


**Research Article**

## Immunization with Formaldehyde Inactivated Whole Cell Vaccine against Multidrug Resistant *Pseudomonas aeruginosa* Produces Protective IgG Antibodies in Mice Model

Modina Ansary Nabonee\*, Asma Rahman, Nafisa Jabin Mishu, Nigha Zannat Dola, and SM Shamsuzzaman

### Summary

The emergence of multidrug resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) are particularly challenging for clinician. Development of an effective vaccine may overcome the problems associated with treatment failure and antibiotic resistance. This study was designed to determine immune response against MDR *P. aeruginosa* using formaldehyde inactivated *P. aeruginosa* in mice model. In this study, twelve Swiss albino mice were used. Intramuscular inoculation was implemented three times at 14 days interval with formaldehyde inactivated MDR *P. aeruginosa*. The mice were given intraperitoneal challenge with live *P. aeruginosa* and observed for 14 days. Tail blood was collected to assess antigen binding capacity and bactericidal capacity of the serum antibody of immunized mice by ELISA and serum bactericidal antibody (SBA) assay, respectively. The survival rate was 100% among the mice of immunized group at 14 days of post challenge. In ELISA, OD values of serum immunoglobulin G of pre and post challenge immunized mice were significantly higher ( $p < .001$ ). The SBA assay of the immunized mice sera revealed 100% bactericidal activity after 3h of incubation with 50% guinea pig complement. It was observed that formaldehyde inactivated MDR *P. aeruginosa* induced protective antibodies in mice model.

**Keywords:** ELISA, Immune response, MDR *P. aeruginosa*, Serum bactericidal antibody assay, Whole cell vaccine

### Introduction

The versatility of *Pseudomonas aeruginosa* (*P. aeruginosa*) allows it to flourish in different environmental situations (soil, aquatic environments or plants) and clinical settings (nosocomial infections and medical equipment, such as inhalers, respirators, and vaporizers etc.) [1]. This opportunistic pathogen is one of the most common causes of nosocomial infections in the intensive care unit (ICU), commonly in mechanically ventilated patients [2,3]. It also causes chronic respiratory infections in people with cystic fibrosis (CF), bronchiectasis or chronic obstructive pulmonary disease (COPD), and acute infections in immunocompromised individuals [4]. Infections with MDR *P. aeruginosa* are associated with high rates of mortality, currently estimated at about 18–61% [5]. Aside from its intrinsic resistance to several antibiotics, its propensity to acquire resistance is responsible for its multidrug resistance profile, rendering the pathogen a therapeutic challenge. Therefore, it is essential to develop an alternative strategy to tackle this highly antibiotic-resistant pathogen (e.g vaccine, antibody therapy etc.) [6,7]. The development of novel immunotherapies and vaccines against *P. aeruginosa* could help to

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prevent infections caused by this highly antibiotic-resistant microorganism [4]. In order to develop an effective vaccine against *P. aeruginosa*, detailed knowledge of the host immune responses and the bacterial defense mechanisms is important. Both innate and adaptive immune responses work in synergy to act against *P. aeruginosa* infection. As *P. aeruginosa* is an extracellular pathogen, humoral, mucosal or systemic opsonizing immunity is most effective to prevent bacterial colonization and infection. However, T-cell responses can also mediate protective immunity in individuals with *P. aeruginosa* infections [8].

Numerous experimental vaccine candidates have been evaluated such as LPS, alginate, pili, flagella, whole cell etc. In order to improve the presentation of multiple antigens to the immune system, whole-cell and live-attenuated *P. aeruginosa* mucosal vaccines were shown to be protective in animal models [4]. As an innovative strategy for development of bacterial vaccine, inactivation method could be simultaneously applied and for which formaldehyde is an effective inactivating agent [9]. The intramuscular (IM) route optimizes the immunogenicity of the vaccine and minimizes adverse reactions at the injection site as because muscles have larger and more numerous blood vessels and absorption rate are usually faster than other routes [10].

However, still no study regarding protective vaccine efficacy for *P. aeruginosa* has been carried out in Bangladesh. Therefore, this study was designed to evaluate the immune response of whole cell vaccine after intramuscular immunization with formaldehyde inactivated multi drug resistant *P. aeruginosa* in murine model.

## Materials and Methods

### Animals

Twelve 6 to 8 weeks old female Swiss albino mice weighing 12-15 gram and two guinea pigs were obtained from ICDDR'B, Breeding house, Dhaka, Bangladesh. All mice were maintained at the animal house of the microbiology department of the affiliated institution providing particular pathogen-free facility. All experimental Protocols and animal care were reviewed and approved in accordance with the regulations of the Ethical Review Committee of Dhaka Medical College.

### Preparation of Bacterial Strains

A mixed solution of MDR *P. aeruginosa* (in PBS) obtained from different samples were chosen to use as a candidate for formalin inactivated whole-cell vaccine preparation. At first culture of bacteria were done in Blood agar and MacConkey agar media. Then subculture was done in Tryptic soy broth for 24 hours at 37°C and washed twice with sterile phosphate-buffered saline (PBS). After that formalin was added to the suspension (final concentration 3%, v/v) and incubated at 37°

C. The inactivated *P. aeruginosa* then washed twice. Then re-suspended with sterile PBS to achieve a concentration of  $3 \times 10^7$  CFU/ml and used for the immunization. Viability of bacteria was checked by observing no growth after overnight incubation at 37° C following streaking on Mueller Hinton agar plate [9, 11].

### Immunization Schedule

The mice were randomly divided into three groups such as Group 1 (experimental group), Group 2 & Group 3 (control group). The Group 1 mice were immunized with formalin-inactivated whole-cell *P. aeruginosa* on day 0, 14 and 28 intramuscularly. The control group received sterile PBS simultaneously [9,12].

### Challenge

Fourteen days after last immunization, Group 1 and Group 2 mice were challenged intraperitoneally with  $3 \times 10^8$  CFU/ml live MDR *P. aeruginosa* suspended in 500 µl of PBS. All mice were monitored for 14 days post challenge for any clinical features like reluctant to feed, lack of movement, weight loss, etc. [9, 13].

### Collection of Blood for SBA and ELISA

Blood was collected from tail end 10 days after 1st inoculation and then 7 days after each booster. Firstly, the tail end was cut about 2-3 mm close to its blunt tip. Then blood was collected into a micro centrifuge tube containing PBS (1:5 dilution). After 2 hours, the blood was centrifuged at 3,000 g for 10 minutes. Clear sera from the top of the tube were taken into another sterile micro centrifuge tube and used for ELISA and SBA assay [12].

### Antibody Detection by ELISA

Indirect ELISA was performed for detection of IgG antibody from mice sera which is specific for *P. aeruginosa* antigen. Antigen was separated by sonication of *P. aeruginosa* [13].

### Procedure of Sonication

Briefly, 100 µL of distilled water was mixed with bacterial pellets and set aside on ice for 30 min. Then sonication was done at 20 kHz for  $2 \times 10$  s (subjected to viscosity of the samples) and centrifugation was done for 20 min at 10,000 g. Then, supernatant was kept at -20° C and used as antigen later on. Antigen concentration was optimized at 10µg/ml by checkerboard titration method [13].

### Optimized ELISA

Antigen coating was done in a 96 well immunoabsorbent plates with 100 µl/well of antigen (10µg/ml) in sodium bicarbonate buffer (pH=9.6) by overnight incubation at 4° C. The wells were washed with PBS three times. Then the wells were blocked with 5% skimmed milk (w/v) in PBS for

30 min at 37°C (200 µl/well). The plate was washed three times with 0.05% PBST (0.05% Tween20 in PBS) and once with PBS. Then sera (100 µl/well) were added at a 100-fold dilution and incubated for 90 min at 37°C followed by whole night at 4°C. Horseradish peroxidase (HRP) conjugated anti-mouse IgG (Thermo Fisher Scientific, USA) in PBST (1:5000) were added (100 µl/well) and incubation was done for 90 min at 37°C. After washing, tetramethyl benzidine (TMB) and urea peroxide (substrate) were added and kept for 10 min under dark condition. Then stop solution (1M H<sub>2</sub>SO<sub>4</sub>) was added. ELISA plate reader (BioTek Inc., USA) was used for measurement of optical density value at 450 nm [9]. Following formula was applied for calculation of cut-off value of OD:

Cut-off value of OD = M (mean) + 2 × Standard deviation

### SBA Assay

#### Preparation of Bacterial Strains

A MDR *P. aeruginosa* strain was secluded and cultured in Mueller-Hinton agar media for 18–20 h at 37°C. Then a bacterial suspension was prepared (1 × 10<sup>8</sup> CFU/ml) and serially diluted in PBS at different concentrations (upto 1 × 10<sup>3</sup> CFU/ml) [13].

#### Complement

Sera was collected from guinea pig and used for complement. Following blood collection, centrifugation was done for 10 min at 3000 g. Then serum was transferred to sterilized cryotubes and preserved at –20°C. To determine the effectiveness of functional antibody of mouse serum, complement was diluted with PBS in a various concentration (i.e. 50%, 25%, and 12.5%) and added along with bacterial suspension. Viability of *P. aeruginosa* was also checked using immunized and unimmunized mice sera and bacterial suspension in absence of complement in a different well of microtiter plate [13].

#### Optimization of SBA Assay

Serum samples of immunized and unimmunized group of mice were inactivated at 56°C for 30 min and serially diluted in PBS (1:10, 1:20, 1:40, and 1:80). Then 10 µl of each of the diluted serum were taken in a microtiter plate. Pooled guinea pig sera of above mentioned dilutions were placed to each well. Then, 10 µl of bacterial suspension of previously prepared concentrations was mixed with the identical wells. Thus final reaction volume of 50 µl was obtained and incubated for 1 h, 2 h, 3 h, and 4 h at 37°C [14]. From every well, 1 µl of suspension was placed in Mueller-Hinton agar plate followed by incubation at 37°C for overnight. Presence of viable bacteria was checked by detecting their growth. All the measures were done in triplicate [15].

#### Optimized Serum Bactericidal Antibody Assay

Optimum SBA finding was attained by 30 µl suspension

of 50% guinea pig sera, 10 µl serum of immune mice (1:10 & 1:20 dilution) and 10 µl of bacterial inoculum (1.5 × 10<sup>4</sup> CFU/ml) followed by incubation at 37°C for 3 h. Viable CFU counts were noted. The negative control well was inoculated with bacteria and complement [16, 17].

### Data Analysis

The data were documented methodically. All statistical analysis was done by SPSS version 25. Statistical significance between groups was compared by unpaired T test. *p*-value < .05 was considered as a minimum level of significance.

## Results

### Bacterial Inactivation by Formaldehyde

Following inactivation, the bacterial suspension was cultured overnight in Mueller Hinton agar plate. No colonies were detected on the plate. Un-inactivated bacteria were used as a control.

### Survival of Mice

After lethal challenge, 100% survival was observed at 14 days post challenge among the experimental group of mice. All mice from Group 2 died within 24 hour of challenge.

### Immune Response against *P. aeruginosa*

OD value of IgG antibodies in sera of experimental and control group of mice were measured following 10 days after 1<sup>st</sup> immunization [data not shown] and 7 days after 1<sup>st</sup> booster [Figure 1] and 2<sup>nd</sup> booster [Figure 2]. OD value of all the immunized mice sera was above the cutoff value (0.139 and 0.144 respectively). After each immunization, significant statistical difference was observed between the OD values of immunized and control mice sera (*p* < .001).

OD value of serum anti-*P. aeruginosa* IgG antibodies following lethal challenge is shown in Figure 3 which was also above the cutoff value (0.142) and statistically significant (*p* value < .001).

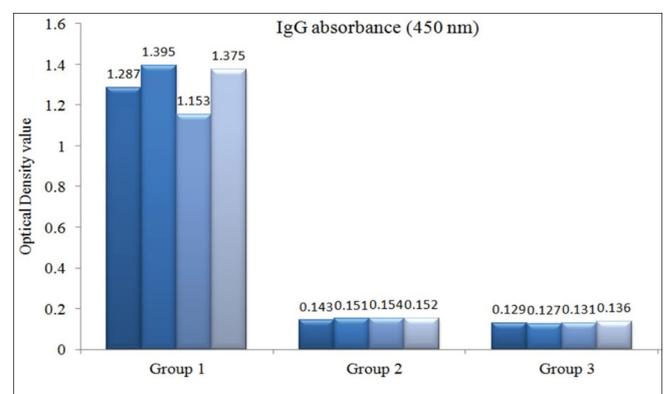
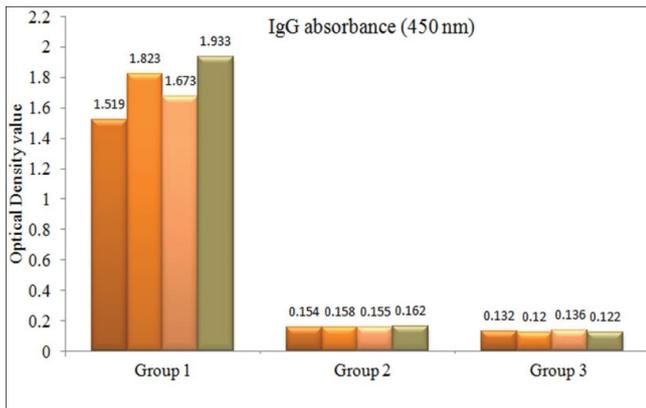


Figure 1: OD value of immunized and control serum after 1<sup>st</sup> booster

There were significant differences of serum IgG antibody response of the experimental group of mice within subsequent doses of immunization schedule (Table 1).

**SBA Assay**

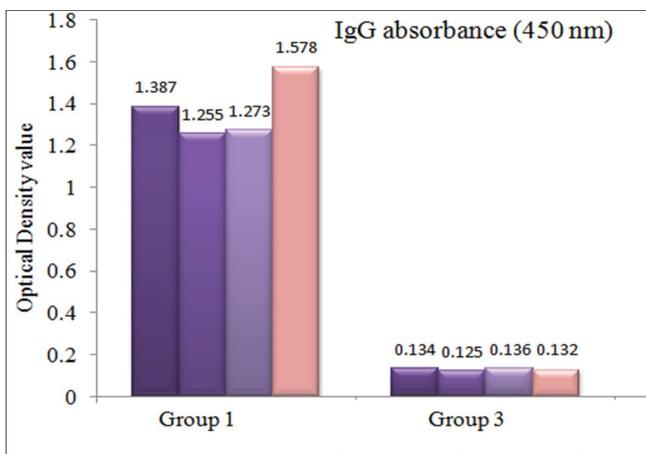
SBA assay of pre challenge immunized mice sera (1:10 dilution) with bacterial inoculum ( $1.5 \times 10^4$  CFU/ml) inhibited bacterial growth using 50% GPC which has been shown [Figure 4]. Serum samples were collected seven days before lethal challenge.



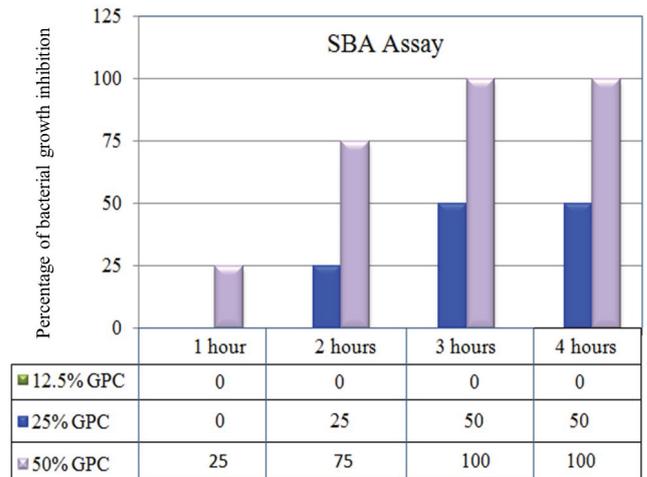
**Figure 2:** OD value of immunized and control serum after 2<sup>nd</sup> booster

**Table 1:** The optical density value of IgG absorbance (450 nm) within the subsequent booster doses of immunization schedule of experimental group of mice were interpreted by ANOVA: single factor

Source of Variation	SS	Df	MS	F	p-value	F crit
Between Groups	4.113928	3	1.371309	78.8597	3.63E-08	3.490295
Within Groups	0.208671	12	0.017389			
Total	4.322599	15				



**Figure 3:** OD value of immunized and control serum after lethal challenge



**Figure 4:** SBA using pre challenge sera (1:10)

Sera at 1:20 dilution also caused bacterial growth inhibition by using with 50% complement after 3 hours of incubation. SBA assay was also done with post challenge sera which also exerted growth inhibition with 1:10 diluted sera.

**Discussion**

The complexity in the pathogenesis of *P. aeruginosa* and the emergence of multidrug-resistant strains are the leading cause of the increased susceptibility of infections by this notorious pathogen to the vulnerable groups. Hence the development of a vaccine for this challenging pathogen might be a hopeful alternative to antibiotics [4]. In the current study, the survival rate of the immunized group of mice was 100% after 14 days of lethal challenge. A study observed that following intramuscular immunization with PcrV<sub>NH</sub> (PcrV<sub>NH</sub> is an artificial PcrV derivative where PcrV is a structural protein of the type 3 secretion system of *P. aeruginosa*), the percentage survival was 100% to 50% from 36 hours to 7 days of post challenge [18]. No data regarding survival rates of mice after lethal challenge with live MDR *P. aeruginosa* is available in Bangladesh. Whole cell vaccine was used for enhancing multiple antigen presentation to the immune system resulting a more complete and effective immune response. Thus may ensure broad coverage and clinical efficacy. Formaldehyde inactivation was chosen because integrity of surface antigens is well maintained on the bacteria while inactivated with it [13, 19]. In the present study, immunization with formaldehyde inactivated whole cell vaccine stimulated production of IgG polyclonal antibody against *P. aeruginosa* after each immunization as well as following lethal challenge which was measured by ELISA. A study conducted in China reported that formaldehyde inactivated bacteria induced both IgG1 and IgG2a immune responses. The same study also reported that the IgG titer was highest at 56<sup>th</sup> days after first immunization [9]. A different study showed that immunization with heat-inactivated whole cell *P. aeruginosa* stimulates significant serum IgM, IgG and

lung supernatant IgA antibody titers against *P. aeruginosa* that induced both mucosal as well as systemic humoral immune responses [20]. In this study, the highest OD values of the IgG antibody were noticed following the second booster. This might be due to the fact that memory cells produced more IgG antibody following second booster than first booster. After lethal challenge, IgG antibodies were slightly decreased in mice sera due to removal of the offending pathogen from the body [12].

An assay was performed in this study to determine the serum bactericidal activity of antibody produced against *P. aeruginosa* in immunized mice. In the present study, immunized mice sera were able to kill MDR *P. aeruginosa* using 50% guinea pig complement. A study found that SBA for *P. aeruginosa* revealed bacterial killing by activation of classical pathway of complement indicating presence of anti-*pseudomonas* functional antibody in human serum [15]. The serum antibodies might be essential for the initiation of the classical pathway of complement system in the early period of infection and confers killing of bacteria. Besides this, antibodies also can cause opsonization of infectious microorganisms followed by stimulating effector functions on neutrophils [21]. A study reported the presence of sufficient IgG2b and IgG3 isotypes against *P. aeruginosa* in CD4+ T cell deficient whole cell vaccinated mice sera which may be enough to enhance bacterial clearance [20]. The whole-cell vaccine-mediated humoral immune response can play the vital role in immunity for Gram negative bacteria [21].

## Conclusion

Development of an effective vaccine against this pathogen is a burning issue that can reduce the rate of infection as well as the sufferings of the vulnerable groups and decreases the selective pressure on antibiotics. Although other measures of immunological responses are needed to be evaluated in a larger group of animal model, the present study delivers a hope that formaldehyde inactivated whole cell vaccine against MDR *P. aeruginosa* induces protective antibodies in Swiss albino mice.

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## Statement of Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

## List of Abbreviations

ELISA, Enzyme Linked Immunosorbent Assay; GPC, Guinea pig complement; MDR, multidrug resistant; OD, optical density; SBA, Serum bactericidal antibody

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**Data Availability:** All the raw data and specific materials are presented in the manuscript.

**Ethics Approval:** Ethical clearance was obtained by the Ethical Review Committee (ERC) of Dhaka Medical College.

**Consent to Participate:** Not applicable

**Consent to Publish:** Not applicable

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