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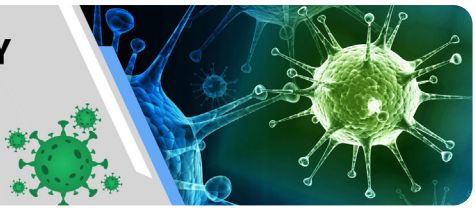

Intradermal Immunization with Formaldehyde Inactivated *Pseudomonas Aeruginosa* Leading to the Production of Protective Immunoglobulin G against Protein of Specific Molecular Weight in Swiss Albino Mice

Saoda U*[1], Shamsuzzaman SM[2], Alam JAJ[3], Lopa NM[4]

Abstract

Pseudomonas aeruginosa, an extremely adaptive organism is liable for a number of acute and chronic infections with a wide range of predisposing conditions. Irrational use of antibiotics has increased the crisis by raising MDR strains and also prompted the development of effective vaccine against it. This study was carried out to detect the specific protein as per molecular weight against protective antibodies in serum and splenic cell culture supernatant by formalin inactivated whole cell vaccine against multidrug resistant *P. aeruginosa.* In this study, MDR *P. aeruginosa* was isolated from various clinical samples and used for the intradermal immunization of 15 Swiss albino mice. Fourteen days following immunization with the third dose of vaccine, the mice were challenged intradermally with live *P. aeruginosa* and observed for 14 days. Sera from tail blood and supernatant from mice spleen cell culture were collected. *P. aeruginosa* antigens used in the preparation of vaccine were sonicated and separated using SDS-PAGE electrophoresis as per molecular size. Protective antibodies that bind with antigen were assessed by ELISA. In this study, 14 days after post challenge, 100% survival rates were seen among the immunized mice. ELISA showed all serum from the pre- and post-challenge immunizations had considerably greater optical density values of IgG in comparison to the control mice and highest absorbance was recorded against antigen eluded from band with MW 34-42 kDa in both serum as well as splenic cell culture supernatant. In this study, formalin inactivated intradermal immunization with MDR *P. aeruginosa* produced protective antibodies in Swiss albino mice against antigen of specific molecular weight. Use of this target antigen could be applied as an innovative strategy for bacterial vaccine development.

Keywords: *Antibody, formalin inactivation, Immunization, P. aeruginosa, SDS-PAGE*

Background

Pseudomonas aeruginosa is a pervasive organism belonging to the family *Pseudomonadaceae* that is able to cause both acute as well as chronic infections in healthy and non-immune people. Its variability enables it to thrive in a wide range of environments, including soil, water reservoirs, and even human bodies and medical equipment.

Because of its remarkable ability to produce resistance, *Pseudomonas aeruginosa* has become a "superbug" and is frequently resistant to several antibiotics. The development of novel antibiotics and other therapeutic approaches for *P. aeruginosa* infections has not proceeded so well, despite the

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great interest in the disease. This is because drug resistance mechanism is complex and variable, and a thorough understanding of *P. aeruginosa* pathogenic mechanisms is lacking. There is a growing urgency to develop efficient therapeutic strategies to thwart *P. aeruginosa* invasion, necessitating diverse efforts [1]. Given how fast resistance has developed to each new class of antibiotic introduced and the hurdles in producing new effective drugs, focusing on research into the underlying resistance mechanisms and the development of new antibiotics alone is insufficient [2]. Vaccines have a remarkable impact on both prevention and cure of a disease and can be used for decades with a much lower probability of resistance emergence compared with antibiotics [3]. In preclinical *P. aeruginosa* vaccine development whole-cell killed vaccines have been considered as attractive candidate formulations, as pathogen-associated molecular patterns (PAMPs) serve as adjuvants, and the antigen presenting cells (APCs) deliver protective antigens in a variety of ways [4]. Although the development of first-generation vaccines is financially profitable but they are unstable and present problems associated with standardization, and there is also a lack of knowledge about their composition [5]. To overcome these adversities in general and elicit more specific protection there are better alternatives to these formulations. As *P. aeruginosa* exhibits several virulence mechanisms and adapts to host environments it is important to consider using multiple vaccine candidates in combination. Recent reverse vaccinology approach identified multiple antigens that in combination are effectively considered to be responsible for control of *P. aeruginosa* infection [6]. For the construction of effective vaccines, it is of paramount importance to identify all antigens immunologically recognized by a patient population infected with a pathogen [7]. A successful approach used to identify the best antigen candidates for vaccines against pathogens is the use of immunoproteomics, which is based on a two-dimensional electrophoresis [8]. Widely employed in biochemical investigations, polyacrylamide gel electrophoresis (PAGE) is a promising fractionation method for structural proteomics and an efficient approach for protein separation in a straightforward procedure [9]. Using SDS polyacrylamide gel electrophoresis (SDS-PAGE), certain native proteins can be extracted in their pure form from cell lysates or tissue preparations. This type of refined antigen frequently elicits favorable antibody responses [10]. ELISA test has always been extensively used for serological diagnosis of various diseases and evaluation of immunity in vaccinated animals [11]. In seromonitoring of vaccinated individuals and identification of antigen-antibody interaction in them this method provides both precision and specificity [12]. No approach to identify antigen against protective antibodies developed from vaccine for *P. aeruginosa* has been taken so far in Bangladesh. Hence, this study has been done to identify the protein antigen and evaluate development of protective antibody from vaccine after intradermal immunization of formaldehyde inactivated MDR *P. aeruginosa* in murine infection model.

Materials and Methods

This study was conducted from November 2022 to June 2023 at the Department of Microbiology at Dhaka Medical College, Dhaka, Bangladesh.

Culture of Bacteria

To get solutions for vaccination, organisms from various samples were cultured for 24 hours at 37°C in MacConkey agar medium.

Immunization of Mice

Animals

Fifteen Swiss-albino female mice of 6-8 weeks of age were collected from Animal Resources Facility of icddr,b and were retained under precise pathogen free environments in the animal house of Dhaka Medical College.

Preparation of Formalin Inactivated whole cell *P. aeruginosa* Vaccine

Group-1 was vaccinated against formalin inactivated solution prepared from a mixed solution of *P. aeruginosa* isolated from wound swab, pus urine, blood, endotracheal aspirate and sputum. For this, a loop full of organisms was inoculated into trypticase soya broth (TSB) and was incubated at 37°C overnight. Following incubation, centrifugation was done at 2,000 g for 20 min at 4°C and the supernatant was discarded. Phosphate buffered saline (PBS) was then used to wash twice the pelleted bacteria. To formulate formalin inactivated *P. aeruginosa*, 37% formalin was included to the suspension to achieve a final conc. of 3% (v/v) and incubated for 2 hours at 37°C. After incubation, the suspension was again two times washed with sterile PBS and resuspended in PBS to achieve conc. of 1.5 X 10^8 CFU/ml. 134 µl of inoculum was mixed with 866 µl of sterile PBS to achieve a conc. of 2 X10^7 CFU/ml [13].

Immunization Schedule

Three doses of intradermal immunizations were performed with 20 µl of formaldehyde inactivated bacterial solution (2X10^7 CFU/ml) on day 0, 14 and 28 with the help of insulin syringe in group-1 experimental group. The mice were sedated with intraperitoneal ketamine injection (100mg/kg) prior to each immunization and sterile PBS of 20 µl was also given to the control group-2 on same days. Control group-3 was kept uninfected and uninoculated.

Blood Collection for ELISA

Blood from the tail of mice was collected on 13th, 27th and 41st days following first immunization. The tail was stretched

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and 70% alcohol was used to clean it. The tail was cut 2 mm before its rounded end by a sterile scalpel (22 FR) and 50µl of fresh blood was kept in a micro centrifuge tube containing 200µl of phosphate buffer saline to maintain a dilution of 1:5. The diluted sera was retained at vertical position for 2 hours, after which centrifugation at 3000 g for 10 minutes was done and clear sera was moved into a separate micro centrifuge tube [14].

Intra-peritoneal Challenge

Two weeks after the last inoculation, the mice from experimental group (group- 1) and control group (group-2) were challenged intra-peritoneally with 3X10^8 CFU/ml live MDR *Pseudomonas aeruginosa* suspended in 100µl PBS. All mice were observed for 14-days post challenge for any clinical manifestations such as weight loss, lack of movement, reluctant to feed or death.

Separation of mononuclear cells from spleen

The spleen was collected from each of the Group-1 (Experimental) and Group-3 (Negative control) mice under aseptic conditions in order to detect the antibody production. Under a sterile biosafety cabinet, each spleen was put into a petri dish containing 5ml of complete RPMI medium (RPMI-1640, 10% FBS, 200U/ml Penicillin, 200 µg/ml). Spleen was the crushed between two frosted glass slide followed by filtering the cell suspension through a 70 µm Nylon cell strainer and centrifuged at 350 rpm for 10 minutes at 4°C, supernatant was discarded and cells were resuspended in 5ml of complete RPMI medium [15].

Determination of viability of Splenic mononuclear cell

The cell pellet was resuspended in one ml PBS. One part of 0.4% trypan blue and one part cell suspension (dilution of cells) were mixed. Mixture was allowed to incubate for 3 min at room temperature. A drop of cell mixture was applied to a hemacytometer on the stage of a microscope and focused on the cells. The unstained (viable) and stained (nonviable) cells were counted separately in the hemacytometer. The percentage of viable cells was calculated as follows:

Viable cells % = (total number of viable cells per ml of aliquot /total number of cells per ml of aliquot)× 100. The sample was considered appropriate if ≥ 50 % cells were found viable.

Antibody Detection by ELISA

To determine the optical density (OD) by ELISA for the existence of immunoglobulin G (IgG) specific for *P. aeruginosa* antigen, mice sera and splenic cell culture supernatant were used.

Sonication of Whole-Cell P. aeruginosa

About 100 µl of distilled water was used to dilute the bacterial pellets and set aside for 30 mins on ice and sonicated at 20 kHz for 2 × 10 s (reliant on samples and thickness of the samples), and retained on ice for 5 min. Then, this solution was centrifuged at a 10,000 g for 20 min to pellet debris (debris may contain unbroken cells or organelles and nuclei). Then, to a fresh microcentrifuge tube supernatants were transferred and were stored at −20°C for usage as antigen. Checkerboard titration was done to optimize the antigen, and for ELISA 10 µg of antigen was used [14].

SDS-PAGE Electrophoresis of sonicated protein

For SDS-PAGE the separation gel (10%) was first prepared by mixing H2O, Acrylamide/bis, Tris–HCl, SDS, 10% N,N,N′,N′-tetramethylethylene-diamine (TEMED) and Ammonium persulfate in the exact mentioned order. After adding TEMED and APS to the SDS-PAGE separation gel solution, the gel will polymerize quickly, so these two reagents were added when ready to pour. Gel was poured leaving ~2 cm below the bottom of the comb for the stacking gel. In ~30 min, the gel was completely polymerized. The stacking gel was prepared in the same manner with different composition of same constituents. The stacking gel was poured on top of the separation gel and combs were added to make wells. In ~30 min, the stacking gel also became completely polymerized. After clamping gel into apparatus, both buffer chambers were filled with gel running buffer according to the instructions for the specific apparatus. Finally, samples and molecular mass protein markers were loaded into wells for separation by electrophoresis. Following electrophoresis, the recovered gel was stained with Coomassie brilliant blue for a minimum of 4 hours at room temperature. And then de-stained with distilled water over night. After electrophoresis, using a clean scalpel several sections or strips of the gel that include protein samples corresponding to their respective bands shown in molecular weight marker was cut off. This included four protein bands ranging from molecular weight 26 to 33 kDa, 34 to 42 kDa, 43 to 55 kDa and 56 to 72 kDa. For elution of proteins from gel matrix the cut sections were transferred to micro centrifuge tubes containing elution buffer in which they were completely crushed and incubated overnight using a rotary shaker followed by centrifugation for 10,000 x g for 10 min. the supernatant was preserved for ELISA [16-17].

ELISA

Coating with bicarbonate-coating buffer (pH 9.6) and overnight incubation at room temperature of ELISA plates were done with 100 µl/well of antigen (10 µg/ml) followed by washing two times with PBS and blocking with 200 µl/well skimmed milk in PBS, incubation at 37°C for 1 hr 30 min, then three times washing with PBS-tween and once with PBS. Samples of serum (100 µl/well) and splenic cell culture supernatant (300 µl/well) then were added and

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incubated for 1 hour at 37°C and then at 4°C overnight. The next day, plates were washed according to above mentioned procedures, horseradish peroxidase-labeled anti- mouse IgG antibody (Thermo Fisher Scientific, USA), diluted (1:5000) conjugate, was mixed in PBS-Tween (100 µl/well) and added to the plates and incubated at 37°C for 90 min and after washing, 100 µl/well tetramethylbenzidine (50 µl) and urea peroxide (50 µl) substrate were added. Fifty microliter of 1M sulfuric acid was included to these plates to halt the reaction. Measurement of absorbance was done at 450 nm with the help of ELISA plate reader (BioTek Inc., USA).

Calculation of cutoff OD value was done by the following formula:

$$OD = M\ (mean) + 2 \times Standard\ deviation$$

Data Processing

Data were assembled and revised precisely by examination and reexamination. All errors and discrepancies were fixed and were aloof methodically.

Data Analysis

The study results were documented scientifically. Data were evaluated and compared by t test & ANOVA Statistical analysis of all data was carried out by Microsoft Excel 2020. P value of 0.05 was taken as lowest level of significance.

Ethical Approval

This study was approved by the Research Review Committee (RRC) and the Ethical Review Committee (ERC) of Dhaka Medical College. Approval for animal experimentation was obtained from Animal Experimentation Ethics Committee (AEEC) of DMC.

Results

Immunized and unimmunized mice survival rates in *P. aeruginosa* following lethal challenge were 100% in vaccinated group of mice following challenge and Group 3 control mice that were not challenged. Death of every Group 2 control or non-immunized mice following challenge was also noted. OD values in 3 serum samples from immunized group (Group-I) collected on 13th, 27th and 41st days after 1st immunization were analyzed (Figure 1). Significant difference was also observed among the OD values of experimental and control mice sera (P <0.001 after each inoculation. Mean of negative control was 0.132; standard deviation 0.006, cutoff value was 0.144. OD value range after the 1st inoculation was 0.317-0.44, 2nd inoculation was 1.23-1.45 and after 3rd inoculation 1.53-1.89. OD value difference in immune response produced after each booster of formaldehyde inactivated whole cell P. aeruginosa was observed after each inoculation and after intraperitoneal challenge in vaccinated group of mice interpreted by ANOVA.

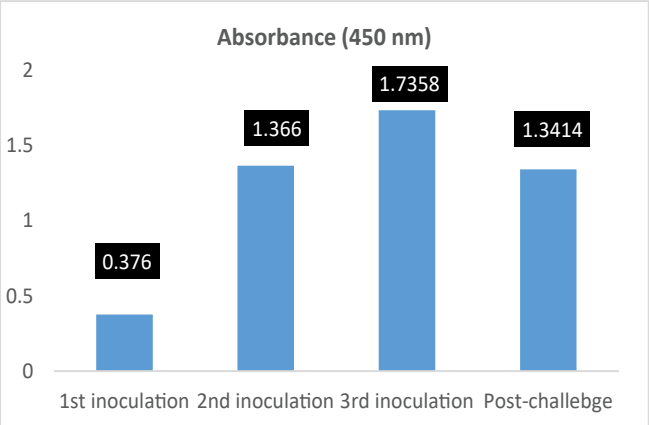

Figure 1: OD values in serum samples from immunized group of mice after each inoculation and challenge by ELISA

Table 1: the OD value of IgG absorbance (450 nm) within the different inoculation schedule of experimental group interpreted by ANOVA: single factor

	Sum of square	df	Mean square	F	P value
Between groups	5.070893	3	1.69		
Within groups	0.161069	16	0.01	167.906	2.66E+12
Total	5.231962	19			

There was also significant difference between OD values of cell culture supernatant of vaccinated mice group and negative control mice group (P < 0.0001) (Figure 2).

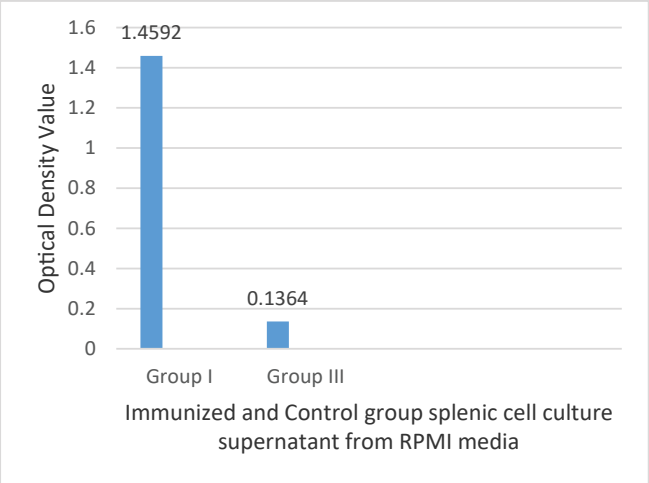

Figure 2: Optical density of anti-Pseudomonas aeruginosa antibodies in splenic cell culture supernatant depicting remarkable production of antibodies in experimental group mice spleen compared to control group evidenced by significant p value <0.0001

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The OD value of IgG absorbance (450 nm) by ELISA showing reaction of the polled mice sera & splenic cell culture supernatant produced from vaccination with inactivated vaccine against eluded antigens of different molecular weight separated by SDS demonstrated highest absorbance recoded against band with MW 34-42 kDa (OD value 1.8376 & 1.562 respectively).

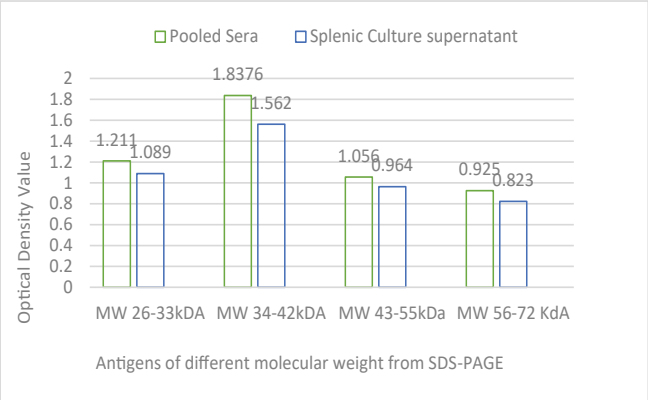

Figure 3: OD values of the pooled sera and splenic cell culture supernatant of vaccinated group against eluded antigens of different molecular weight bands from SDS-PAGE

Discussion

As a noteworthy bacterial pathogen, P*seudomonas aeruginosa* is known to cause both community acquired as well as nosocomial infections in hospitalized patients and immunocompromised hosts. Observations of nosocomial infections caused by *P. aeruginosa* over the years has revealed a trend of increasing anti-microbial resistance particularly of MDR [18]. With nearly 49 million sepsis cases and 11 million deaths worldwide, an effective vaccine against it could prevent the morbidity and mortality and decrease our overuse of antibiotics [19]. Intradermal (ID) vaccination induces a more potent immune response and requires lower vaccine doses as compared with standard vaccination routes [20].

This study shows following immunization with formaldehyde inactivated MDR *P. aeruginosa* in experimental group of mice resulted in 100% survival post-challenge whereas all mice from non-immunized group died. All pre and post challenge sera showed significant antibody production in vaccinated compared to control group. In another single-center, open-label, phase 1 study conducted in healthy human volunteers to assess the safety and immune response to Pseudostat, an oral preparation of whole-cell, formaldehyde-inactivated *P. aeruginosa* serum IgA levels increased by day 14 after the first dose, and remained above the day 0 level for the remainder of the observation period [21]. Splenic cell culture supernatant from the vaccinated and uninfected-unimmunized group also revealed higher antibody production in vaccinated group. Standardized serological techniques, such as ELISA are used to compare the vaccine-specific antibody levels as well as to detect binding antibody titers These methods result in a single value threshold to represent the full immune response.

In this study SDS-PAGE of sonicated MDR *P. aeruginosa* lead to identification of four intense molecular bands of weight ranging from 26 to 33 kDa, 34 to 42 kDa, 43 to 55 kDa and 56 to 72 kDa. Following which ELISA was done using pooled mice sera from vaccinated group which revealed the highest absorbance recorded against protein eluded from molecular weight 34-42 kDa. In a study by [22] a range of candidate proteins were selected from whole-cell protein extracts by identifying those proteins which bound antibodies from rabbit, the rabbit having been vaccinated subcutaneously with Pseudostat™, a whole-cell vaccine, the cells being inactivated. It disclosed an antigen from P. aeruginosa which has a molecular weight in the range of about 60 to 65 kDa which was similar to the molecular weights obtained from current study. In another study by [23] which aimed to investigate the whole genome sequence of *P. aeruginosa 24Pae112* to introduce novel and putative vaccine candidates it was observed that 16 proteins (seven OMPs and nine secreted) were ideal according to the defined criteria (surface-exposed, antigenic, non-allergenic, and non-homologous human proteins). These proteins had a molecular weight of 110 kDa and were prevalent in 75% of *P. aeruginosa* genomes. The variation in the result from current study might be due to differences in the selection criteria that involved more features other than immunogenicity. In this study, specific protein of molecular weight 34-42 kDa was identified to be the target protein against which majority antibody production occurred clearly stating the significance of role of the protein of this molecular weight in vaccine production and future researches.

Conclusion

As the world's population struggles with the epidemic era healthcare facilities are overburdened with patients in the face of medical supply shortages, health care worker exhaustion, an excess of critically ill patients and comprehensive infection control protocols. In the face of all these adversities vaccine has become of paramount importance among most treatment options. A more thorough understanding of the proteins that lead to and arise as a result of infection and immunization is one method to direct the development of an effective vaccine. The present study provides a clue for evaluation of antigens against which protective antibodies are developed in animal models vaccinated with MDR *P. aeruginosa* but it is also important to co-relate the immunological protection using assays other than ELISA. Nevertheless, using this antigen of fixed molecular weight recovered from current study can

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prove to be a useful adjunct in field of vaccine development against MDR *P. aeruginosa.*

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Nil.

Conflict of Interest

There are no conflicts of interest in this study.

List of Abbreviations

MDR, multidrug resistance; ELISA, Enzyme Linked Immunosorbent Assay; SDS, Sodium Dodecyl Sulphate; OD, optical density.

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