated by the Membrane Attach Complex which, employed after the activation of the

complement system (Berends *et al.*, 2015). We are interested to study the role of humoral immune response and evaluate the potential of the serum raised against *P. aeruginosa* biofilm antigen in reducing and removing *P. aeruginosa* biofilms. This may pave the way for vaccine development against pathogenic drug-resistant bacteria.

## Materials and Methods

### Cultural conditions

*P. aeruginosa* culture was inoculated in Tryptone Soy Broth for 24 hours at 37°C. The next day, white matt at the air-liquid interface was pipetted out, transferred to saline tubes and vortexed for 1 minute to break the meshwork. Subsequently, 5ml formaline-saline was added and left overnight. Next morning, the cell suspension was centrifuged at 3000 RPM for 5 minutes. Supernatant was discarded and the pellet was washed twice and the pellet was suspended in 5-6 ml saline, and matched with 0.5 McFarland to obtain 10<sup>8</sup> cells /ml. The content was innoculated on Nutrient Agar, Sabourauds dextrose Agar and Thioglycolate broth to check sterility.

### Sera against *Pseudomonas aeruginosa*

To obtain the sera, 0.1, 0.1, 0.1, 0.1, 1, 2, and 2 ml of antigen was administered into an adult, healthy rabbit at first, third, fifth, seventh, tenth, thirteenth, and twentieth day with, respectively, through intravenous route, except for the last two days for which intraperitoneal route was adapted. Two days after the last dose, the rabbit was heart bled. The blood was collected in sterile tubes; serum was separated and frozen at -20°C.

### Slide agglutination test

Culture of *P.aeruginosa* was inoculated in Tryptone Soy Broth for 24 hours at 37°C.

Next day, the white matt formed at the Air-Liquid interface was pipette out and a drop of suspension was placed on a clean slide. A drop of rabbit serum was placed on a slide. Content of the slide was mixed and observed for clump formation. Agglutination occurring within five minutes was marked as strong response, while the delayed one was considered as weak response.

### Disinfection and Removal of *Pseudomonas aeruginosa* Biofilms

Disinfection and removal of biofilm was determined in two ways as described previously (Abidi *et al.*, 2014). In the first experiment, potential of the sera to inhibit *P. aeruginosa* biofilm formation was evaluated. For this, the culture was inoculated in 5-ml TSB and grown to stationary phase and diluted 1:100 in the Tryptone Soy Broth. 100 µl of diluted culture was pipetted in 34 wells. 100 µl of sera was inoculated in each well, and plate was covered and incubated at 37°C for 24 hours.

In the second experiment, potential of the sera to remove formed biofilms was evaluated. For this, microtitre plates were inoculated as mentioned above and incubated at 37 °C for 24 hours. After incubation plates were washed to remove planktonic cells. 200 µl of rabbit serum was inoculated in each well and incubated for 1.5 hours.

Subsequently, four small trays were set up in a series and autoclaved tap water up was added in the last three, while first served as waste. Planktonic bacteria were removed from the microtiter plates by submerging the plates in water tray. For biofilm staining, 125 ulof 0.1% crystal violet solution was added to each well and incubated for 10 min at room temperature. Post-incubation, the stain was emptied over the waste tray and

plates were washed successively in each of the next two water trays with vigorous shaking to remove all liquid. Subsequently, the plates were inverted and vigorously tapped on paper towels to remove all the contents and left to air dry. Finally, the dye was solubilized by adding 200 µl of 95% ethanol and plates were covered and incubated for 10 to 15 min at room temperature. Next, contents of each well were mixed by repeated pipetting. 125 µl of crystal violet-ethanol solution was transferred from each well to a separate well of a new optically clear flat-bottom 96-well plate. Optical densities (OD) of each of these 125-µl samples were measured at a wavelength 630 nm.

Measurement of anti-biofilm efficacy called Percentage Reduction was calculated from blank, control and test OD using equation:

$$\text{Percentage Réduction} = ((C-B) - (T-B) / (C-B)) * 100 \%$$

Where  $B$  = absorbance of blank,  $C$  = absorbance of control and  $T$  = absorbance of test.

## Results and Discussion

### Slide Agglutination Test

Pre-immunized rabbit sera showed agglutination within 5 minutes when incubated with *P. aeruginosa* biofilm content, indicating a strong response against biofilm proteins.

### Anti-biofilm Activity of Raised Sera against *P. aeruginosa* Biofilm

Rabbit serum exhibited 46.16 % reduction in biofilm formation. Similarly, rabbit serum also displayed 54.7 % reduction in preformed biofilms.

The present study found that the anti-sera

from rabbit showed strong immune response against biofilm antigens. Our study supports the understanding that innate immunity may prove helpful in disruption of biofilm and allowing antibiotics to penetrate inside the inflammation (Ciofu, 2003).

Many studies have shown that the immune system may fail to eradicate *P. aeruginosa* biofilm (Mathee *et al.*, 1999), especially in indwelling medical devices. Cases have been reported in which indwelling devices were removed and shown colonization by *P. aeruginosa* despite patients were being treated with antibiotics (Jesaitis *et al.*, 2003). The antibody protective mechanism against biofilm by the host immunity may be mediated by Outer membrane protein (OMP). OMP belonging to the Omp85 family have been identified as antigen in pathogenesis and immunity against *P. aeruginosa* (Tashiro *et al.*, 2008; Duchesne *et al.*, 2013). It has been found that Opr86 acts as a protective antigen to inhibit biofilm formation by *P. aeruginosa* PAO1 and several other isolates. Furthermore, *Pseudomonas aeruginosa* OMPs have also been studied for potential vaccine antigens (Lee *et al.*, 2000). OMPs have been found more suitable as antigens than lipopolysaccharides, exopolysaccharides, or isolated flagella for the clinical use.

The present study identifies a potential immunologic biofilm inhibition potential against clinical strains. Opr86 has also been proposed as a *Pseudomonas aeruginosa* would be a good target for design of both new antibiotics and vaccine (Tashiro *et al.*, 2008).

In conclusion, in this study, we have demonstrated that the rabbit serum inhibited and removed *P. aeruginosa* biofilms. Therefore, passive immunotherapies that target this particular pathogen should be of

value in attenuating acute infection and interfering with chronic infection.

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