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Research Article

Antibody Responses Against Proteins of Different Molecular Weight Following Inoculation of Formalin Inactivated *Acinetobacter baumannii* in Mice

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Abstract

Acinetobacter baumannii is one of the most significant threats to global public health with a seemingly limitless capacity for antimicrobial resistance. Developing preventive vaccines to combat these pathogens can provide long-term protective immunity. This study was conducted to evaluate the specific protein against which the protective antibody is produced in serum and splenic cell culture supernatant after vaccination in mice. The vaccine used was a formaldehyde-inactivated whole-cell vaccine against multidrug resistant (MDR) *A. baumannii.* MDR *A. baumannii*, isolated from different samples, used in formaldehyde-inactivated vaccine and inoculated intradermally in experimental mice. Serum was collected from tail blood on days 13, 27 and 41 following 1st immunization. Two weeks after 3rd immunization, mice were challenged with live *A. baumannii* intraperitoneally and observed for 14 days. Sera from tail blood and mouse spleens were collected. MDR *A. baumannii* antigen was sonicated and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis was performed. Protective antibodies that bind with antigens in serum and culture supernatant were evaluated by ELISA. Survival rates were observed to be 100% at 14 days post-challenge among the immunized mice. Serum from pre- and post-challenge immunizations showed considerably greater optical density (OD) values of IgG than control mice. Although antibodies are developed against several antigens of different molecular weights, the protein fraction of 30-40 kDa showed a higher OD value. Formalin-inactivated intradermal immunization with MDR *A. baumannii* produced protective antibodies in mice against proteins of specific molecular weights. Proteins of 30-40 kDa molecular weights could be used as vaccine candidates to prevent *A. baumannii* infection.

Keywords

A. baumannii, Antibody, Bangladesh, Dhaka, Immunization, SDS-PAGE, Whole Cell Vaccine

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1. Introduction

The global emergence of multidrug-resistant strains of *Acinetobacter baumannii* worldwide which is a major nosocomial pathogen has become a challenge for developing effective treatment alternatives [1]. This gram-negative, multidrug-resistant (MDR) pathogen causes nosocomial infections that are particularly common in intensive care units (ICUs) and among immunocompromised patients having central venous catheters [2]. It is frequently found in healthcare settings and is highly effective at colonizing humans in hospital [3]. *Acinetobacter* has been shown to colonize the skin, also isolated from respiratory and oropharynx secretions in high numbers. In recent years, it has been designated as a "red alert" human pathogen due to its extensive antibiotic resistance spectrum [4].

Acinetobacter baumannii has developed a broad spectrum of antimicrobial resistance compared to other non-baumannii species, which is associated with a higher mortality rate among infected patients [2]. Many studies have identified the risk factors for the acquisition of A. baumannii infection, including older age, prolonged hospital or ICU stay, mechanical ventilation, previous antimicrobial therapy, low birth weight or prematurity, major or emergent surgery, use of devices, dialysis, and prolonged use of total parenteral nutrition or intravenous lipids [5]. The world health organization (WHO) has recently published a warning list of 12 bacteria for which there is an urgent need to develop new drugs. Carbapenem resistant *Acinetobacter baumannii* is ranked first on the list [6].

Vaccination against *A. baumannii* would eliminate or reduce the need for antibiotics and overcome many of the problems associated with antibiotic resistance. To improve the presentation of multiple antigens to the immune system, we might focus on development of whole-cell killed or live-attenuated vaccines [7]. Considering the prevention of drug-resistant infections, deactivation method could be simultaneously applied as an innovative strategy for the development of bacterial vaccines [7]. To identify potential vaccine candidates, identifying disease-associated antigens that elicit immune responses by combining separated proteins and immunological detection can be a good method [8]. For proteome separations, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is often used for size-based separations of proteins [9]. Data regarding antibodies against different antigen of different molecular size of *A. baumannii* in animal models are lacking. Therefore, this study was designed to develop protective antibodies against formaldehyde-killed whole-cell *Acinetobacter baumannii* in immunized mice sera and splenic lymphocyte culture supernatant using sonicated crude antigens as well as antigens of different molecular size separated by SDS-PAGE.

2. Materials and Methods

This in vivo study was conducted on Swiss albino mice in the department of Microbiology of the Medical College. Clinical samples from the admitted patients in Medical College Hospital were cultured on MacConkey agar media. From the growth culture plate, *Acinetobacter baumannii* was identified by observing pale colony on MacConkey's agar media, Gram-negative coccobacillary form on Gram stain, negative oxidase test, non-motile, urease negative and non lactose fermentation on TSI media. The isolated *A. baumannii* was preserved. Four to six weeks old 15 Swiss albino female mice were collected from icddr, b and kept, cared in animal house facility of Microbiology Department of Medical College. Mice were randomly divided into 3 groups: five mice in the experimental group (group-I), five mice in the control group (group-II), and five mice in the negative control group (group -III). Mice were provided a non-medicated and precise pathogen-free environment throughout the experiment.

2.1. Bacterial Culture

All bacterial strains were subcultured in Mueller Hinton agar and maintained at 37 ℃ for 24 hours to ensure that bacteria were at the same growth stage in all experimental steps before preparing vaccine.

2.2. Formalin Inactivated *A. baumannii* Whole-cell Vaccine Preparation

A loopful of the organism was inoculated into tryptic soy broth (TSB) and incubated at 37 ℃ overnight. Centrifugation was performed at 2000g for 20 minutes at 4 ℃ and supernatant was discarded. To wash twice the pelleted bacteria, Phosphate buffered saline was added. Then, 37% formalin was added to the suspension to achieve a final concentration of 3% (v/v) and the mixture was incubated for 2 hours at 37 ℃. Again, washed with sterile PBS two times to achieve conc. of 1.5× 10^8 CFU/ml. Subsequently, 134 µl of the inoculum was mixed with 866 µl of sterile PBS to achieve a concentration of 2×10^7 CFU /ml. Complete inactivation of the bacteria was confirmed by plating the supernatant onto culture plates and observing no growth after overnight incubation at 37 ℃.

2.3. Immunization Schedule of Mice with Formaldehyde-inactivated Whole-cell *A. baumannii* Vaccine

Group-I was vaccinated with formalin-inactivated solution prepared from a mixed suspension of *Acinetobacter baumannii* isolated from blood. Three intradermal immunizations were done with 20 µl of the bacterial solution (2×10^7 CFU /ml) on day 0, 14, and 28 in the upper and outer quadrant of

the alternate thigh of the experimental group-I mice, while 20 µl of sterile PBS was administered to the unimmunized control group-II on the same schedule. Control group-III was kept uninoculated. Prior to each immunization, an intraperitoneal ketamine injection (100mg/kg) was given. Intradermal inoculation was done with an insulin syringe BD Ultra-Fine^{TM} (31G) [10].

2.4. Collection of Serum from Mice for ELISA and Intraperitoneal Challenge of Mice

Blood was collected on 13th, 27th and 41st days from the tail of mice following first immunization. For this, mice were exposed to ketamine to reduce their consciousness level. The tail was then stretched, cleaned with 70% alcohol, and cut 2mm proximal to its blunt end using a sterile scalpel (22FR). Initially, 50 µl of fresh blood was collected into a microcentrifuge tube containing 200 µl phosphate buffered saline (PBS) to yield a dilution of 1:5. The cut end of the tail was then kept under pressure with sterile cotton for about 5 minutes to prevent oozing of blood. The diluted sera were kept upright for 2 hours followed by centrifugation at 3000g for 10 minutes. Blood cells settled to the bottom, and clear sera from the top of the tube were transferred into a separate sterile microcentrifuge tube [10].

On day 42, the mice from the experimental group and control group (group-II) were challenged intraperitoneally with 3 ×10^{8} CFU/ml of live MDR *Acinetobacter baumannii* in 100 µL of PBS. All mice were observed for 14 days of post-challenge for any clinical manifestations, such as lack of movement, weight loss, reluctance to feed, or death. Pooled sera were prepared by mixing 10 µl of sera from immunized mice collected on days 13, 27, 41 and after challenge.

2.5. Splenic Mononuclear Cell Processing

2.5.1. Separation of Splenic Mononuclear Cell from Mice Spleens

From each of the Group-1 (Experimental) and Group-III (control) mice, spleens were collected under aseptic conditions in order to detect antibody-producing cells against the inoculated antigen. Under a sterile biosafety cabinet, each spleen was kept into a petri dish containing 5 ml of complete RPMI medium (RPMI-1640, 10% FBS, 200 U/ml penicillin, 200 µg/ml gentamicin). The spleen was crushed between two frosted glass slides then filtered the cell suspension through a 70 µm Nylon cell strainer and centrifugation was done at 350g for 10 minutes at 4 ℃. Supernatant was discarded, and cells were resuspended in 5ml of complete RPMI medium [11].

2.5.2. Ensuring Presence of Viable Mononuclear Cells

An aliquot of the cell suspension was centrifuged for 5 min at 100g. The cell pellet was resuspended in 1 ml of PBS. Then, 20 µl of 0.4% trypan blue was mixed with 20 µl of cell suspension, incubate for 3 minutes at room temperature. A drop of trypan blue /cell mixture to a hemocytometer was applied. Unstained (viable) and stained (nonviable) cells were counted separately in the hemocytometer. Calculation of the percentage of viable cells was as follows [12]:

$$\text{Viable cell \%} = (\text{total number of viable cells per ml of aliquot} / \text{total number of cells per ml of aliquot}) \times 100$$

2.5.3. Culture of Splenic Cell in RPMI Media

Splenic mononuclear cells were taken in a 24-well culture plate containing 1 ml of RPMI media in each well. Then, 20 µl of diluted antigen was added to the culture plate and incubation of the plate was done at 37 ℃ for 6 days [11].

2.6. Sonication of Whole-cell *Acinetobacter baumannii*

Bacterial cells used in the preparation of the inactivated vaccine were resuspended in 100 µl of distilled water and incubated on ice for 30 minutes. Sonication was done at 20 kHz for 10 seconds. An additional 10 seconds of sonication was required depending on the thickness of the samples, followed by incubation on ice for 5 minutes. Centrifugation was done at 10000 g for 20 minutes. Supernatants were transferred to a new microcentrifuge tube and stored at -20 ℃ for use as antigen. Checkerboard titration was done to optimize the antigen concentration [10].

2.7. Procedure and Preparation of SDS-PAGE

SDS-polyacrylamide gel electrophoresis were carried out using 10 ml of 10% separating gel and 5ml of 5% stacking gel containing the desired concentration of H_2O, 30% acrylamide mix, 1.5 M Tris-HCl, 10% SDS, 10% ammonium persulfate and 10% N, N, N', N'-Tetramethylethylenediamine (TEMED). After polymerization (30 minutes) of separating gel and stalking gel sonicated samples (30 µl each) and molecular mass protein markers were loaded in wells for electrophoresis. Electrophoresis apparatus was poured with Tris-glycine electrophoresis buffer in the top and bottom reservoirs, and a voltage of 8 V/cm was applied to the gel to allow proteins to be separated by molecular size. Staining was done with Coomassie Brilliant Blue for 4 hours at room temperature followed by de-staining with distilled water for 24 hours. Identification and excision of the bands of interest

were done by using clean scalpel to cut sections of the gel that included the molecular weight marker and lanes of the protein sample corresponding to their respective bands which ranged from 11-17 kDa, 30-40 kDa, 60-70 kDa, 100-120 kDa [13]. For elution, excised gel pieces were placed in 0.5−1 mL of elution buffer in a microcentrifuge tube and crushed then incubated on a rotary shaker at 30 ℃ overnight. Centrifugation was done at 10,000 g for 10 minutes, and for ELISA, supernatant was stored at -20 ℃ [14].

2.8. ELISA

2.8.1. Detection of IgG Antibodies by ELISA with Sonicated & SDS - PAGE - Separated Antigens

Sonicated antigens and separated protein bands of different molecular weights were diluted to a final concentration of 10 µg/ml in a bicarbonate-coating buffer (pH 9.6) separately and ELISA plates were coated with 100 µl/well of diluted antigen (10µg/ml), incubated at 4 ℃ overnight, then washed two times with PBS followed by blocking with 200 µl/well of 5% w/v skimmed milk in PBS, incubated at 37 ℃ for 90 minutes, then washed 3 times with PBS-tween and once with PBS. Serum samples (100 µl/well) and splenic cell culture supernatants (300 µl/well) for sonicated antigens and pooled serum samples (100 µl/well) and splenic cell culture supernatants (300 µl/well) for separated protein bands as primary antibodies were added and incubated for 1 hour at 37 ℃, followed by overnight incubation at 4 ℃. After washing the plates, horse radish peroxidase-labeled anti-mouse IgG antibody (Thermo Fisher Scientific, USA) mixed with blocking buffer (1:5000), 100 µl/well was added and incubated at 37 ℃ for 90 minutes. After using substrate solution, absorbance (OD) was measured using an ELISA reader plate at 450nm (Biotek Inc, USA).

2.8.2. Calculation of cut off for Positive Value

Cut off value of OD was calculated by the following formula:

$$OD = Mean + 2 \times Standard\ deviation.$$

2.9. Ethical Approval

This study was approved by the Research Review committee and the Ethical Review Committee of the institution. Approval for animal experimentation was obtained from Animal Experimentation Ethics Committee (AEEC) of this institution.

2.10. Data Analysis

The results of the study were methodically documented. Data analysis was done by using 'Microsoft Office Excel 2020'. A t-test was used to analyze and compare the data and $p < 0.05$ was considered the minimal level of significance.

3. Result

The survival rate of immunized mice was 100% in vaccinated group, while 100% mortality was observed in the unvaccinated group. OD values in serum samples from immunized and unimmunized groups collected on days 13 (Figure 1a), 27(Figure 1b), 41 (Figure 1c) after 1st immunization and post challenge (Figure 1d) were analyzed. All the pre- and post-challenge sera from immunized group mice showed significantly higher IgG OD values compared to sera from unimmunized control mice ($P<0.001$) after each inoculation. A statistically significant difference ($p<0.001$) between the OD values of immunized group-I and unimmunized group-III mice splenic cell culture supernatant was observed (Figure 2a). In SDS-PAGE of sonicated *Acinetobacter baumannii*, protein bands of *Acinetobacter baumannii* were identified according to the range of molecular weight (11-17 kDa, 30-40kDa, 60-70kDa and 100-120kDa). OD values of IgG of the pooled sera and splenic lymphocyte culture supernatant of immunized group-I against eluted antigens of different molecular weight separated by SDS-PAGE shown with highest absorbance recorded against protein with MW 30-40 kDa (Figure 2b).

Table 1. *Optical density (OD) value range of IgG absorbance, mean of negative control, standard deviation and cut off value of mice sera collected during different vaccination schedule.*

Vaccine schedule	OD value range	Mean of negative control	Standard deviation	Cut off value
1st inoculation	0.517-0.735	0.131	0.008	0.147
2nd inoculation	1.298-1.392	0.129	0.002	0.132
3rd inoculation	1.519-1.930	0.129	0.227	0.142
Post challenge	1.253-1.651	0.133	0.005	0.144

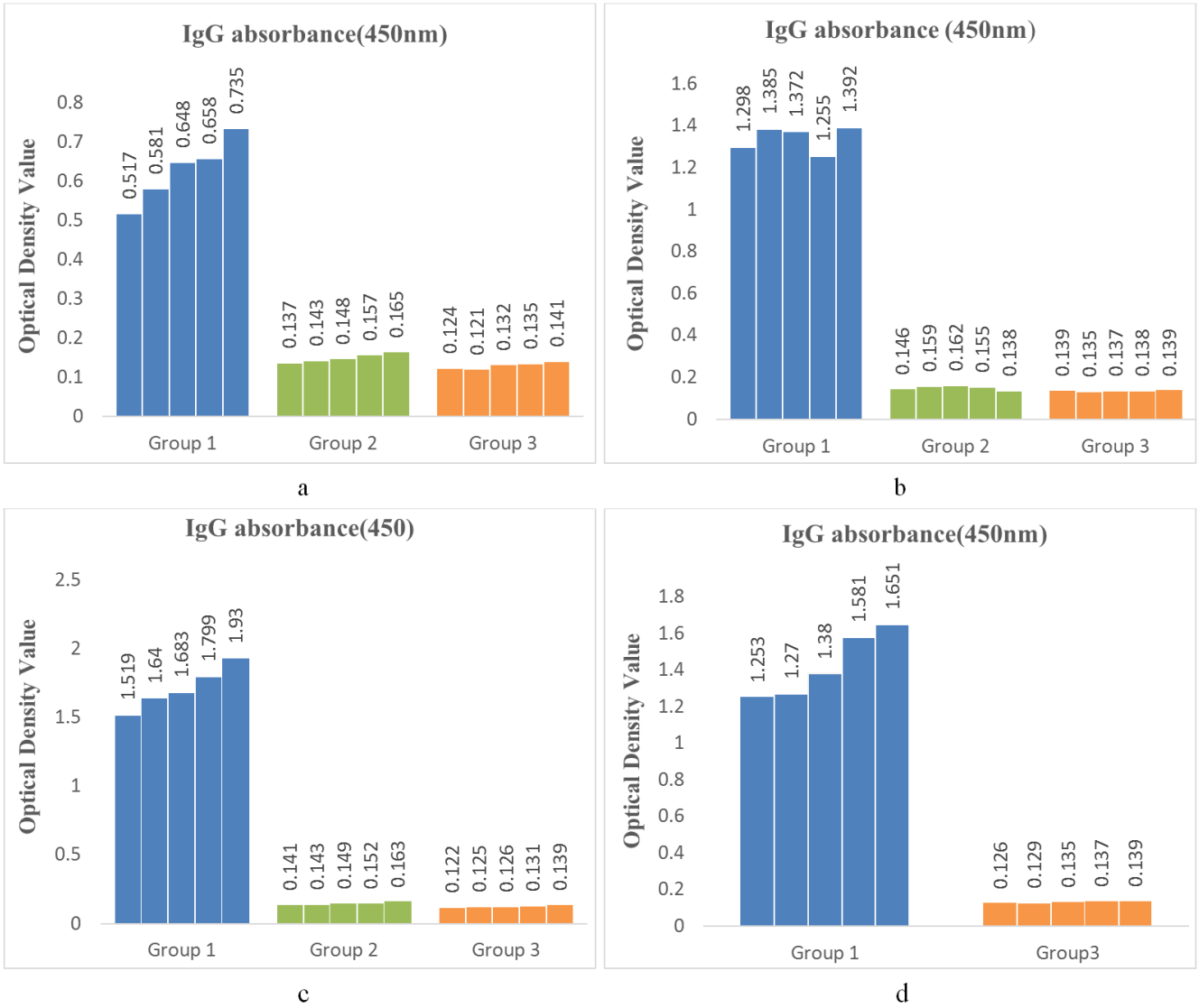
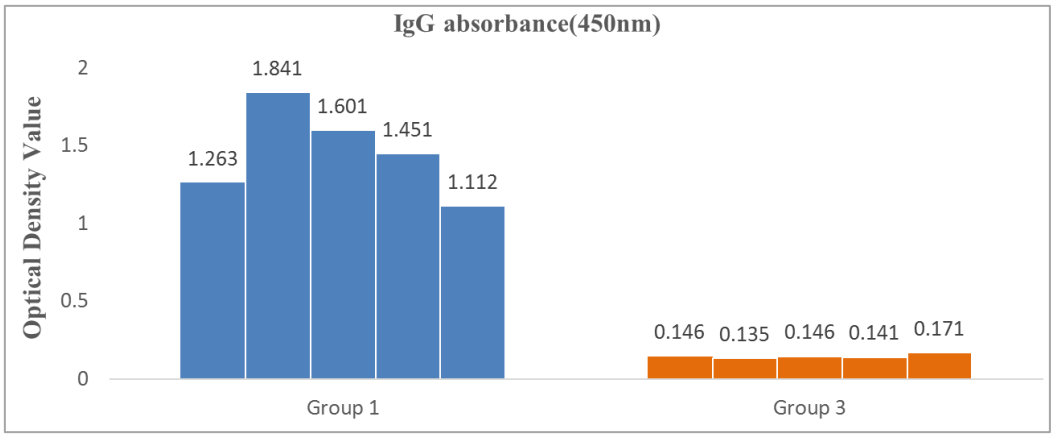
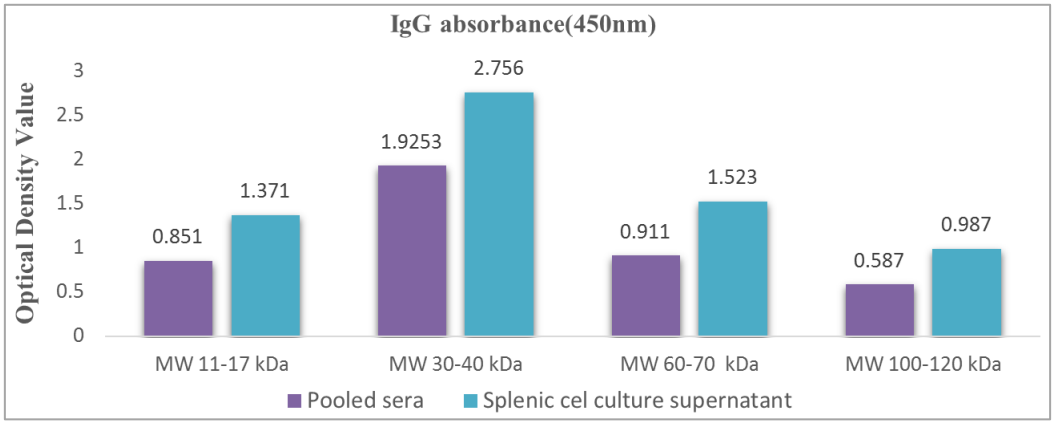


Figure 1. *Optical density (OD) of immunized and unimmunized control serum samples after the first (a), second (b), third (c) inoculation, and post-challenge (d) by ELISA. Here, the OD values of IgG in serum samples collected on the 13th day following the first immunization showed that Group I had OD values above the cutoff value of 0.147 (a). On the 27th day following the first immunization, Group I had OD values above the cutoff value of 0.132 (b). On the 41st day following the first immunization, Group I had OD values above the cutoff value of 0.142 (c). After the lethal challenge, Group II mice did not survive, while Group I had OD values above the cutoff value of 0.144 (d). Here, Group I = immunized mice, Group II = unimmunized mice with PBS inoculation, and Group III = unimmunized and uninoculated mice. The cutoff values are mentioned in Table 1.*



(a)



(b)

Figure 2. *(a) IgG titer in immunized and control group mice splenic cell culture supernatant after incubation in RPMI media. (b) ELISA reaction of the pooled sera and splenic lymphocyte culture supernatant of immunized group against eluted antigens of different molecular weights separated by SDS-PAGE shown with highest absorbance recorded against protein with MW 30-40 kDa.*

4. Discussion

In this study, 100% vaccinated mice survived after lethal challenge, which is similar to other studies [15, 16] where mice were immunized intramuscularly with the formalin-inactivated whole-cell vaccine against *Acinetobacter baumannii* demonstrated full protection and 80% survival rate after challenge respectively. A study by Kawser *et al* reported the survival proportion among the immunized mice was 80% at 14 days post-challenge with *Klebsiella pneumoniae* [10].

In the current study, OD values of serum IgG absorbance showed a significant increase in IgG antibody absorbance ($p<0.001$) within the immunized group of mice after 2nd and 3rd inoculations. After all inoculations, IgG values in the sera of unimmunized and negative control group mice were similar which is significantly lower than those in sera and splenic cell culture supernatant of immunized mice.

The Survival of 100% of vaccinated mice after the lethal challenge might be due to the fact that protective antibodies were produced after vaccination in experimental group. This is further supported by the presence of high titer of IgG antibodies in the sera and splenic cell culture supernatant of immunized mice. On the other hand, the death of all the control mice (group II) after the lethal challenge can be explained by the absence of protective antibodies, as these mice were not immunized, which was further evidenced by the presence of very low titers of IgG antibodies in both the sera and splenic cell culture supernatant of these mice.

Consistent with the present findings, formalin-inactivated whole-cell vaccination resulted in significantly higher levels of IgG in mice that received the vaccination compared to the control group [17]. The results of the current study demonstrated that the pre-challenge mouse sera's IgG OD values reached their maximum following the third inoculation. Post challenge sera IgG OD values were slightly lower than the pre-challenge sera IgG OD values in experimental mice and this can be explained by the possibility that some of the antibodies were utilized in killing the live bacteria after the challenge in immunized group of mice. When immunized mice were challenged intraperitoneally by live *A. baumannii*, specific IgG antibodies developed against antigens bound to the bacteria, leading to compliment activation via classical pathway, which may result in bacterial lysis. The other mechanism might be, once IgG binds with the bacterial antigen (opsonization), phagocytosis occurs.

In the splenic cell culture supernatant, immunized group had an optical density of IgG antibody above the cut off value. There was a statistically significant difference ($p< 0.01$) between the optical density values of the immunized and unimmunized control mice's cell culture supernatant. This can be explained by robust production of antibodies by sensitized B lymphocytes as well as memory B cells present in the spleen of immunized mice once, they were stimulated by the sonicated antigens of *A. baumannii* in the splenic cell culture media.

The IgG antibody absorbance of the crushed band's supernatant with pooled serum and splenic cell culture supernatant showed that OD value of the antigen fraction with a molecular weight of 30-40 kDa was higher than the OD values of other bands used. This indicates that protein bands of *Acinetobacter baumannii* ranging from 30-40 kDa were more antigenic and could induce more antibodies from B cell followed by 60-70 kDa bands. A study by Sheweita et al also showed that proteins of *Acinetobacter baumannii* with molecular weights 70, 60, 40, and 30 kDa may be responsible for the production of immunoglobulins [18]. Therefore, according to the results of this study, the protein band ranging from 30-40 kDa could be used as a candidate vaccine against *Acinetobacter baumannii. A. baumannii* can survive in different conditions and cause multidrug-resistant infections, especially in hospitalized, patient and vaccine could reduce mortality and morbidity of these patients. However, limitation of

this study was antibody responses against individual antigens within the 30-40 kDa range were not assessed.

5. Conclusion

This study showed that, inoculation of formalin-inactivated *Acinetobacter baumannii* in experimental group of Swiss albino mice produced a higher level of protective antibodies (IgG) evidenced by 100% survival rate of the immunized mice compared to unimmunized control mice. Additionally, although antibodies were developed against several antigens of different molecular weights, the protein fraction of 30-40 kDa separated by SDS-PAGE showed a higher OD value when reacted with both the pooled serum and splenic cell culture supernatant of the vaccinated group of mice compared to other proteins. Therefore, this protein with a molecular weight ranging from 30-40 kDa may be used as good vaccine candidate to prevent *Acinetobacter baumannii* infection. Identification of specific molecular weight antigen within this range could be evaluated in the future, which could be a new step in future research.

Abbreviations

AEEC	Animal Experimentation Ethics Committee
CFU	Colony Forming Unit
ELISA	Enzyme-Linked Immunosorbent Assay
ERC	Ethical Review Committee
Icddr, b	International Center for Diarrheal Disease Research, Bangladesh
ICU	Intensive Care Unit
MDR	Multidrug Resistant
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RPMI	Roswell Park Memorial 1640 Medium
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	N, N, N', N'-Tetramethylethylenediamine
TSB	Tryptic Soy Broth
WHO	World Health Organization

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Author Contributions

Kohinur Hasan: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing

SM Shamsuzzaman: Conceptualization, Formal Analysis, Methodology, Project administration, Supervision, Writing – review & editing

Mohammad Harun Ur Rashid: Formal Analysis, Methodology, Software, Validation, Visualization, Writing – review & editing

Farhana Parveen: Formal Analysis, Investigation, Resources, Writing – review & editing

Informed Consent Statement

Informed consent was obtained from all studied subjects in the study.

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Conflicts of Interest

The authors declare no conflicts of interest.

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