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Positive Memory B Cell Response After Injecting Intradermal Formalin Treated Enterobacter Cloacae: An Experimental Study In Mice

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Abstract
Introduction
Multi-drug resistant Enterobacter cloacae is one of the major causes of nosocomial infection. A vaccination approach would be effective to prevent the burden of disease related to this organism. Hence, the present study aimed to evaluate the memory B cell response after intradermal immunization of formaldehyde-inactivated Enterobacter cloacae in a murine infection model.
Methods
The present in vivo study was conducted on swiss albino female mice in the department of Microbiology of Dhaka Medical College. A total of 15 mice were randomly divided into 3 groups: Experimental mice (inoculated with formaldehyde inactivated whole cell Enterobacter cloacae emulsified with PBS on day 0, 14 and 28 followed by a lethal challenge with live MDR Enterobacter cloacae), Placebo controlled mice (inoculated with PBS) and Negative control mice (uninoculated and uninfected). Serum from the tail blood was collected 10 days after 1st inoculation then 7 days after each inoculation and finally by cardiac puncture after 14 days of lethal challenge. Blood from cardiac puncture of group-1 and group-3 was used to separate peripheral blood mononuclear cells (PBMC) by density gradient centrifugation on Ficoll-isopaque and culture these cells in RPMI media containing 10% heat-inactivated fetal bovine serum. After that, bacterial antigen was introduced into the media to induce memory B cells to become antibody secreting cells (ASC). Optical density (OD) value of IgG antibody absorbance by ELISA was done for serum sample and cell culture supernatant.
Results
The OD value of IgG absorbance during the different inoculation schedule of experimental group demonstrated significant increase above the cut off value of serum IgG antibody titre (p-value <0.001). Moreover, a statistically significant positive difference was observed between the OD values of experimental and control mice sera during the whole immunization schedule as well as after lethal challenge (p-value <0.05). Regarding the survival rates, all the immunized mice survived during 14 days observation period after lethal challenge while all the control mice died within 24 hours of challenge. There was a statistically significant difference between the optical density values of experimental and negative control mice cell culture supernatant (p-value of <0.05).
Conclusions
Our study showed that immunization with formaldehyde inactivated Enterobacter cloacae elicited a positive memory B cell response in mice which might guide further strategy of development of vaccines using inactivated whole-cell gram-negative bacteria to protect against these organisms.

Keywords: *Vaccine, Gram negative bacteria, Enterobacter cloacae, Memory B cell response, MDR bacteria*

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I. Introduction

Enterobacter cloacae, a facultatively anaerobic, Gram-negative bacteria of the family *Enterobacteriaceae*, is one of the commonest pathogens causing a range of nosocomial infections, including pneumonia, urinary tract infections, and septicemia [1]. Among the *Enterobacteriaceae* isolated from the patients with hospital-acquired infections, after *Escherichia coli* and *Klebsiella species*, *Enterobacter cloacae* is listed as the third most common isolate [2]. This species attributes to more than 10% of the nosocomial infection worldwide with a mortality rate ranging from 6% to as high as 40% [3].

In recent years, the emerging threat of multidrug resistant (MDR) *Enterobacter* species, including resistance to the new generation antibiotics like carbapenems (meropenem, imipenem, and ertapenem) has led to a greater challenge in the management of these organisms. *Enterobacter cloacae* is one of the most common carbapenem-resistant bacteria from the *Enterobacteriaceae* family [4]. Several intrinsic and acquired

mechanisms are responsible for the antimicrobial resistance in these organisms. For instance, extended-spectrum β-lactamase (ESBL) genes causes resistance to most of the β-lactam antibiotics including cephalosporins and monobactams. *Enterobacter cloacae* is resistant to penicillin and early generation cephalosporins by its intrinsic mechanism, regardless of AmpC-lactamase production [5]. Moreover, resistance to third- and fourth generation cephalosporins could result from mutations, usually in ampD, leading to constitutive hyperproduction of ampC [6,7].

New-age strategies of therapeutics are necessary to mitigate the global treat of antibiotic resistance including boosting human immune responses through immunotherapeutics (using pathogen specific antibodies) and immunological interventions (modulating innate immunity, and vaccination) [8]. Moreover, vaccination against specific organisms might be an effective strategy to protect vulnerable group of people. However, despite being one of the major organism causing nosocomial infection, no immunoprophylactics or vaccines are approved for clinical use at present for combating *Enterobacter cloacae* [9]. In this context, introduction of such interventions against this organism could highly reduce disease burden of hospital-acquired infection.

Majority of the vaccine candidates against gram-negative organisms causing nosocomial infection are based on outer membranes vesicles [9]. However, using the whole cell of the bacteria for the designing a vaccine would provide sufficient supply of antigens resulting in a more robust inflammatory stimulus for adequate immune response [10]. Hence, with a view to present multiple antigens to the immune system, a whole cell killed, or live attenuated vaccines approach would be appropriate.

Success of a vaccine depends on the initiation and maintenance of immunological memory [11]. Memory B cell is responsible in this regard by acting rapidly and precisely to antigenic re-stimulation which results in both short and long-lived plasma cell pool and, as a result maintaining a high level of serum antibody. These cells may persist for lifetime and contribute to the rapid clearance of pathogens following re-exposure [12]. However, there is hardly any study exploring the memory B cell response in mice after injecting inactivated *Enterobacter cloacae*. Hence, the present study aimed to evaluate the memory B cell response after intradermal immunization of formaldehyde-inactivated *Enterobacter cloacae* in a murine infection model.

II. Methods

Study design

The present study was designed as an in vivo study conducted on swiss albino female mice in the department of Microbiology of Dhaka Medical College. The study was approved by the ethical review committee of Dhaka Medical College.

Animals

A number of fifteen 4 to 6 weeks old Swiss albino female mice collected from animal resource facility of icddr,b were used for the present study. These mice were randomly divided into 3 groups: Experimental mice (Group 1), Placebo controlled mice (Group 2) and Negative control mice (Group 3). Group-1 was inoculated with formaldehyde inactivated whole cell *Enterobacter cloacae* emulsified with PBS, Group-2 was inoculated with PBS and Group-3 was kept uninoculated and uninfected.

Immunization of mice

Bacterial culture and CFU determination

A total of six multi drug resistant (MDR) *Enterobacter cloacae* strains collected from different clinical samples of human were selected to use as candidate for inactivated whole cell vaccine preparation . All bacterial strains were sub cultured onto Mueller-Hinton agar plate and were maintained at 37°C for 24 hours before each use to ensure that the bacteria were at same growth stage in all experimental steps.Preparation of vaccine dosage

Group-1 underwent immunization using a formalin-inactivated solution derived from a combination of *E. cloacae* strains isolated from various sources including urine, blood, endotracheal aspirate, and wound swabs. To achieve this, a loopful of these organisms was introduced into a culture medium known as tryptic soy broth (TSB), which was then left to incubate at 37°C overnight. Following the incubation period, a centrifugation step was performed at 2,000 times the force of gravity (2,000 g) for 20 minutes at a temperature of 4°C, resulting in the removal of the liquid supernatant. The bacterial pellet was subsequently subjected to two washes using phosphate buffered saline (PBS). To render the E. cloacae non-viable for use in immunization, a solution containing 37% formalin was combined with the bacterial suspension, resulting in a final concentration of 3% (v/v). This mixture was then allowed to incubate for 2 hours at 37°C. Following incubation, the bacterial suspension underwent two additional washes using sterile PBS, after which it was resuspended in PBS to achieve a concentration of 1.5×10^8 colony-forming units per milliliter (CFU/ml). Subsequently, 134 µl of this inoculum was combined with 866 µl of sterile PBS to attain a concentration of 2×10^7 CFU/ml for immunization purposes. The effectiveness of bacterial inactivation was confirmed by culturing the supernatant

on agar plates and observing no growth after overnight incubation at 37°C. The final product was then stored at a temperature of -20°C until it was ready for use in the immunization process.

Immunization Schedule of mice

Three intradermal inoculations were performed on day 0, 14 and 28 in the alternate thigh of the group-1 mice with 20 µL of formalin-inactivated whole-cell *Enterobacter cloacae* and the group-2 mice with 20 µL of sterile PBS on the same schedule. The intradermal inoculation was done with an insulin syringe BD Ultra-Fine TM (31G) in the upper and outer quadrant of the thigh after proper anesthesia with intra-peritoneal injection of Ketamine (dose 100 mg/kg).

Collection of Serum for ELISA

Serum from the tail blood was collected 10 days after 1st inoculation and then 7 days after each inoculation to detect optical density (OD) value of IgG antibody absorbance by ELISA. For this purpose, mice were exposed to ketamine and then the tail was stretched and cleaned with 70% alcohol. With the help of sterile scalpel (22 FR) the tail was cut 2mm proximal to its blunt end. At first 10 µl of fresh blood was collected into a micro centrifuge tube containing 40 µl PBS to yield a dilution of 1:5 by using yellow pipette from the cut tail end. The cut end of the tail was then kept pressurized 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 3,000g for 10 minutes. Blood cell settled down bottom and clear sera from the top of the tube were taken into a separate sterile micro centrifuge tube and were kept at -20C for further use.

Intra-peritoneal Challenge

Two weeks after the last inoculation, the mice from experimental group (group 1) and placebo control group (group 2) were challenged intraperitoneally with 1.5X10^8 CFU/mL live MDR *Enterobacter cloacae* suspended in 100µl PBS. All mice were observed for 14 days post challenge for any clinical manifestations or death.

Collection of Blood after Cardiac Puncture

Fourteen days following the lethal challenge, a cardiac puncture technique was employed to extract 2-3ml of blood from mice belonging to both group 1 and group 3. To prepare for this procedure, the chest region was shaved using a razor, and subsequently cleansed with povidone iodine and a solution consisting of 70% alcohol. By palpating the cardiac pulsations, the needle of an insulin syringe was then inserted into the heart at a 45-degree angle after appropriate anesthesia. Approximately one milliliter of blood was withdrawn and transferred undiluted into a sterile test tube, where it was allowed to naturally coagulate. The test tube was subjected to centrifugation at a force of 3000g for a duration of 10 minutes, resulting in the separation of serum which was then carefully extracted from the upper layer and placed into a distinct micro centrifuge tube. The obtained serum samples, acquired via cardiac puncture, were subsequently preserved at a temperature of -20°C for subsequent utilization.

Separation of Peripheral blood mononuclear cell (PBMC)

Around 1ml blood collected from each mouse by cardiac puncture was taken in a heparinized blood collection tube and diluted with PBS in 1:1 ratio. Then the diluted blood was poured slowly in the falcon tubes where 1ml ficoll-isopaque has been taken. The diluted blood made a separate layer above ficoll-isopaque. Then the falcon tubes were centrifuged at 4000g for 30 minutes to separate PBMC. After centrifugation a clear layer of PBMC cell was seen above the ficoll-isopaque.The PBMC from the clear layer was taken with the help of micropipette.

Harvesting PBMC

Clear layer of PBMC was taken with the help of micro pipette and added in a 24 well culture plate, where each well contained 1ml RPMI media. The RPMI 1640 media supplemented with 10% fetal bovine serum, 200U/ml Penicillin and 200µgm/ml Gentamicin. After that 50µl diluted Ag was added to the culture plate and incubation of the plate was done at 370c for 6 days.

Detection of IgG antibody from serum sample and cell culture supernatant by ELISA

Mice sera and cell culture supernatant were used for the evaluation of optical density (OD) value of IgG antibody by indirect ELISA. Antigenes were separated after sonicating the whole cell *Enterobacter cloacae*. Absorbance was measured at 450nm using ELISA plate reader (Biotek Inc., USA). Indirect ELISA for serum sample 10 µg/ml of antigen was considered as ideal quality to use in indirect ELISA study.

Measurement of OD

Cut off value of optical density (OD) was calculated using given below formula:
OD= M (mean) + 2 X SD (Standard deviation)

Statistical analysis

All statistical analyses were done by SPSS version 24.0. Statistical significance of serum antibody titer within different inoculation schedule of experimental group interpreted by ANOVA. Statistical significance of serum antibody and memory B cell response between experimental group and control group was determined by Student's t-test. P value <0.05 was taken as minimal level of significance

III. Results

Regarding the survival rates of immunized and unimmunized mice, all the mice of group-1 survived for 14 days observation period after lethal challenge while all the mice of group-2 died within 24 hours of challenge.

The optical density value of IgG absorbance (450nm) during the different inoculation schedule of experimental group demonstrated significant differences of serum IgG antibody titre (p-value <0.001) (Table 1). Besides, all the serum samples from the experimental group had optical density of IgG polyclonal antibody above the cut off value of 0.141, 0.141 and 0.142 on 10th, 7th and 7th days after the first, second and third booster respectively. Moreover, there was statistically significant difference between the optical density values of experimental and control mice sera with p-value of <0.05 (Table 1 and Figure 1a, 1b, 1c). Similarly, the optical density of IgG antibodies in serum samples collected after the lethal challenge showed that all the serum samples from the experimental group had optical density of IgG polyclonal antibody above the cut off value of 0.138 with a statistically significant difference between the optical density values of experimental and control mice sera with p-value of <0.0001 (Table 1 and Figure 1d).

Table 1: Optical density (OD) of IgG absorbance by mice sera collected during different vaccination schedule

Vaccine schedule	Optical density (OD), mean (SD)			p-value
	Group 1	Group 2	Group 3	
1st dose	0.63 (0.08)	0.15 (0.01)	0.13 (0.01)	<0.001
2nd dose	1.34 (0.06)	0.15 (0.01)	0.13 (0.01)	0.003
3rd dose	1.72 (0.17)	0.15 (0.01)	0.13 (0.01)	<0.001
Lethal challenge	1.39 (0.14)	-	0.13 (0.01)	<0.001
p-value	<0.001	0.874	0.958	

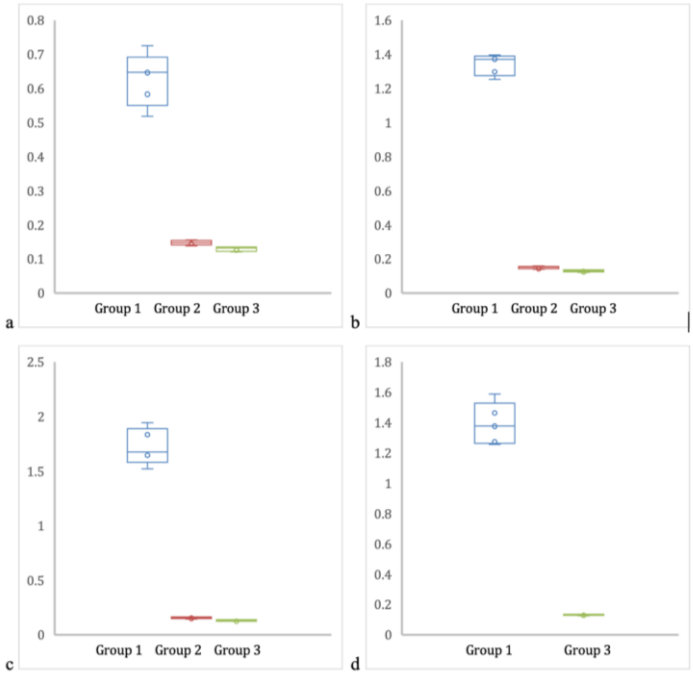

Figure 1: Optical Density (OD) of serum samples by ELISA (a. after 10 days of first dose, b. after 7 days of second dose, c. after 7 days of third dose, d. after 14 days of lethal challenge)

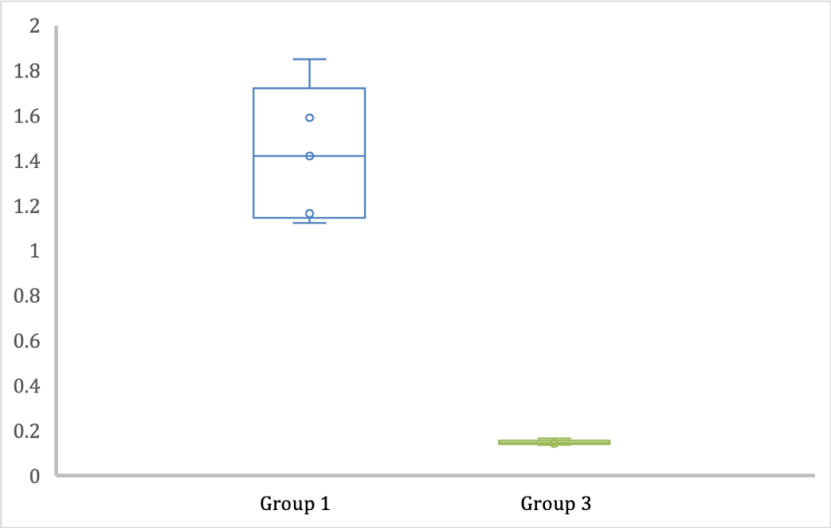

Figure 2: Optical Density (OD) of cell culture supernatant after harvesting Peripheral blood mononuclear cell (PBMC) in RPMI media, by ELISA

Figure 2 shows optical density of IgG antibodies in cell culture supernatant collected after harvesting PBMC in RPMI media. All the samples from experimental group had optical density of IgG polyclonal antibody above the cut off value of 0.167. The cut off value was calculated as mean + 2SD. Here, the mean optical density values of cell culture supernatant of negative control mice was 0.147 and SD was 0.01. There was statistically significant difference between the optical density values of experimental and negative control mice cell culture supernatant with P value of 0.007.

IV. Discussion

In the current era of widespread antimicrobial resistance, approaches to enhance host immunity using pathogen specific antibodies, modulating innate immunity, novel vaccine platforms etc. have shown promising results [8]. Among these approaches, vaccination might be considered as one of the most effective method to prevent infectious diseases. However, despite being one of the most common organisms causing hospital acquired infection, there is hardly any evidence regarding development of vaccine against *Enterobacter cloacae*. Gram-negative bacteria like *Enterobacter cloacae* are predominantly killed by complement cascade rather than opsonic phagocytosis owing to their structural complexity [13]. In the present study, an assay to determine the memory B cell response after intradermal immunization of formaldehyde-inactivated *Enterobacter cloacae* in a murine infection model was established.

We observed a significant rise in OD of IgG antibody absorbance within the experimental group of mice after the second and third inoculation of inactivated bacteria which might be due to the production of more IgG antibodies by memory cells after second booster onward. However, the antibody level slightly decreased following the lethal challenge which might be due to the utilization of antibodies to clean up offending pathogens from the body. By contrast, the sera from the placebo control group contained a very low level of detectable antibody after all inoculation which was close to the negative control group. There is hardly any previous in vivo study evaluating the IgG antibody level after inoculation of formaldehyde-inactivated *Enterobacter cloacae*. However, a previous study showed immunization with formalin inactivated whole cell *citrobacter rodentium* vaccine elicited significant levels of IgG in the immunized mice group compared to the control group [14]. This raised antibody titre in the experimental group, probably, boosted up the survival rate after lethal challenge. In our study, the survival rate among the experimental group mice was 100% at 14 days post lethal challenge while it was null in control group. Still there is hardly any previous evidence to compare with regarding the survival rates of the mouse after lethal challenge following intradermal immunization with formalin inactivated *E. cloacae*. However, a similar higher survival rates in experimental groups were reported by some studies investigating vaccines against other gram negative organisms including *Escherichia coli* and *Klebsiella pneumoniae* [15–17]. The raised antibody titre of IgG in the serum of the experimental group mice can be crucial for activation of the classical pathway of the complement system as well as opsonization of the infectious microorganisms through binding of their paratopes at bacterial surface antigens [16]. Thus, antibodies can stimulate the effector functions of neutrophils, which recognize the antigen-antibody complex via Fc receptors [18].

In our study, after 14 days of lethal challenge with MDR *Enterobacter cloacae*, blood sample was collected by cardiac puncture to evaluate the memory B-cell responses against the organism using a stimulation method, which enhanced the detection of memory B cells in the culture supernatant by promoting their proliferation and differentiation into plasma blasts that secrete antibodies. It was observed that all the samples from the experimental group had an optical density of IgG polyclonal antibody above the cut-off value with a statistically significant difference when compared to the control group.

The present study provided a baseline evidence regarding positive memory B cell response in mice after injecting inactivated *Enterobacter cloacae*. It provides new insight for the strategy of vaccine development using inactivated whole-cell gram-negative bacteria to protect against hospital acquired bacterial infections, especially those caused by drug resistant organisms. However, the study has several limitations. Firstly, the experiment included a small number of mice sample to provide a robust evidence. Besides, detailed mechanisms of memory B cell response to the inactivated *Enterobacter cloacae* could not be explored in the present study.

V. Conclusions

Our study evidenced that immunization of the experimental mice with formaldehyde inactivated *Enterobacter cloacae* resulted in production of higher titres of IgG antibodies both in serum and PBMC culture supernatant compared to the control mice. This finding points to antibody synthesis in serum and memory B cell differentiation into antibody-secreting cells in PBMC culture supernatants which would guide further attempts of designing whole cell vaccine against this organism.

Declarations

Ethics approval:

The study was approved by the Ethical Review Committee of Dhaka Medical College. Informed signed consent was obtained from all eligible participants who agreed to participate. The authors declare that the procedures followed the regulations established by the Helsinki Declaration of the World Medical Association.

Consent for publication:

Not applicable.

Availability of data and materials:

Patient-level data will be available on request from the corresponding author.

Conflict of interest:

The authors declare that they have no competing interests.

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Investigation: Arpita Goutam
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Supervision: S. M. Shamsuzzaman
Writing – original draft: Arpita Goutam
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