ORIGINAL ARTICLE

# Immunogenicity and Efficacy of Heat Inactivated Whole-Cell Vaccine to Increase Murine Survival after Extensively Drug-resistant *Acinetobacter baumannii* Infection

Nazmun Sharmin[1], Md. Mahbub E Khoda[2], Mohammad Nazim Uddin[3]

## ABSTRACT

***Background:*** Due to the rapid emergence of extensively drug-resistant strains of *Acinetobacter baumannii* worldwide, there is a necessity for greater consideration of the role of preventive vaccines in combating these pathogens. ***Objective:*** The study aims to assess the effectiveness of a heat-inactivated whole-cell vaccine against *A. baumannii.* ***Methods:*** Eleven Swiss albino mice were divided into Experimental (n=4), Placebo-controlled (n=4), and Negative Control (n=3) Groups. Clinically isolated extensively drug-resistant *A. baumannii* species emulsified wih Complete Freund's adjuvant was used for intramuscular vaccination in the Experimental group in three different phases on 14-day intervals whereas the Placebo-controlled group received phosphate-buffered saline emulsified with CFA in the same schedule and Negative Control group was kept unimmunized. Serum was collected from the tail blood of each mouse on the 10th day after each immunization, by cardiac puncture on the 14th day after the lethal dose from the experimental group, and after the death of the placebo-controlled group. ***Results:*** Mice inoculated with heat-inactivated whole-cell vaccine survived and showed a higher neutralizing (IgG) antibody response after 2nd (>7-fold titer) and 3rd inoculation (>12-fold titer) whereas all mice from the Placebo-controlled group and Negative Control group did not survive after lethal challenge with different extensively drug-resistant strain of *A. baumannii.* Sera from the experimental group of mice collected after 3rd inoculation resulted in a higher serum bactericidal killing index after three hours of incubation. ***Conclusion:*** These results suggest intramuscular vaccination with heat-inactivated whole-cell vaccine survival after extensively drug-resistant *A. baumannii* infection.

**Keywords:** Acinetobacter baumannii, heat inactivated whole cell vaccine, serum bactericidal antibody

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## INTRODUCTION

*Acinetobacter* species are non-fermentative, non-fastidious, catalase-positive, oxidase-negative, non-motile, and strictly aerobic gram-negative coccobacilli[1]. Among these species, *A. baumannii* causes serious infections that are associated with high morbidity and mortality rates[2] particularly, in immunocompromised patients, elderly, premature neonates, and patients who had recently undergone surgery or experienced major trauma or were previously admitted to contaminated ICUs[3] causing septicemia, endocarditis, meningitis, skin and soft tissue infection, wound infection, respiratory tract infections and urinary tract infections[4]. The World Health Organization declared that A. baumannii is one of the most serious ESKAPE organisms *(Enterococcus faecium, Staphylococcus aureus, Klebsiella*

1. Department of Microbiology, National Institute of Cardiovascular Diseases (NICVD), Dhaka-1207, Bangladesh.
2. Department of Respiratory Medicine, Dhaka Medical College & Hospital, Dhaka-1000, Bangladesh.
3. Department of Pharmacology & Therapeutics, Shaheed Monsur Ali Medical College, Uttara, Dhaka-1230, Bangladesh

**Correspondence to:** Dr. Nazmun Sharmin, Department of Microbiology, National Institute of Cardiovascular Diseases (NICVD), Dhaka-1207, Bangladesh. Email: dr.nazmun17@gmail.com

*pneumoniae, A. baumannii, Pseudomonas aeruginosa, and Enterobacter species)* that effectively escape the effects of antibacterial drugs[5]. Now, multidrug-resistant (MDR) and extensively drug-resistant (XDR) A. baumannii have emerged worldwide as critical problems and have been increasingly reported[6] due to the widespread and indiscriminate use of antibiotics.

*Acinetobacter baumannii* exhibits an intrinsic resistance to a range of antibiotics such as amoxicillin, narrow-spectrum cephalosporins, ertapenem, trimethoprim, and chloramphenicol[7]. In addition, *A. baumannii* has managed to acquire resistance genes against several classes of antibiotics through the transfer of plasmids, transposons, and integrons from other gram-negative bacteria[8]. Currently, there are a small number of drug candidates like colistin, tigecycline, eravacycline[9], cefiderocol and other therapeutic options such as monoclonal antibodies[10], phages[11] that may be used to treat XDR *A. baumannii* infections. Several researchers adopted the idea of solving the A. baumannii problem by vaccination[12]. To date, there are no licensed vaccines against XDR *A. baumannii*.

Among the inactivated vaccine trials some single antigen candidates have been tested, the outer membrane protein (OmpA), the K1 capsular polysaccharide, the biofilm-associated protein (Bap), and the membrane-associated polysaccharide (poly-N-acetyl-β-1-6-glucosamine)[13]. However, heat-inactivated whole-cell vaccines are easy to prepare, inexpensive, and do not require expensive denaturation of antigens, that may induce conformational changes of the epitopes. Previous studies have shown that emulsification with CFA and IM immunization in mice generates hyper-immune antibodies against its multi-antigenic components, mostly those belonging to the outer membrane[14], providing diverse protection against several A. baumannii species with a complete bacterial clearance from the infected tissues resulting in increased mice survival rates[15]. Furthermore, the vaccine proved to induce a protective level of immunoglobulins and reduced the pro-inflammatory cytokines serum levels of IL-1β, TNF-α, and IL-6 that are normally associated with sepsis and helped to secure high survival rates in vaccinated mice associated with both pneumonia and sepsis[16] that could be lifesaving in case of outbreaks or critical conditions[17].

We, therefore, attempted to develop a HI-WCV derived from clinically isolated XDR A. baumannii species and investigated the potential efficacy of enhancing protective immunity against other XDR A. baumannii, by evaluating the ability of the vaccine to improve the complement-mediated serum bactericidal activities of the immunized mice sera after intramuscular inoculation.

## METHODS

***Sample collection:*** Clinical samples like endotracheal aspirate, blood, and wound swabs were collected from patients admitted in different departments, especially the intensive care unit and burn units of Dhaka Medical College Hospital, Dhaka, Bangladesh.

***Phenotypic identification:*** *Acinetobacter spp.* was identified by observing colony morphology on blood agar (non-pigmented, white or cream-colored, smooth to mucoid colonies, 1-2 mm in diameter, non-hemolytic), on MacConkey agar (generally form colorless colonies), Gram staining (gram negative coccobacilli) and biochemical tests- oxidase test (negative), and catalase tests (positive), urease production (variable), indole test (negative), motility (non-motile) and citrate utilization test (positive)[18].

***Genotypic identification:*** PCR with a blaoxa-51-like gene was used to identify *A. baumannii* from the isolated Acinetobacter spp.[19].

***Antimicrobial susceptibility test:*** Susceptibility was determined by a modified Kirby-Bauer disc diffusion technique using Mueller-Hinton (MH) agar plates and zones of inhibition were interpreted according to Clinical Laboratory Standard Institute guidelines[20]. Criteria of the United States Food and Drug Administration[21] were used for the interpretation of the zone of inhibition of tigecycline. The antimicrobial discs (Oxoid Ltd, UK) -amoxiclav (amoxicillin 20µg and clavulanic acid 10µg), piperacillin-tazobactam (100µg)/10µg), ceftazidime (30µg), ceftriaxone (30µg), cefepime (30µg), cefoxitin (30µg), aztreonam (30µg), ciprofloxacin (5µg), imipenem (10µg), amikacin (30µg), gentamycin (10µg), doxycycline (30µg), tigecycline (15µg) and colistin (8mg/ml) and *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains[22]. The isolates that were resistant to all 14 antibiotics were further isolated for preparation of HI-WCV.

***Preparation of HI-WCV:*** A loop of XDR A. baumannii was inoculated into TSB and cultured overnight at 37˚C and then centrifuged at 3,000g for one minute. The supernatants were discarded, and the cell pellets were washed twice with PBS. Subsequently, the bacterial concentrations were determined by serial dilution with normal saline and plating onto MacConkey agar plates followed by incubation at 37˚C for 24 hours. The bacterial cells corresponding to 1×107 CFU/ml were heat-inactivated in a water bath by incubating for 30 minutes at 56˚C. Complete inactivation of the bacteria was confirmed by plating the supernatant onto Mueller-Hinton agar plates[23] and then storing it at -20˚C until inoculation.

***Mice and inoculation schedule:*** Eleven 6-8 weeks-old male Swiss-albino mice were collected and kept under specific pathogen-free conditions with a non-medicated diet. The mice were randomly divided into 3 groups where EG and PC groups contained 4 mice and the negative control (NC) group contained 3 mice.

***Inoculation schedule of mice:*** Three Intramuscular (IM) inoculations were performed on days 0, 14, and 28 (Figure 1A) in the alternate thigh of the EG group of mice with 20µl of heat-inactivated whole-cell A. baumannii emulsified with 20µl CFA (1:1), and with 20µl of PBS emulsified with 20µl CFA (1:1) for the PC group of mice on the same day. The IM injection was done with an insulin syringe BD Ultra-Fine TM (31G). CFA (Sigma-Aldrich, Missouri, 50 United States) was used for the first immunization. The prepared mixtures were inoculated after intraperitoneal injection of ketamine (ketamine 100 mg/kg) and chloroform by inhalation.

***Survival analysis:*** All the mice from each group were observed continuously for fourteen days after lethal challenge (1.5×108 CFU/ml) with a live different strain of A. baumannii. Mice from the NC Group and PC group died within 72 hours due to the development of septicemia. On the other hand, Mice from the EG Group survived and were fully active for all fourteen days of observation.

***Serum collection:*** Tail blood was collected after sterilization with 70% alcohol before inoculation and on the 10th day after each inoculation and about one ml blood was collected in a sterile test tube by cardiac puncture from euthanized NC group, immediately after death from the PC group and 14 days after the lethal challenge from EG group (Figure 1A).

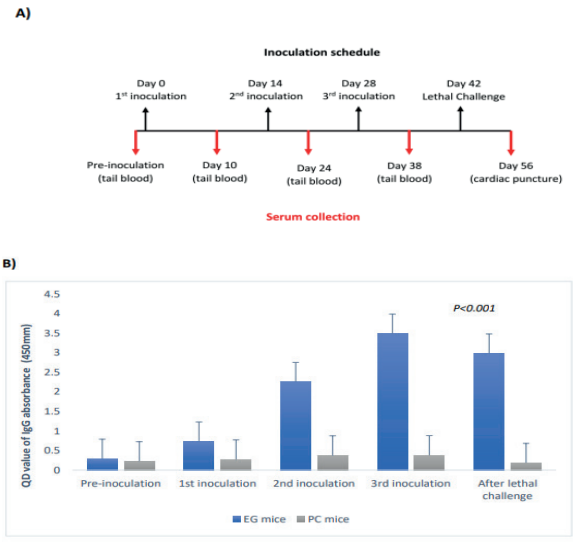


**Figure 1: A)** Both EG and PC groups (n=4) were inoculated with HI-WCV on Day 0, 14, and 28 intramuscularly, and a lethal challenge was given intraperitoneally on Day 42. Serum was collected from tail blood before inoculation and on Day 10, 24, 38. Blood was collected by cardiac puncture from the euthanized NC group, immediately after death from the PC group and on Day 56 after the lethal challenge from the EG group. **B)** Mean Optical Density of serum samples before and after each inoculation (Day 0, 14, 28) and after lethal challenge (Day 42) of both EC Group and PC Group of mice (n=4). Here, $p<0.001$ except in pre-inoculation $(p=2.77)$.

***Detection of IgG antibody:*** Bacterial cells used in the preparation of HI-WCV of A. baumannii (1×107 CFU/ml), were used as antigens after sonication. Protein concentration was detected by a nanodrop spectrophotometer (ThermoFisher Scientific). Immunoplates (Maxisorp, Nun) were coated with 100 µl/well of 10µg/ml antigen overnight at 4°C in the carbonate buffer. Subsequently, wells were blocked with 200µl blocking buffer (5% w/v non-fat dry milk in PBS-0.05% tween 20) and incubated at 37˚C for 1-2 hours (s) after covering with adhesive plastic and washed 3 times with a wash buffer (1×PBST). 100µl of diluted serum sample 1:100 in 1% bovine serum albumin in 1×PBS containing 0.1% Tween (PBST) were added to duplicate wells and incubated at 37°C for one hour. To each well, 100µl of a 1:5000 dilution of goat anti-mouse IgG peroxidase-conjugated antibodies (Thermo Fisher Scientific, USA) was added and incubated at 37°C for one hour. The plates were washed three times with wash buffer post all incubations. The plate was developed

using TMB, following 1M sulfuric acid addition to stop the reaction, and read at 450/630nm by ELISA plate reader (Biotek Inc., USA). Cut off value of OD was calculated by following the formula:

OD= M (mean) + 3 × SD (Standard deviation)

***Serum Bactericidal Antibody Assay:*** An XDR *A. baumannii* isolated stock was made by using PBS to determine the dilution (1:1,250) necessary to yield ~100 CFU/spot on MH agar plates. The serum samples from EG of mice were heat-inactivated in a water bath by incubating at 56˚C for 30 minutes. 3-4 weeks old two guinea pigs' sera were used as a source of complement and to heat-inactive, serum was incubated at 56˚C for 30 min. SBA was performed with some modifications[24]. After diluting the bacteria in 20ml of PBS according to the pre-determined optimal dilution factor (1:1250), 10µl of diluted bacteria was added to each well of the assay plate. 50µl of 20% solution heat-inactivated guinea pig complement was added to all wells in column 1 (control A wells) and 50µl of 20% solution native guinea pig complement was added to all wells in columns 2 through 12 (Control B and test sample wells). The plate was incubated at 37˚C for 3 hours after brief mixing. 10µl of the reaction mixture was taken from each well onto an MH agar plate, tilted, and allowed the spots to run for ~1cm and incubated in the incubator upside-down for 16-18 hours.

Photograph of the plates was taken using a digital camera and transferred images to a computer to count the number of colonies in each spot. Calculation of non-specific killing and 50% Killing Index was done by the following formula:

Non-specific killing (NSK) = 1-Control B/ Control A,

50% Killing Index (KI) = Control B/2

Because a serum dilution would rarely yield exactly this 50% KI value, it was interpolated from two sequential serum dilutions, one that kills less than 50% and one that kills more than 50%, (Figure 2a, b, c & d)

The formula for calculating the interpolated SBA KI is shown below:

SBA KI=10^({logX1 ((Y50-Y1)×(logX2-logX1))/((Y2-Y1))})

***Statistical analysis:*** Data from the antibody response and complement-mediated serum bacteriolysis were expressed as the mean± standard deviation from repeated assays. The data were subjected to a two-way analysis of variance (ANOVA). Statistical significance was considered as $p<0.05$. All the statistical analyses were performed using Stata software version 14. (StataCorp LLC, USA).

## RESULTS

***In vivo assay:*** Immunized mice sera were collected before 1st inoculation and on the 10th day after each vaccine inoculation from both EG and PC Group mice and humoral immune response was evaluated by the mean Optical Density (OD) values of duplicates of serum IgG absorbance at 450 nm in 1:160 dilution by indirect ELISA (Figure 1B). Among EG mice mean OD values of pre-inoculation and after 1st inoculation (Day 10) were 0.29±0.028 and 0.330±0.002 respectively. After 2nd inoculation (Day 24) mean OD value was 2.251±0.262 showing a rise in serum antibody titer resulting in a rapid memory response peak ($p<0.001$). Moreover, the mean OD value was highest (3.487±0.275) after 3rd inoculation where serum was collected on day 38 ($p<0.001$). However, there was a decrease in mean OD value (2.981±0.119) after lethal dose (Day 56) ($p<0.001$). The sera from PC Group mice contained very low levels of detectable antibodies at any time-point with an average OD value of 0.0376±0.012 which was very similar to NC Group mice serum OD value and all the mice from this group died within 24 hours after injection of the lethal dose.

***In vitro assay:*** Immunized mice sera collected on days 10, 24, 38, and 56 were tested for the ability to promote in vitro complement-mediated serum bactericidal activity of the test XDR *A. baumannii.* Table 1 shows the organized colony counts per spot from MH agar plates after overnight incubation at 37˚C at 8 different dilutions. Bacterial killing is seen for all samples tested in the first three dilutions and as samples are diluted further up the plate, a decrease in bacterial killing is seen where serum is less concentrated. For control A the mean CFU count was 36 in all dilutions. For control B the mean CFU count was 29 in all dilutions. For pre-inoculation, the mean CFU count was between 24 to 35 in all dilutions indicating no inhibition of bacterial survival. The calculated non-specific killing (NSK) was 23%.

**Table 1:** The organization of CFU counts/spot on MH agar plates

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Dilution |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 41 | 35 | 36 | 34 | 43 | 45 | 32 | 30 | 23 | 21 | 15 | 35 | Dil **8** **1:17496** |
| B | 38 | 33 | 31 | 37 | 39 | 35 | 24 | 21 | 12 | 15 | 19 | 8 | Dil **7** **1:5832** |
| C | 39 | 31 | 37 | 33 | 25 | 29 | 17 | 18 | 8 | 10 | 10 | 9 | Dil **6** **1:1944** |
| D | 35 | 34 | 32 | 36 | 14 | 19 | 5 | 6 | 4 | 8 | 7 | 6 | Dil **5** **1:648** |
| E | 32 | 26 | 21 | 27 | 9 | 7 | 0 | 1 | 1 | 1 | 1 | 1 | Dil **4** **1:216** |
| F | 33 | 28 | 23 | 25 | 2 | 3 | 3 | 1 | 1 | 0 | 2 | 1 | Dil **3** **1:72** |
| G | 38 | 26 | 24 | 28 | 2 | 3 | 1 | 0 | 1 | 2 | 0 | 1 | Dil **2** **1:24** |
| H | 34 | 22 | 29 | 27 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | Dil **1** **1:8** |
| | **control A** | **control B** | **Pre-inoculation** | | **1st inoculation** | | **2nd inoculation** | | **3rd inoculation** | | **After lethal challenge** | | |

**Table 2:** Comparison of the differences of rising in IgG antibody and SBA titers

| Immunization schedule | Day | PC Group (mean) | ± SD | EG Group (mean) | ± SD | Titre fold rise | 50% SBA titre | SBA KI | Titre fold rise |
|---|---|---|---|---|---|---|---|---|---|
| **After 1st inoculation** | 10 | 0.0127 | 0.0011 | 0.029 | 0.0284 | 2 | 15 | 19.1 | 1.27 |
| **After 2nd inoculation** | 24 | 0.0376 | 0.0104 | 0.330 | 0.0026 | 8 | 10 | 20.04 | 2 |
| **After 3rd inoculation** | 38 | 0.080 | 0.0077 | 2.252 | 0.2629 | 27 | 6 | 42.12 | 7 |
| **After lethal dose** | 56 | 0.078 | 0.0121 | 3.488 | 0.2759 | 42 | 4 | 19.27 | 4.8 |

Here, the 50% killing index (KI) cut-off value was 14.5. During the 1st, 2nd, 3rd inoculation and after the lethal challenge, the mean CFU count for 1st three dilutions (1:8, 1:24, 1:72) at 25% and 50% guinea pig complement concentration was between 1.5 to 2.5, 0 to 2, 0 to 1.5 and 0 to 1.5 respectively, which indicated bactericidal efficacy of the immunized serums. The highest SBA KI of 42.12 was found after the 3rd inoculation, whereas the lowest SBA KI of 19.1 was found after the 1st inoculation (Figure 2a, b, c & d).

***Comparison of in vivo and in vitro assay:*** The neutralizing (IgG) antibody titer was significantly increased after the 2nd (7-fold) and 3rd vaccination (12-fold). On the other hand, a 7-fold rise in SBA KI from the 50% SBA cutoff value was only shown after the 3rd inoculation (Table 2).

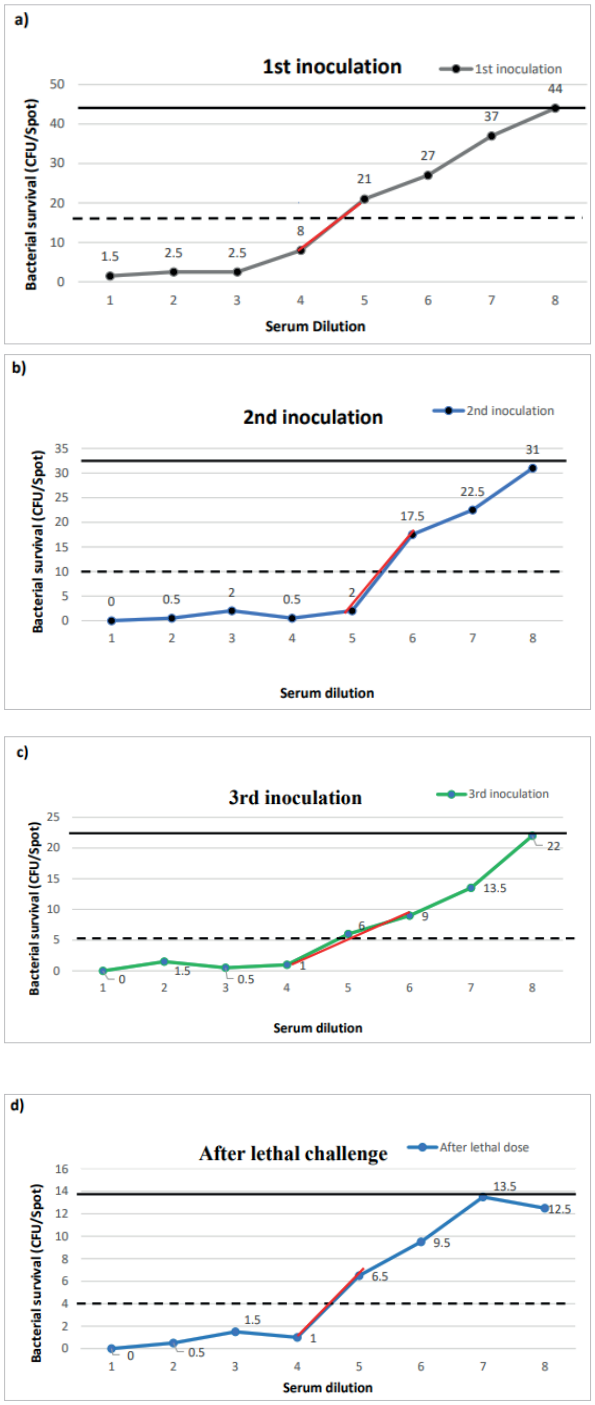


**Figure 2: a), b), c), d),** shows a schematic of linear interpolation after each inoculation. The number of surviving bacteria (y-axis) at each dilution of serum tested (x-axis) is plotted (balls) and individual points were connected by a thin line. The solid and dashed horizontal lines indicated 0% and 50% killing, respectively. The serum dilutions above (e.g., Dilution 5) and below (e.g., Dilution 4) the 50% killing line were connected by a red line, and bactericidal titer (KI) was indicated.

## DISCUSSION

Over the past few decades, *A. baumannii* has emerged as a major healthcare facility-acquired bacterial infection because of resistance to many classes of antibiotics by chromosome-mediated genetic elements[25], leading to the rapid emergence of MDR, XDR, and pan-drug-resistant (PDR) strains that cause the consideration for preventive vaccines to combat. Though some single antigen candidates have been tested, these are still under trial. Various studies regarding heat-inactivated whole-cell vaccines showed that these were easy to prepare, inexpensive, and did not require expensive denaturation of antigens, which may induce conformational changes of the epitopes and produce protective immunity (IgG Ab) against XDR *A. baumannii*, in murine models after intramuscular administration[23].

In the current study, sera collected from each mouse after each inoculation were analyzed for IgG antibody absorbance by indirect ELISA at 450 nm. The OD values of serum IgG absorbance at 1:160 dilution showed that there was a significant rise ($p<0.001$) in the OD value of IgG antibody absorbance within the EG Group of mice after 2nd and 3rd inoculation though the level was slightly decreased after intraperitoneal injection of lethal dose with 1.5×108 CFU/ml live XDR A. baumannii which might be due to utilization of the previously formed IgG antibody to neutralize A. baumannii antigen. The sera from PC mice contained very low levels of detectable antibodies after all inoculation which was very similar to NC group mice. Hence the mice from the EG Group survived whereas all the mice from the PC Group were dead after lethal challenge as the serum neutralizing antibody was insufficient for them to survive. A study done by Shu et al.[23] showed that heat-inactivated whole-cell vaccine-immunized mice sera collected on days 0 to 36 had an increase in IgG antibodies. The OD values of IgG absorbance of immunized mice sera collected after the second inoculation on day 22 resulted in a rapid memory response peak. However, the OD values on day 36 in immunized mice sera, showed a slight decrease in the antibody response. The sera from placebo-inoculated control mice contained very low levels of detectable antibodies at any time point. The findings suggested that inoculation with heat-inactivated whole-cell vaccine stimulated high levels of serum IgG antibodies recognizing the XDR *A. baumannii* strain as the target antigen.

Activation of the complement pathway is an important immune defense mechanism present in serum. Therefore, complement activation could play an important role in minimizing the ability of A. baumannii to escape immune clearance. The present study showed that the sera obtained from mice inoculated with heat-inactivated whole-cell vaccine were able to activate complement-mediated bacteriolysis in the presence of 25% guinea pig complement at 1:216 serum dilution after three hours of incubation with XDR A. baumannii. It was noted that the bacteriolysis effects in sera of mice from the 1st and 2nd inoculation with HI-WCV collected on the 10th day and 24th day showed only a slight increase from the 50% SBA titer, suggesting that sera from two subsequent immunizations did not significantly increase the complement-mediated serum bacteriolysis activity. However, the SBA KI of the EG Group after 3rd inoculation suggested that they were able to mount effective responses, resulting in the inactivation of the virulent XDR A. baumannii although it was decreased after injecting the lethal dose which might be due to utilization of the previously formed antibody to kill the virulent A. baumannii injected by intraperitoneal route.

The comparison of the rising fold of IgG antibody titers of the EG Group with the PC Group and analysis of SBA titers in post-vaccinated sera showed that the IgG antibody titer was significantly increased after the 2nd and 3rd inoculation. On the other hand, a rise in SBA KI from 50% SBA titer was only shown after the 3rd inoculation. These variations might be due to several factors including source and quantity of exogenous complements, bacterial strain, test sera, and antigen expression in targeted bacteria. A study done by McConnell et al.[26] also suggested that HI-WCV can aid in the clearance of and accord protection against *A. baumannii* infections.

## CONCLUSION

All these results conclude that, though a single immunization of heat-inactivated whole-cell vaccine did not significantly increase immune response and the complement-mediated bacteriolysis activity, the results after the booster immunization were able to mount effective responses, resulting in inactivation of the XDR A. baumannii. This vaccine development approach may further the development of viable vaccines against XDR A. baumannii infections.

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**Competing Interests:** All authors have no competing interests.

**Financial Support:** Nil

**Ethical Clearance:** The study was performed according to the protocols and guidelines approved by the Ethical Review Committee of Dhaka Medical College, Dhaka, Bangladesh (MEU-DMC/ECC/2019/256).

**Authors' Contribution:** Conception and design of the study: N Sharmin, data collection, analysis, validation and visualization: N Sharmin, MN Uddin, Manuscript preparation, review, editing and submission: N Sharmin, MME Khoda, MN Uddin

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