Efficacy of a Vaccine Against Multidrug Resistant Escherichia coli 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. **Objective:** To determine immune response against MDR E.coli using formaldehyde inactivated E.coli in mice model. **Methods:** 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. **Results:** 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.

Key words: MDR E. coli, ELISA, Immune response, Whole cell vaccine.

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 microorganism[3]. 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* infections[7].

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 models[3]. As an innovative strategy for development of bacterial vaccine, inactivation method could be simultaneously applied and for which formaldehyde is an effective inactivating agent[8]. 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 routes[9].

However, possibly 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.

Immmunization of mice

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.

Preparation of formalin inactivated whole cell *Esch.coli*

A loop full of Esch.coli was inoculated into TSB (Trypticase soya broth) in micro centrifuge tube and was incubated at 370C 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 cone. 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.[10]

Immunization schedule

Three Intradermal (I/D) inoculations were performed with 250 µl bacterial solution (2X107 CFU/ml) on day 0, 14 and 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.[10]

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 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 seperate sterile micro centrifuge tube and were kept at -200C for further use.[11]

Intra-peritoneal challenge

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 2X108 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.[10]

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 method[12].

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 40 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 370C (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 370C followed by whole night at 40 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 H2¬SO4) 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 showed 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 showed 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 showed 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 showed 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)

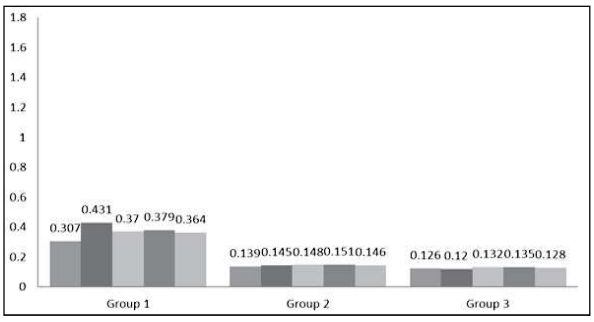

Figure 1: Optical Density (OD) of serum samples after 1st booster by ELISA

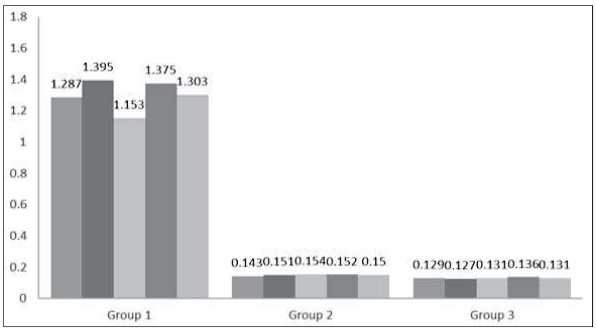

Figure 2: Optical Density (OD) of serum samples after 2nd booster by ELISA

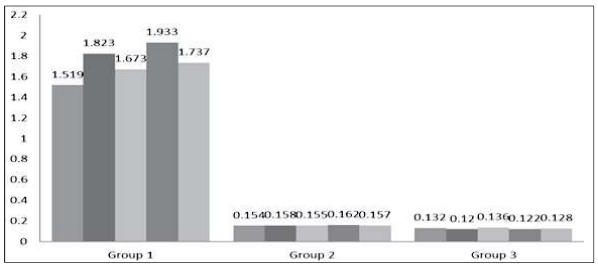

Figure 3: Optical Density (OD) of serum samples after 3rd booster by ELISA

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

Source of 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				

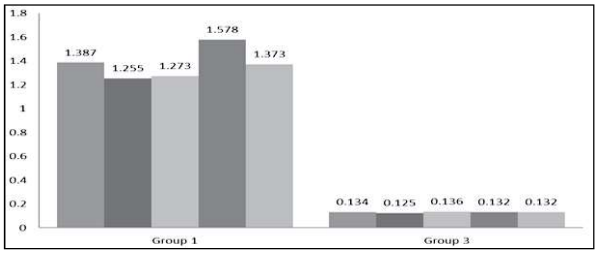

Figure 4: Optical Density (OD) of serum samples after lethal challenge by ELISA

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 antibiotics[3].

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.

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. 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.[14]

In this study, the highest OD values of the IgG antibody also 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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References

1. Chen Y, Liu Z, Zhang Y, Zhang Z, Lei L Xia Z. Increasing Prevalence of ESBL-Producing Multidrug Resistance Escherichia coli From Diseased Pets in Beijing, China. Front Microbiol, 2019; 10:2852.
2. Gallet AVC, Ocampo AM, Barry N. Molecular epidemiology of carbapenem resistant Esch.coli infection uncovers high frequency of non-carbapenemase producers in five tertiary care hospitals in Colombia. Colombia J Med Microbiol 2018; 32 (4): 12-21.

3. Mejías MS, Jurado-Martín I, McClean S. Understanding E.coli–host Interactions: The ongoing quest for an efficacious vaccine. Cells. 2020 Dec 5;9(12):2617. doi: 10.3390/cells9122617. PMID: 33291484; PMCID: PMC7762141.

4. Ramphal R. Infectious Due to Psudomonas, Burkholderia, and Stenotrophomonas Species. In Harison's Prinsiples of Internal Medicine, 20th ed Jameson JL, Kasper DL, Longo DL, Fauci AS, Hauser SL, Loscalzo J, Eds McGraw-Hill Education: New York, NY, USA 1 (2018): 1167-1173

5. Kocsis B. The investigation of colistin resistance mechanism in clinical isolate of Enterobacter asburiae. in 25th European Cong Clin Microbiol Infect Dis 2015; 1007.

6. Juan C, Peña C, Oliver A. Host and pathogen biomarkers for severe- Pseudomonas aeruginosa infections. Journal of Infectious Disease 2017; 215 : S44-51.

7. Sharma A, Krause A, Worgall S.Recent developments for E.coli vaccines. Human Vaccines 2011; 7 : 999-1011.

8. Fan Y, Mu Y, Lu L, Tian Y, Yuan F, Zhou B, *et al.* Hydrogen peroxide- inactivated bacteria induces potent humoral and cellular immune responses and releases nucleic acids, International Immunopharmacology 2019;69: 389-97.

9. Taylor CR, Lamone P, Lillis C, Lynn P. In: Fundamentals of Nursing Care: The Art and Science of Nursing Care. Walters Kluwer, 2011.

10. Rahman A, Shamsuzzaman SM, Dola NZ, Nabonee MA. Protective Effects of Immunization with Formalin Inactivated Oral Whole Cell Vaccine against MDR C.freundii in Murine Model. American Journal of Infectious Diseases and Microbiology 2022; 10(3): 92-7.

11. Kawser Z, Shamsuzzaman SM. Intradermal Immunization with Heat-Killed Klebsiella pneumoniae Leading to the Production of Protective Immunoglobulin G in BALB/c Mice. Int J Appl Basic Med Res 2021;11(3):160-5. doi: 10.4103/ijabmr.IJABMR_63_20. Epub 2021 Jul 19. PMID: 34458118; PMCID: PMC8360222.

12. Rahman A, Shamsuzzaman SM, Dola NZ, Nabonee MA. Protective Effects of Immunization with Formalin Inactivated Oral Whole Cell Vaccine against Multidrug Resistant Citrobacter freundii in Murine Model. American Journal of Infectious Diseases and Microbiology 2022;10: 92-7.

13. Karam A, Badmasti F, Ahmadi. Vaccination of mice with hybrid protein containing Exotoxin S and PcrV with adjuvants alum and MPL protects Esch.coli infections. Sci Rep. 2021; 12:1325.

14. 14. Fan Y, Mu Y, Lu L, Tian Y, Yuan F, Zhou B, Yu C, Wang Z, Li X, Lei S, Xu Y. Hydrogen peroxide- inactivated bacteria induces potent humoral and cellular immune responses and releases nucleic acids. Int Immunopharmacol 2019; 69: 389-97.