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Evaluation of Protective Antibody Response following Vaccination with Formaldehyde Inactivated *Enterobacter cloacae*

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Abstract

Background: Multi-drug-resistant (MDR) strains of Enterobacter cloacae are becoming increasingly prevalent worldwide, which is reducing treatment options. **Objective:** The purpose of the present study was to evaluate protective antibody response following vaccination with formaldehyde inactivated *Enterobacter cloacae*. **Methodology:** This was an animal study which was conducted in the Department of Microbioloogy at Dhaka Medical College, Dhaka, Bangladesh for a period of one year from January 2022 to December 2022. The study used formaldehyde inactivated MDR Enterobacter cloacae obtained from various clinical samples to immunize 10 Swiss albino mice through intradermal injection. After the third dose of immunization, the mice were challenged with live Enterobacter cloacae through intraperitoneal injection and were monitored for 14 days. Blood was collected from the tail 7 days after each booster and through cardiac puncture 14 days after the challenge. The serum antibody produced by the vaccine was evaluated for bactericidal activity and antigen-binding capacity using ELISA. **Results:** In this study, it was observed that 100% of the immunized mice survived after 14 days of challenge. The ELISA showed that all the immunized mouse sera had 100% bactericidal activity. Additionally, all the pre- and post-challenge immunized serum immunoglobulin G antibodies had significantly higher optical density values compared to the control mice in ELISA. **Conclusion:** Our study showed that when formaldehyde inactivated MDR *Enterobacter cloacae* was used for intradermal immunization in Swiss albino mice, it produced antibodies that offered protection. Using a first-generation vaccine can provide a larger pool of antigens to elicit the desired immune response.

Keywords: Immunization; Enterobacter cloacae; inactivated vaccine; protective immunoglobulin G; ELISA

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Introduction

Enterobacter species commonly cause nosocomial infections such as UTIs, respiratory and soft tissue infections, osteomyelitis, and endocarditis[1]. *Enterobacter spp*. is increasingly resistant to multiple drugs, including last-resort carbapenems[2].

Infections caused by multidrug-resistant *Enterobacter cloacae* are becoming more common, and the associated morbidity, mortality, and financial costs are unacceptably high[3-4]. The development of new antibacterial agents has slowed down, causing significant concern5. As MDR GNR infections rise, collaborative efforts for prevention and antibiotic alternatives are crucial[5]. One way to enhance the immune response to antigens is through vaccines using whole cells, killed or attenuated[6]. In order to prevent drug-resistant infections, a promising strategy could

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involve using the deactivation method in tandem with formaldehyde, which is an effective agent for inactivating bacteria during the development of a bacterial vaccine[6].

Immunological memory is crucial for the effectiveness of vaccines to be maintained over time[7]. In the case of many infectious diseases, a single infection is sufficient to establish immunological memory[8]. B cells respond quickly and accurately to antigens, creating short-lived and long-lived plasma cells and extending elevated antibody levels. Memory B cells persist for life, rapidly eliminating pathogens upon re-encounter[9].

No studies in Bangladesh have developed antibodies against *Enterobater species* of different molecular sizes. This study was aimed to develop antibodies against formaldehyde-treated *Enterobacter cloacae* in mice and measure them against antigens of varying molecular sizes.

Methodology

This was an animal study which was conducted in the Department of Microbioloogy at Dhaka Medical College, Dhaka, Bangladesh for a period of one year from January 2022 to December 2022.

Animals: Fifteen female Swiss albino mice aged 4-6 weeks were housed and cared for in the animal facility of the microbiology department at our affiliated institution. The mice were randomly divided into three groups, with Group 1 being experimental groups consisting of five mice each, positive control mice were in group-2 containing 5 mice, while Group 3 was negative control group consisting of five mice. Non-medicated feed and water were provided to all animals throughout the experiment. The animal study was approved by the ethical review committee of the corresponding institution.

Immunization of Mice

Bacterial Culture: All bacterial cultures were incubated at 37°C for 24 hours to synchronize growth stages before each experiment.

Preparation of the Dosage of Vaccine Formulation: A mixed solution of *Enterobacter cloacae* (in normal saline), which was isolated from urine, pus, wound swab, endotracheal aspirate, and blood, was prepared for Group 1. Subculture was performed aerobically at 37°C overnight in Mueller-Hinton agar media. Using a sterile wire loop, 8-10 well-isolated colonies of organism were emulsified in 10ml of sterile normal saline. The colony-forming unit (CFU) was obtained by spectrophotometry. Once the desired bacterial load (1.5 × 107 CFU/ml) was achieved, the bacteria were inactivated with 37% formalin and incubated for 2 hours at 37°C. The formaldehyde-inactivated inoculums or bacterial solutions were used for the immunization of mice. After immunization, the viability of bacteria was checked by streaking again on the culture plates, which was confirmed by observing the absence of any growth after overnight incubation at 37°C.

Immunizations Schedule: The mice were given anesthesia before each immunization. This was done by injecting ketamine into the peritoneal cavity of the mice, with the amount adjusted according to their body weight (100 mg/kg). Ketamine is a muscle relaxant, and chloroform was used to maintain the proper anesthetic state. Three intradermal immunizations were administered in the alternate thigh of the mice using an insulin syringe BD Ultra-Fine™ (31G). Each time, the mice were given 20 µL of bacterial solution (prepared on the same day) with a concentration of 1.5 × 107 CFU/ml in 20 µL sterile normal saline. Groups 2 were given 20 µL of sterile saline instead. The immunizations were given on days 0, 14, and 28.

Collection of Serum for ELISA: Blood serum was collected from the tail of the mice on the 13th and 27th days after immunization, as well as two weeks after the third dose was administered. The collected blood (50 µl) was mixed with 200 µl of PBS in a micro centrifuge tube to create a dilution ratio of 1:5. This was achieved using a yellow pipette to collect the blood from the cut end of the tail. After 2 hours, the diluted sera was centrifuged at 3,000 g for 10 minutes, separating the blood cells from the serum. The clear sera from the top of the tube was collected into a new micro centrifuge tube and stored at -20°C for further use.

Intra-peritoneal Challenge: After two weeks since the last inoculation, the mice from both the experimental group (group-1) and the positive control group (group-2) were challenged by an intraperitoneal injection of 3X108 CFU/ml live MDR Enterobacter cloacae that was suspended in 100µl of PBS. All the mice were then observed for 14 days' post-challenge for any clinical signs such as weight loss, reluctance to move, refusal to feed, or death.

Collection of blood by Cardiac Puncture: After two weeks of the lethal challenge, blood was collected by cardiac puncture from Groups 1 and 3. The chest area of mice was shaved and washed with povidone-iodine and 70% alcohol. Blood was drawn from the heart using an insulin syringe inserted at an angle of 45°

after feeling the cardiac pulsation by finger. Approximately 2-3 ml of blood was collected and kept undiluted in a sterile test tube. Serum was collected from the blood as described above and stored at −20°C for further use.

Separation of Lymphocyte from Spleen: Spleens were collected from mice in two groups - Group-1 (experimental) and Group-3 (negative control) - under sterile conditions. Each spleen was placed in a Petri dish containing 5ml of complete RPMI medium and crushed using frosted glass slides. The resulting cell suspension was then filtered through a Nylon cell strainer into a 15ml sterile conical tube and centrifuged at 350 rpm for 10 minutes at 4°C. After discarding the supernatant, the cells were re-suspended in 5ml of complete RPMI medium.

Antibody detection by ELISA: Immunoglobulin G (IgG) specific for *Enterobacter cloacae* antigen was measured in mouse sera and splenic culture supernatant by ELISA.

Microbiological Procedure

Sonication procedure of *Enterobacter cloacae*: The bacterial pellets were suspended in 100 µL of distilled water and kept on ice for 30 minutes. They were then sonicated at 20 kHz for 2 rounds of 10 seconds each, depending on the samples and their viscosity. After that, the samples were kept on ice for 5 minutes and then centrifuged at 10,000 g for 20 minutes to remove debris, which may contain unlysed cells, nuclei, or unlysed organelles. The supernatants were then transferred to a new microcentrifuge tube and stored at −20°C to be used as an antigen. The antigen was optimized by checkerboard titration, and 10 µg of antigen was used in ELISA.

Preparation and pouring SDS-Polyacrylamide gels: 10 ml solution of Acrylamide was prepared for the separating gel in a 50 ml falcon tube. The components were mixed and start polymerization as soon as the TEMED is added. The acrylamide solution was poured to the gap between the glass plates and left sufficient space for the stacking gel. The gel was placed in a vertical position at room temperature until polymerization is complete. The stacking gel was prepared in a disposable plastic tube or falcon tube and poured it directly onto the surface of the polymerized separating gel. A clean Teflon comb was inserted into the stacking gel solution and the spaces of the comb was filled completely. After polymerization is complete, the Teflon comb was removed and the wells were washed immediately. The gel was mount in the electrophoresis apparatus and ran it until the bromophenol blue reaches the bottom of the separating gel. Glass plates was removed from electrophoresis apparatus and placed on paper towel. Gel was immersed in Coomassie Brilliant Blue staining solution and placed on rotating platform for 4 hours at room temperature. The gel was soaked in distilled water overnight on a rocking platform, changing the solution 3-4 times. 24-hour de-staining detected 0.1 µg protein in a single band.

Identification and excision of the band of interest: After electrophoresis, a gel section with the molecular weight marker and one protein sample lane using a clean scalpel, preferably adjacent to the excised protein band(s) lanes was cut.

Elution of Protein from the Gel Matrix: Gel pieces were immersed in elution buffer and crushed with a pestle. After overnight incubation and centrifugation, the supernatant was pipetted into a new microcentrifuge tube and stored at -20°C.

Optimized ELISA: OD value was measured in mice sera and splenic cell culture supernatant using ELISA. Crude antigen and antigens of different molecular weight from Enterobacter cloacae were used in separated plates. ELISA plates were coated with 100 µ l/well of antigen (10 µg/ml) in a bicarbonate-coating buffer (pH 9.6). The plates were then left to incubate overnight at room temperature. After incubation, the plates were washed twice with PBS and blocked with 200 µl/well of 5% w/v skimmed milk in PBS. The blocking process was carried out by incubating the plates at 37°C for 30 minutes. The plates were then washed thrice with PBS-tween (0.05% tween 20) and once with PBS. Next, the serum samples and splenic cell culture supernatant were added at different dilutions (100 µl/well) in separated plates. The plates were then incubated at 37°C for 90 minutes and then at 4°C overnight. After washing the plates as described earlier, diluted (1:5000) conjugate, horse radish peroxidase-labeled anti-mouse IgG antibody (Thermo Fisher Scientific, USA) in PBS-Tween was added (100 µl/well) and incubated at 37°C for 90 minutes. Again after washing, 100 µl/well tetramethylbenzidine (50 µ l) and urea peroxide (50 µl) substrate were added. Thereafter, 50 µl of 1M sulfuric acid was added to stop the reaction. Finally, absorbance was measured at 450 nm using an ELISA plate reader (BioTek Inc., USA). Cutoff value of OD was calculated by the following formula: OD = M (mean) +2 × Standard deviation.

Data Processing: The data collected for the study was carefully compiled and edited through multiple rounds

of thorough checking and rechecking. Any omissions or inconsistencies were corrected in a systematic manner.

Data Analysis: The study results were systematically recorded and analyzed, followed by comparison using the Z-test. All statistical analysis was done by SPSS version 16, SPSS Inc (SPSS for Windows, Version 16.0. Chicago, SPSS Inc; 2007). P = 0.05 was considered as the minimum level of significance.

Results

During the study on the survival rates of immunized mice, it was observed that all the mice in Group 1 survived the 14-day observation period after being challenged with a lethal Enterobacter cloaca. On the other hand, all the mice in Group 2 (positive control group) died within 24 hours of the challenge. The optical density (OD) of anti-Enterobacter cloacae antibodies was measured in 15 serum samples. Five samples were from Group 1 mice, five were from Group 2 mice, and five were from Group 3 mice. The samples were collected from tail end at 13th, 27th and 41st days following 1st inoculation (Figure I) (Figure II), (Figure III). After 14 days of challenge, blood collection by cardiac puncture from group-1 and group-3. All the serum samples from the immunized mice had OD values of anti-Enterobacter cloacae IgG polyclonal antibody above the cutoff value. There was a statistically significant difference between the OD values of experimental and control mouse sera (P < 0.001 after the first inoculation and P < 0.0001 after the second, 3rd inoculation and after lethal challenge). There was statistically significant difference between the optical density values of experimental and negative control mice cell culture supernatant with P value of 0.0007 (Figure IV, V).

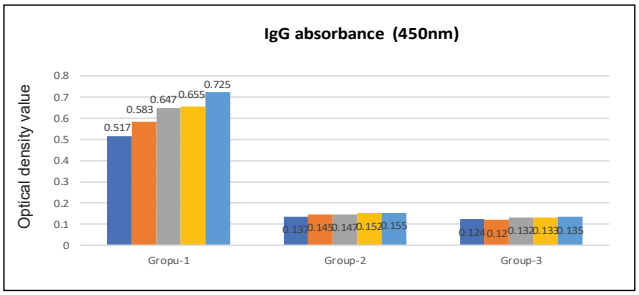

Figure I: Optical density of serum samples after the first booster by ELISA. Here, mean of negative control 0.129, standard deviation 0.006, cutoff value for prechallenge sera 0.141, range after the first booster0.517-0.725, P <0.001

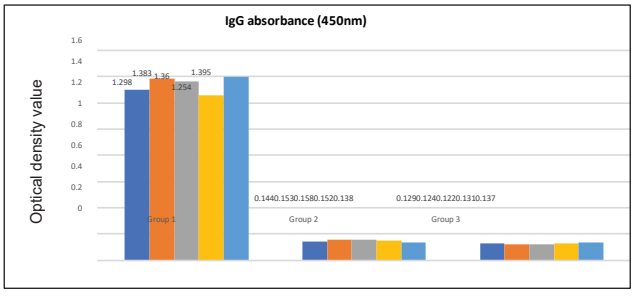

Figure II: Optical density of serum samples after the second booster by ELISA. Here, mean of negative control 0.128, standard deviation 0.006, cutoff value for prechallenge 0.0140, range after the second booster 0.1254-0.1395, P <0.0001

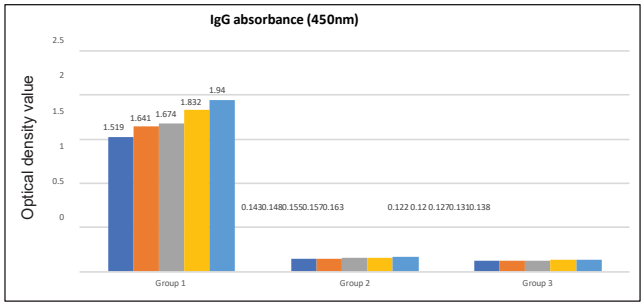

Figure III: Optical density of serum samples after the third booster by ELISA. Here, mean of negative control 0.128, standard deviation 0.007, cutoff value for prechallenge 0.142, range after the second booster 1.519-1.94, P <0.0001

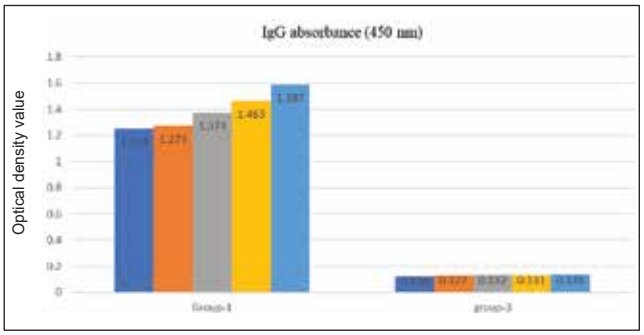

Figure IV: Optical density of serum samples after lethal challenge by ELISA. Here, mean of negative control 0.128, standard deviation 0.004, cutoff value for prechallenge 0.138, range after the second booster 1.254-1.587, P <0.0001

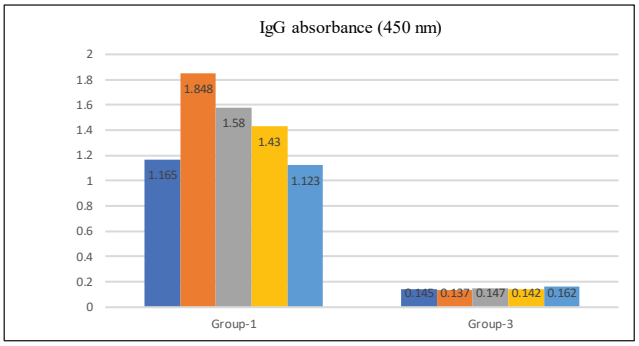

Figure V: Optical density of splenic cell culture supernatant after incubation in RPMI media by ELISA. Here, mean of negative control 0.147, standard deviation 0.01, cutoff value for prechallenge 0.167, range after the second booster 1.123-1.848, P = 0.0007

Discussion

Vaccination is the most effective and affordable way to prevent infectious diseases. Adenoviral gene therapy, pro-inflammatory cytokines, and cyclic di-GMP have potential but are costly and lack specificity. Active vaccination and passive antibody therapy look promising for targeting specific pathogens[10]. The present study found that all experimental mice survived a 14-day challenge. Another study conducted by Goutam et al. at DMCH reported that all mice survived after being immunized with formalin-inactivated intradermal Enterobacter cloacae[11]. Additionally, Kurupati et al[12] discovered that injecting mice with a plasmid containing the OmpK36 gene led to a mixed Th1/Th2 response, which protected the mice against a lethal bacterial challenge.

In the current study, the immunization protocol resulted in the production of *Entrobacter cloacae*-specific IgG polyclonal antibodies in all of the mouse sera after the first and second boosters, as well as the lethal challenge and in selenic cell culture supernatant, as measured by ELISA. The experimental group of mice displayed a higher concentration of IgG antibodies. This increase indicates an indirect sign of B cell maturation and differentiation after immunization. Zorgi et al[13] also observed higher levels of IgG antibodies in the experimental group of mice.

In this study, researchers categorized *Enterobacter cloacae* protein bands based on their molecular weight range. The ranges were 11-17 kDa, 26-43 kDa, 55-72 kDa, and 95-130 kDa. The bands were crushed, and the resulting supernatants were used for ELISA tests. The pooled serum from the experimental mouse group (Group 1) was subjected to these tests. The ELISA outcomes showed that the OD value of the band falling within the 26 to 43 kDa range was notably higher than the OD values of other bands. This suggests that Enterobacter cloacae protein bands with molecular weights ranging from 26 to 43 kDa possess greater antigenicity. They can induce a more robust antibody response from B cells.

A separate study conducted by Pan et al[14] also found that an Enterobacter cloacae protein with a molecular weight of 35 kDa triggered the most significant immune response. Consequently, based on the findings of this investigation, protein bands within the 26 to 43 kDa molecular weight range could serve as a potential basis for an Enterobacter cloacae vaccination strategy.

Conclusion

Developing an effective vaccine against *Enterobacter cloacae* is a promising approach that requires extensive research. In this study, test mice were vaccinated with formaldehyde-inactivated *Enterobacter cloacae*, which led to significantly higher levels of IgG antibodies in both serum and splenic cell cultures compared to the control group. The immunization triggered amplified growth and specialization of B cells, which contributed to the heightened IgG levels. The study identified a specific antigen of *Enterobacter cloacae* with a molecular weight of 26-43 kDa, which exhibited a high OD value in both mice sera and splenic cell culture supernatant. These findings could provide insight into the development of a vaccine that imparts long-lasting protection.

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None

Conflict of Interest
The authors have no conflicts of interest to disclose

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Authors' contributions
ossain S, Yusuf MA, Nahar S conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Hossain S contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Nahar S, Moureen A, Shahid SB, Shamsuzzaman SM involved in the manuscript review and editing. Hossain S, Yusuf MA, Nahar S as collector of Data and Data Analyst. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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