



Vaccination against Multidrug Resistant *Escherichia coli* Produces Immune Response in Mice Model

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Abstract: *Background:* The emergence of multidrug resistant (MDR) *Escherichia coli* are particularly challenging for clinician. Development of an effective vaccine may overcome the problems associated with treatment failure and antibiotic resistance. *Object:* This study was designed to determine immune response against MDR *E.coli* using formaldehyde inactivated *E.coli* in mice model. *Methods & Results:* In this study, fifteen Swiss albino mice were used. Intradermal inoculation was implemented three times at 14 days interval with formaldehyde inactivated MDR *E.coli*. The mice were given intraperitoneal challenge with live *E.coli* and observed for 14 days. Tail blood was collected to assess antigen binding capacity of the serum antibody of immunized mice by ELISA. The survival rate was 100% among the mice of immunized group at 14 days of post challenge. In ELISA, OD values of serum immunoglobulin G of pre and post challenge immunized mice were significantly higher ($p < .001$). *Conclusion:* In this study, It was observed that formaldehyde inactivated MDR *E.coli* induced protective antibodies in mice model.

Keywords: MDR *E. coli*, ELISA, Immune response, Whole cell vaccine.

Original Research Article

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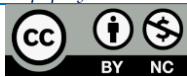
Article at a glance:

Study Purpose: The study aimed to evaluate the effectiveness of a formaldehyde-inactivated multidrug-resistant *Escherichia coli* (MDR *E. coli*) vaccine in inducing immune responses and enhancing survival in mice.

Key findings: The results showed that the vaccinated mice had 100% survival after being challenged with a lethal dose of *E. coli*. Furthermore, a significant increase in IgG antibody levels was observed, indicating a strong immune response to the vaccine.

Newer findings: This research contributes to the understanding of vaccine development for multidrug-resistant bacterial infections. It suggests that formaldehyde-inactivated vaccines could offer a promising strategy for controlling MDR *E. coli* infections, providing new insights into potential treatments for drug-resistant bacterial diseases.

Abbreviations: MDR - Multidrug-resistant, *E. coli* - *Escherichia coli*, IgG - Immunoglobulin G, PBS - Phosphate-buffered saline, LPS - Lipopolysaccharide.



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INTRODUCTION

Antimicrobial resistance (AMR) is a worldwide nuisance to health community and globally 700,000 deaths are annually reported ¹. *Esch.coli* is increasingly associated with multidrug resistance, including the resistance to the last-resort carbapenems ². Infections with MDR *E.coli* are associated with high rates of mortality, currently

estimated at about 18–61% ⁴. Aside from its intrinsic resistance to several antibiotics, its propensity to acquire resistance is responsible for its multidrug resistance profile, rendering the pathogen a therapeutic challenge. Therefore, it is essential to develop an alternative strategy to tackle this highly antibiotic-resistant pathogen (e.g vaccine, antibody therapy etc.) ^{5,6}. The development of novel immunotherapies and vaccines against *E.coli* could

help to prevent infections caused by this highly antibiotic-resistant microorganism³. In order to develop an effective vaccine against *E.coli*, detailed knowledge of the host immune responses and the bacterial defense mechanisms is important. Both innate and adaptive immune responses work in synergy to act against *E.coli* infection. As *E.coli* is an extracellular pathogen, humoral, mucosal or systemic opsonizing immunity is most effective to prevent bacterial colonization and infection. However, T-cell responses can also mediate protective immunity in individuals with *E.coli* infections⁷.

Numerous experimental vaccine candidates have been evaluated such as LPS, alginate, pili, flagella, whole cell etc. In order to improve the presentation of multiple antigens to the immune system, whole-cell and live-attenuated *E.coli* mucosal vaccines were shown to be protective in animal models³. As an innovative strategy for development of bacterial vaccine, inactivation method could be simultaneously applied and for which formaldehyde is an effective inactivating agent⁸. The intradermal (ID) route optimizes the immunogenicity of the vaccine and minimizes adverse reactions at the injection site as because dermal part of muscles have larger and more numerous blood vessels and absorption rate are usually faster than other routes⁹. However, still no study regarding protective vaccine efficacy for *E.coli* has been carried out in Bangladesh. Therefore, this study was designed to evaluate the immune response of whole cell vaccine after intramuscular immunization with formaldehyde inactivated multi drug resistant *E.coli* in murine model.

METHODS

Animals

Fifteen 4-6 weeks old female swiss albino mice were collected from Animal Resources Facility of ICDDRB and were kept under specific pathogen free condition and cared in animal house facility of Microbiology department of Dhaka Medical College. The mice were randomly divided into 3 groups. Experimental mice were in experimental group (group-1) containing 5 mice, control group (group-2) containing 5 mice and negative control group (group-3) containing 5 mice. Group-1 was inoculated with formaldehyde inactivated whole

cell *Esch.coli* emulsified with PBS, group-2 was inoculated with PBS and group-3 was kept uninoculated and uninfected.

Immunization of Mice

A) Bacterial culture and CFU determination

Previously isolated MDR *Esch.coli* strains were collected from different clinical samples and were selected to use as candidate for inactivated whole cell vaccine preparation. All bacterial strains were subcultured 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.

B) Preparation of formalin inactivated whole cell *Esch.coli*¹⁰

A loop full of *Esch.coli* was inoculated into TSB (Trypticase soya broth) in micro centrifuge tube and was incubated at 37°C overnight. Following incubation, the bacterial cultures were centrifuged at 2,000 g for 20 min at 4°C and the supernatant was discarded. The pelleted bacteria were then washed twice (2000 g, 20 minutes, 4°C) with ice cold phosphate buffered saline (PBS). To prepare formalin inactivated *Esch.coli* whole-cell vaccine, 37% formalin was added to the suspension to achieve final conc. of 3% (v/v) (For this, 8.11ml of 37% formalin was mixed with 91.89 ml of sterile distilled water). The suspension containing micro centrifuge tube was then incubated for 2 hours at 37°C. After incubation for 2 hours, the suspension was again washed twice with sterile ice-cold PBS (2000g, 20 minutes, 4°C) and resuspended with ice-cold sterile PBS to achieve conc. of 1.5X10⁸ CFU/ml. Then 134 microlitre of inoculum was mixed with 866 microlitre of sterile PBS in another micro centrifuge tube to achieve conc. of 2X10⁷ CFU/ml. Complete inactivation of the bacteria was checked by streaking again on the Muller Hinton agar plates and confirmed by observing no growth after overnight incubation at 37°C and then stored at -20°C until inoculation.

C) Immunization schedule¹⁰

Three Intradermal (I/D) inoculations were performed with 250 µl bacterial solution (2X10⁷ CFU/ml) on day 0, 14 und 28 in the alternate thigh of the experimental group (group-1) of mice with formalin-inactivated whole-cell *Esch.coli* and the control group (group-2) mice with 250 µl sterile PBS

on the same schedule. The Intradermal inoculation was done with an insulin syringe BD Ultra-Fine TM (31G). The prepared mixtures were inoculated after giving proper anesthesia with intra-peritoneal injection of Ketamine adjusted to the body weight of mice (ketamine 100 mg/kg). Ketamine was acted as muscle relaxant to maintain the proper anesthetic condition. I/D inoculations in the upper and outer quadrant of the thigh were performed after holding on the nape of mice by right thumb, index and middle fingers and tail by little finger so that they cannot move as their skin was stretched and then stretching the thigh a little towards the outer side.

Collection of serum for ELISA¹¹

Serum from the tail blood was collected 10 days after 1st inoculation and then 7 days after each inoculation to detect OD value of IgG antibody absorbance by ELISA. For anesthetic purpose, mice were injected with ketamine to reduce their consciousness level. Then the tail was stretched and cleaned with 70% alcohol. With the help of sterile scalpel (22 FR) the tail was cut 2 mm proximal to its blunt end. At first 10 microlitre of fresh blood was collected into a micro centrifuge tube containing 40 microlitre 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 blood was kept upright for 2 hours followed by centrifugation at 3,000 g 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 -20°C for further use.

Intra-peritoneal challenge¹⁰

Two weeks after the last inoculation/On 42 day, the mice from experimental group (group- 1) and control group (group-2) were challenged intraperitoneally with 2×10^8 CFU/ml live MDR *Esch.coli* suspended in 300 µl PBS. All mice were observed for 14 days post challenge for any clinical manifestations such as weight loss, lack of movement, reluctant to feed or death.

Antibody Detection by ELISA

Indirect ELISA was performed for detection of IgG antibody from mice sera which is

specific for *E.coli* antigen. Antigen was separated by sonication of *E.coli*¹².

Procedure of Sonication

Briefly, 100 µL of distilled water was mixed with bacterial pellets and set aside on ice for 30 min. Then sonication was done at 20 kHz for 2×10 s (subjected to viscidness of the samples) and centrifugation was done for 20 min at 10,000 g. Then, supernatant was kept at -200 C and used as antigen later on. Antigen concentration was optimized at 10µg/ml by checkerboard titration method¹².

Optimized ELISA

Antigen coating was done in a 96 well immunoabsorbent plates with 100 µl/well of antigen (10µg/ml) in sodium bicarbonate buffer (pH=9.6) by overnight incubation at 4° C. The wells were washed with PBS three times. Then the wells were blocked with 5% skimmed milk (w/v) in PBS for 30 min at 37°C (200 µl/well). The plate was washed three times with 0.05% PBST (0.05% Tween20 in PBS) and once with PBS. Then sera (100 µl/well) were added at a 100-fold dilution and incubated for 90 min at 37°C followed by whole night at 4° C. Horseradish peroxidase (HRP) conjugated anti-mouse IgG (Thermo Fisher Scientific, USA) in PBST (1:5000) were added (100 µl/well) and incubation was done for 90 min at 37 °C. After washing, tetramethyl benzidine (TMB) and urea peroxide (substrate) were added and kept for 10 min under dark condition. Then stop solution (1M H₂SO₄) was added. ELISA plate reader (BioTek Inc., USA) was used for measurement of optical density value at 450 nm [9]. Following formula was applied for calculation of cut-off value of OD: Cut-off value of OD = M (mean) + 2 × Standard deviation

Data Analysis

The data were documented methodically. All statistical analysis was done by SPSS version 25. Statistical significance between groups was compared by unpaired T test. p- value < .05 was considered as a minimum level of significance.

RESULTS

Bacterial Inactivation by Formaldehyde

Following inactivation, the bacterial suspension was cultured overnight in Mueller

Hinton agar plate. No colonies were detected on the plate. Un-inactivated bacteria were used as a control.

Survival of Mice

After lethal challenge, 100% survival was observed at 14 days post challenge among the experimental group of mice. All mice from Group 2 died within 24 hour of challenge.

Immune Response against *E.coli*

Figure 1 shows optical density of anti-*Esch.coli* antibodies in serum samples collected 10 days after the 1st booster. Five serum samples were used from the mice of Group-1. All the serum samples had optical density of anti-*Esch.coli* IgG polyclonal antibody above the cut off value of 0.140. Cut off value was calculated as mean + 2SD (standard deviation). Here, the mean optical density values of negative control mice sera were 0.128 and SD was 0.006. There was statistically significant difference between the optical density values of experimental and control mice sera with P value of 0.0003.

Figure 2 shows optical density of anti-*Esch.coli* antibodies in serum samples collected 7 days after the 2nd booster. Five serum samples were used from the mice of Group-1. All the serum samples had optical density of anti-*Esch.coli* IgG polyclonal antibody above the cut off value of 0.137. The cut off value was calculated as mean + 2SD.

Here, the mean optical density values of negative control mice sera were 0.131 and SD was 0.003. There was statistically significant difference between the optical density values of experimental and control mice sera with P value of 0.00001.

Figure 3 shows optical density of anti-*Esch.coli* antibodies in serum samples collected 7 days after the 3rd booster. Five serum samples were used from the mice of Group-1. All the serum samples had optical density of anti-*Esch.coli* IgG polyclonal antibody above the cut off value of 0.142. The cut off value was calculated as mean + 2SD. Here, the mean optical density values of negative control mice sera were 0.128 and SD was 0.007. There was statistically significant difference between the optical density values of experimental and control mice sera with P value of 0.00002.

Figure 4 shows optical density of anti-*Esch.coli* antibodies in serum samples collected after the lethal challenge. Five serum samples were used from the mice of Group-1. All the serum samples had optical density of anti-*Esch.coli* IgG polyclonal antibody above the cut off value of 0.140. The cut off value was calculated as mean + 2SD. Here, the mean optical density values of negative control mice sera were 0.132 and SD was 0.004. There was statistically significant difference between the optical density values of experimental and control mice sera with P value of 0.00003.

IgG absorbance (450 nm)

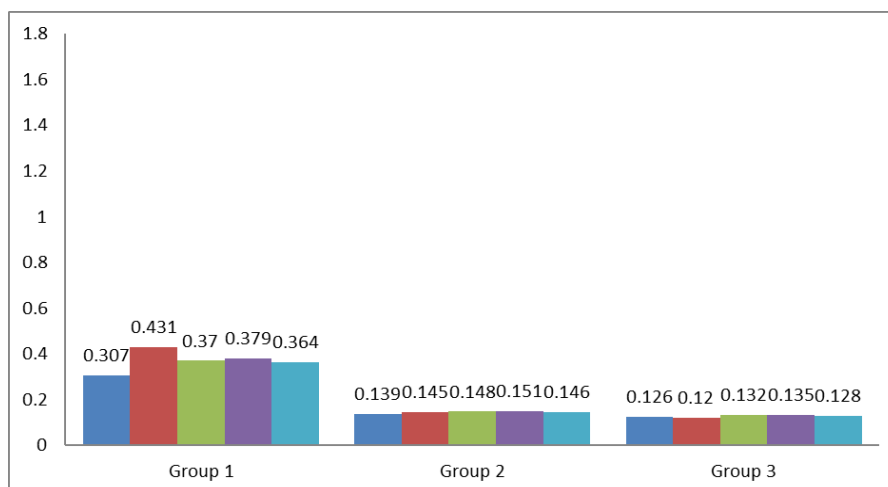


Figure 1: Optical Density (OD) of serum samples after 1st booster by ELISA

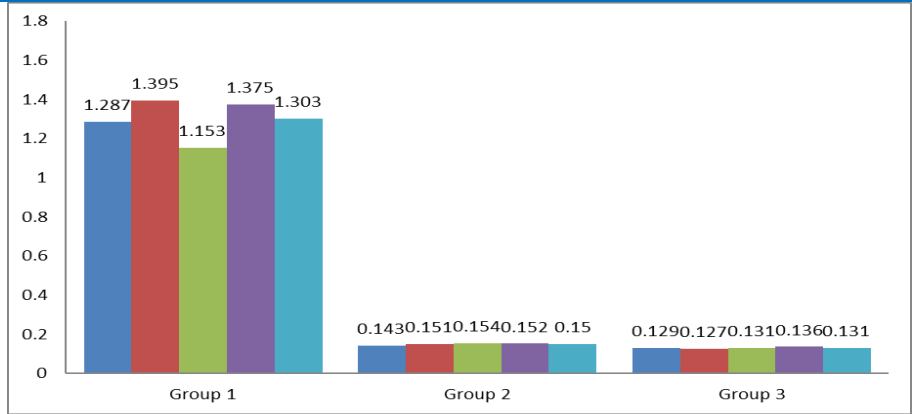


Figure 2: Optical Density (OD) of serum samples after 2nd booster by ELISA

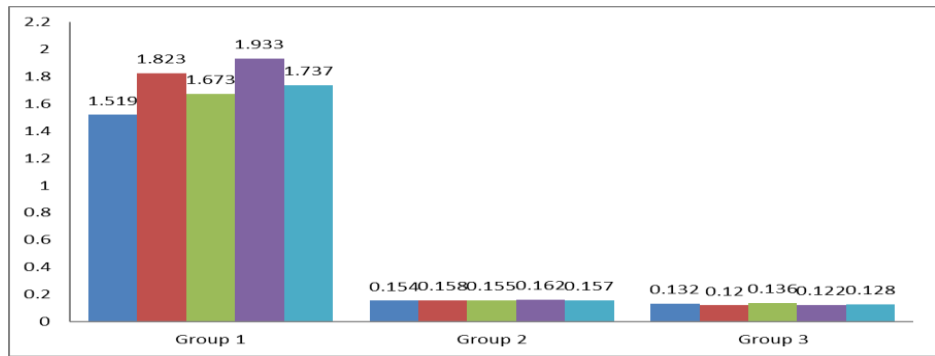


Figure 3: Optical Density (OD) of serum samples after 3rd booster by ELISA

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

variance	SS	df	MS	F	p-value	F-crit
Between groups	5.08695	3	1.695649	130.56842	1.845E-11	3.23887
Within groups	0.20779	16	0.012987			
Total	5.29474	19				

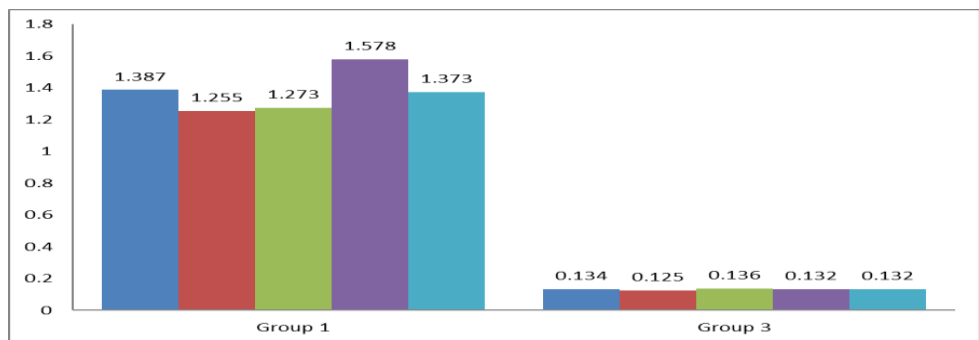


Figure 4: Optical Density (OD) of serum samples after lethal challenge by ELISA

DISCUSSION

The complexity in the pathogenesis of *E.coli* and the emergence of multidrug-resistant strains are the leading cause of the increased susceptibility of infections by this notorious pathogen to the

vulnerable groups. Hence the development of a vaccine for this challenging pathogen might be a hopeful alternative to antibiotics³. In this study, formaldehyde inactivated *Esch.coli* isolated from urine samples were used to immunize five swiss

albino mice (Group-1) and lethal challenge were given to experimental group (Group-1) and control group (Group-2). The survival proportion among the experimental mice was 100% at 14 days post challenge. A study in Iran reported 100% survival of mice after formalin inactivated intradermal *Esch.coli* immunization (13). In the present study, formaldehyde inactivated whole cell bacteria were used in order to improve the presentation of multiple antigens to the immune system, to stimulate broader immune response ¹⁴.

In the present study, after each inoculation, sera were collected for analyzing IgG antibody absorbance by ELISA at 450 nm. The OD value of serum IgG absorbance showed that there was significant rise of OD value of IgG antibody absorbance within the EG (Experimental group) of mice after 2nd and 3rd inoculation. This might be due to production of more IgG antibody by memory cells after 2nd booster onward. However, the antibody level slightly decreased following lethal challenge which might be due to utilization of antibodies to clean up offending pathogens from the body. The sera from control group (Group-2) mice contained very low level of detectable antibody after all inoculation which was close to negative control group (Group-3) mice. Hence, the mice from experimental group (Group-1) survived while all the mice from the Group-2 died after lethal challenge as because the serum IgG was insufficient for them to survive. One study showed immunization with formalin inactivated whole cell vaccine elicited significant levels of IgG in immunized mice group than the control group. Another study conducted by Fan et al (2019) reported that formaldehyde inactivated bacteria induced both IgG1 and IgG2a immune responses but mainly IgG1 (the ratio of IgG2a/IgG1 was always <0.5). The same study also reported that the IgG titre was highest at 56th days after 1st immunization ¹⁵.

A different study showed that immunization with formaldehyde inactivated whole cell *E.coli* stimulates significant serum IgM, IgG and lung supernatant IgA antibody titers against *E.coli* that induced both mucosal as well as systemic humoral immune responses. In this study, the highest OD values of the IgG antibody were noticed following the second booster. This might be

due to the fact that memory cells produced more IgG antibody following second booster than first booster. After lethal challenge, IgG antibodies were slightly decreased in mice sera due to removal of the offending pathogen from the body ¹¹.

CONCLUSION

Development of an effective vaccine against this pathogen is a burning issue that can reduce the rate of infection as well as the sufferings of the vulnerable groups and decreases the selective pressure on antibiotics. Although other measures of immunological responses are needed to be evaluated in a larger group of animal model, the present study delivers a hope that formaldehyde inactivated whole cell vaccine against MDR *E.coli* induces protective antibodies in Swiss albino mice.

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Statement of Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

List of Abbreviations

ELISA, Enzyme Linked Immunosorbent Assay; GPC, MDR, multidrug resistant; OD, optical density.

Declarations

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The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: Md. Sirazum Munir was the main contributor of this research. Md. Ahsanul Haque was the co-investigator during the laboratory works.

Data Availability

All the raw data and specific materials are presented in the manuscript.

Ethics Approval: Ethical clearance was obtained by the Ethical Review Committee (ERC) of Dhaka Medical College.

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