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Intradermal Inoculation of Formalin Treated Escherichia Coli Produced Protective Antibody against Protein of Specific Molecular Weight in Mice Model

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ABSTRACT

Background and aim: Escherichia coli can cause serious infections in humans and animals while constituting a significant portion of the microbiota in many hosts. Due to the increasing global prevalence of multidrug-resistant (MDR) E. coli bacteria, it is vital to explore alternate therapeutic approaches, such as vaccines. This study was designed to detect a specific protein based on molecular weight in serum and splenic cell culture supernatant using a formalin-inactivated whole-cell vaccine against multidrug-resistant (MDR) E. coli.

Material and methods: This study used MDR E. coli obtained from clinical samples to immunize 15 Swiss albino mice intradermally. Fourteen days after the third immunization dose, mice were injected with live E. coli intraperitoneally and monitored for 14 days. Sera and spleen cell culture supernatants were obtained. E. coli antigens used in vaccine production were sonicated, and their molecular weights were determined by SDS-PAGE. ELISA was used to measure protective antibodies against antigens of different molecular weights.

Results: 100% of immunized mice survived after the lethal dose of live E. coli. The experimental group mice's pre- and post-challenge serum and splenic cell culture immunoglobulin G antibodies had noticeably greater optical density (OD) values than the control mice. E. coli proteins ranging from 11 to 17 kDa showed higher OD values in sera and splenic cell culture supernatant.

Conclusions: This indicates that E. coli antigens ranging from 11 to 17 kDa are more immunogenic and can be used as a potential vaccine formulation.

1. Introduction

Escherichia coli is a diverse bacterium that can cause infections in the urinary tract, bloodstream, prostate, and other non-intestinal sites.[1] It is the most common cause of urinary tract infections (UTI) and invasive bacteremia, as well as the second most common cause of neonatal meningitis.[2] Approximately 80–90% of community-acquired UTIs are caused by Uropathogenic E. coli.[3] The emergence and rising prevalence of MDR E. coli strains are regarded as one of the key reasons for the global antimicrobial resistance (AMR) crisis.[4] The emergence and spread of MDR E. coli, which contributes to the dissemination of antibiotic resistance throughout the human microbiome, are significant factors in treatment failures and increased hospitalization costs. Vaccination programs effectively reduce antibiotic use by avoiding infections, disrupting transmission chains, and preventing the evolution of resistant strains.[5] Despite nearly four decades of clinical trials, there is currently no vaccine to prevent extraintestinal pathogenic E. coli (EPEC) because its pangenome exhibits remarkable diversity among pathotypes, clades, and strains, with hundreds of genes linked to pathogenesis, including adhesins, nutrient acquisition systems, and toxins.[6] The whole-cell vaccine contains a natural form of many immunogens, resulting in a powerful and long-lasting immune response. Formalin and heat inactivation are the most commonly used techniques for preparing inactivated whole-cell vaccines.[7] Formalin inactivation of E. coli resulted in a stronger antigenic response compared to heat inactivation, as surface antigens retained their conformations on formalin-inactivated bacteria but not heat-inactivated ones.[8] Intradermal (ID) immunization is an emerging approach to stimulate a strong immune response. Skin is densely populated with antigen-presenting cells (APC), which act as vital guardians against infections along with harboring a large number of T lymphocytes.[9] A particular antibody-antigen interaction is the most crucial part of the detection strategy. ELISA facilitates diagnosis by detecting microbial antigens in a sample, establishes prior exposure to the pathogen through the detection of antibodies, and promotes epidemiologic monitoring through serologic surveys.[10] The identification of particular bacterium antigens is essential for the development of an effective vaccination. SDS-PAGE is commonly used to separate proteins based on their electrophoretic mobility (a function of the length of the polypeptide chain, molecular weight, and other factors.[11] There is a lack of data on the antibody

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response to E. coli antigens of various molecular weights after immunization in mice. Therefore, this study was conducted to determine the presence of protective antibodies in sera and splenic lymphocyte culture supernatant against a formalin-inactivated E. coli vaccine using sonicated antigens and antigens of different molecular weights.

2. Material and Methods

Ethical approval

This study was carried out from January to December of 2023 at the Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh. Ethical permission for this study was obtained from the Research Review Committee (RRC) and Ethical Review Committee (ERC) of Dhaka Medical College (Ref: Memo No. ERC-DMC/ECC/2022/321).

Bacterial culture

Clinical samples were collected from admitted patients at Dhaka Medical College Hospital and cultured on MacConkey agar and blood agar media. They were then subcultured on Motility Indole Urea, Triple Sugar Iron, and Simmons citrate agar media. E. coli was identified by observing lactose fermenting colony on MacConkey agar media, motile, indole positive, urease negative on MIU agar media, butt, and slant yellow with gas production on TSI agar media, citrate negative, oxidase negative, and gram-negative rods on Gram staining. Six MDR E. coli strains from urine samples were selected and subcultured onto Mueller-Hinton agar plates at 37°C for 24 hours so that the bacteria were in the same development stage throughout the experiment.

Preparation of formalin-inactivated E. coli

A loop full of organisms was inoculated into tryptic soya broth (TSB), incubated overnight at 37°C, centrifuged at 2000 g for 20 minutes at 4°C, and the supernatant was discarded. The pelleted bacteria were washed twice with phosphate-buffered saline (PBS), and then 37% formalin was added to the suspension to achieve a final concentration of 3% (v/v) and incubated for 2 hours at 37°C. This suspension was washed twice with sterile PBS and resuspended in PBS to a concentration of 1.5 × 10^8 CFU/ml. 134 µL of inoculum was mixed with 866 µL of sterile PBS to obtain a concentration of 2 × 10^7 CFU/ml. The supernatant was cultured at 37°C overnight, and the efficacy of the vaccine was confirmed by no growth.[12]

Animals

Ethical permission for animal experimentation was obtained from the Ethical Committee for Animal Experimentation (ECAE) of Dhaka Medical College (Ref: Memo No. ERC-DMC/ECC/2022/321) Four to six weeks old, 15 Swiss albino female mice were collected and cared for in the animal house facility of the microbiology department at the affiliated institute. Water and nonmedicated feed were supplied throughout the trial.

Immunization Schedule

The mice were randomly divided into three groups, each containing five mice. Experimental or immunized mice were in group 1, control mice were in group 2, and negative control mice were in group 3. Three intradermal inoculations were conducted on days 0, 14, and 28 in the alternate thighs of the group-1 mice with formalin-inactivated E. Coli and the group-2 mice with sterile PBS on the same schedule. The negative control group consisted of 3 uninfected and uninoculated mice. An insulin syringe BD Ultra-Fine TM (31G) was used for intradermal inoculation. Before each immunization, mice were anesthetized with an intraperitoneal dose of ketamine (100 mg/kg body weight).

Collection of Serum for ELISA

On days 13, 27, and 41 after the initial inoculation, serum from the tail blood was collected. The tail was cleaned with 70% alcohol, and the tail tip was cut 2 mm proximal to its blunt end using a sterile 22 FR scalpel. 50 µl of fresh blood was collected into a microcentrifuge tube containing 200 µl PBS to make a dilution of 1:5. The diluted blood was kept upright for two h and centrifuged at 3000 g for 10 min. Clear sera were then transferred to a separate microcentrifuge tube and preserved at - 20°C for further use.[13]

Intra-peritoneal Challenge

The mice from the experimental group and control group 2 were challenged intraperitoneally with 3 × 10^8 CFU/ml live MDR E. coli in 100 µl PBS two weeks after the last inoculation. All mice were monitored for 14 days to look for any clinical signs, such as weight loss, immobility, reluctance to feed, or even death. Pooled serum of immunized mice was made by mixing serum collected on the 13th, 27th, and 41st days and after the lethal challenge. (Fig. 1)

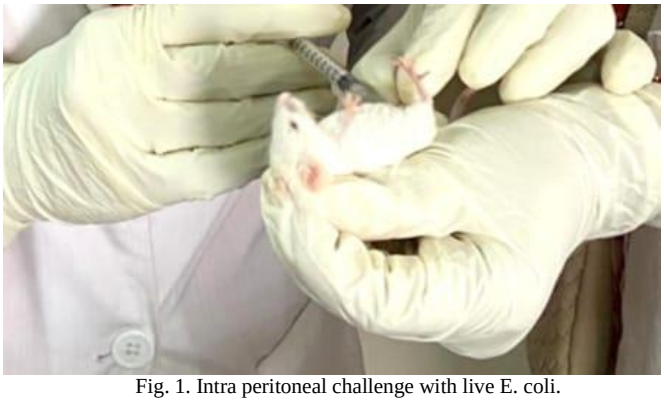

Fig. 1. Intra peritoneal challenge with live E. coli.

Separation of mononuclear cell from spleen

Under aseptic conditions, the spleens of each group-1 (experimental) and group-3 (negative control) mice were collected to detect antibody-producing cells against the inoculated Ag. Each spleen was placed in a Petri plate with 5 ml of complete RPMI medium containing RPMI-1640, 10% FBS, 200U/ml penicillin, and 200µgm/ml gentamicin under a sterile biosafety cabinet. The spleen was crushed between two frosted glass slides, and the cell solution was filtered into a 15 ml sterile conical tube using a 70 µm Nylon cell strainer and centrifuged at 350g for 10 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in 5ml of complete RPMI medium.[14]

Viability of Splenic cell determination

The mononuclear cell pellet was resuspended in one ml PBS. 20µl cell suspension and 20µl 0.4% trypan blue were mixed. The mixture was incubated at room temperature for 3 minutes. A drop of cell mixture was placed on a hemacytometer on a microscope stage. Viable (unstained) and non-viable (stained) cells were counted individually. The percentage of viable cells was determined as follows:

Viable cells % = (total number of viable cells per ml of aliquot /total number of cells per ml of aliquot) x 100

The sample was appropriate if at least 50% of the cells were viable.

Culturing of splenic cells in RPMI media

A clear layer of the mononuclear cells of the spleen was taken in a 24-well culture plate, and 50µl diluted antigen was added to each well and incubated at 37°C for 6 days.[14] (Fig. 2)

Fig. 2. 24 healthy culture plates for culturing the splenic cell.

Sonication of whole-cell E. coli

Bacterial cells used in the formation of the vaccine were suspended in 100 µl distilled water and incubated on ice for 30 minutes. Pellets were sonicated at 20 KHz for 2 x 10 seconds to minimize viscosity, incubated on ice for 5 min, and centrifuged at 10,000g for 20 minutes. The supernatants were transferred to a new microcentrifuge tube and stored at -20 °C.[13]

SDS-Electrophoresis of sonicated protein

10% separating gel was made with H_2O, Acrylamide, 1.5 M Tris-HCl (pH 8.8), SDS, Ammonium persulfate, and 10% N′ -tetramethyl ethylene-diamine (TEMED) in the specified sequence. 5% stacking gel was made using the same ingredients but with varying compositions (pH 6.8). The stacking gel was poured onto the surface of the polymerized separating gel, and a comb was inserted into it to create wells. After completing polymerization, tris-glycine electrophoresis buffer was added to the apparatus. 15µl of the sample and an equivalent volume of SDS gel loading buffer was loaded into the well for electrophoretic separation. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for 4 hours at room temperature and then destained with distilled water overnight.[15] E. coli protein bands ranged from molecular weight 11-17kDa, 34-43kDa, 55-72kDa, and 90-130kDa. A clean scalpel was used to cut off a section or strip of the gel. These bands were excised, eluted with elution buffer, and then crushed and incubated at 30°C overnight. The mixture was then centrifuged at 10,000 g for 10 min, and the supernatant was stored at -20°C.[16]

ELISA

ELISA was performed to detect the presence of IgG specific for E. coli antigen in Mice sera and spleen cell culture supernatant. Antigen (10µg/ml) in a bicarbonate-coating buffer (pH 9.6) was applied in 100µl/well to ELISA plates and incubated overnight at 4°C. Plates were washed with wash buffer, blocked with 200 µL/well of 5% w/v skim milk in PBS, and incubated at 37°C for 90 minutes. They were then washed three times with PBS-Tween. Serum (100µl/well) and splenic cell culture supernatant (300µl/well) samples at different dilutions were added and incubated for one hour at 37°C and then at 4°C overnight. After washing the plates, 100 µL of diluted (1:5000) conjugate, horseradish peroxidase-labeled anti-Mice IgG antibody (Thermo Fisher Scientific, USA) in PBS-Tween, was added to each well and incubated at 37°C for 90 minutes. After washing, substrate solution was added. The absorbance (OD) of each well was measured using an ELISA reader plate at 450nm (Biotek Inc. USA). A cut-off value of optical density was obtained by mean + 2 x standard deviation.

Data Analysis

All statistical analyses were conducted by Microsoft Excel (2020). Results were analyzed and compared by t-test, and p-value <0.05 was taken as a minimal level of significance.

3. Results

The survival rate of immunized mice was 100% after lethal challenge. All five mice in the control group 2 died within 24 hours of the challenge. The mean OD of IgG absorbance of the immunized group showed a gradual increase following the first, second, and third inoculation and a slight decrease following the lethal challenge (Fig. 3). OD value range of immunized mice after the first inoculation was 0.437-0.610; after the second inoculation was 1.253-1.396; after the third inoculation was 1.529-1.966 and 1.280-1.535 after the lethal challenge. There were statistically significant differences ($p<0.001$) between the optical density of anti-Escherichia coli antibodies of experimental and control mice sera after each inoculation (Fig. 3).

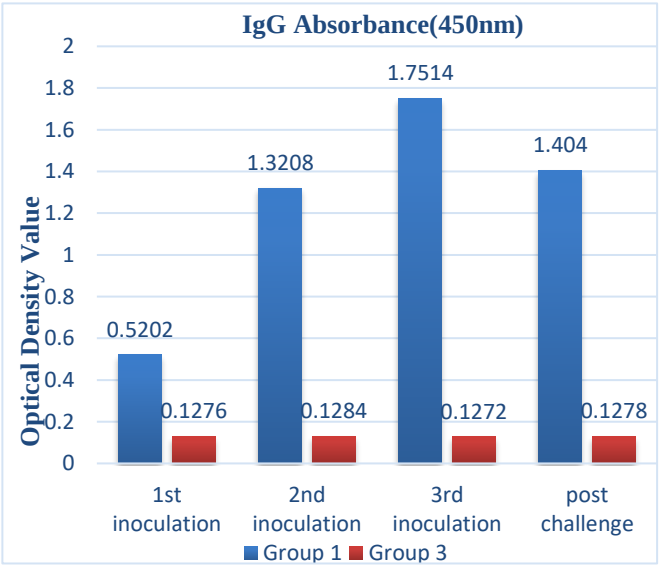

Fig. 3. Mean Optical density values in serum samples within the different inoculation schedules of experimental group 1 and control group 3 and p value<0.001.

Mean optical density values of the cell culture supernatant of experimental and negative control mice differed significantly ($p<0.001$) (Fig. 4). The highest IgG titer in pooled sera and splenic lymphocyte culture supernatant was observed against the protein of molecular weight 11-17kDa that was separated by SDS PAGE. (Fig. 5)

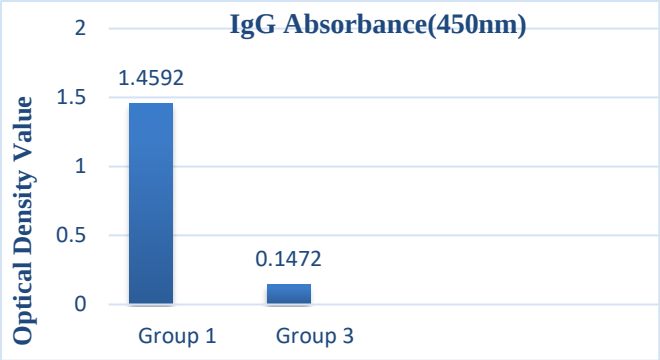

Fig. 4. Mean optical density (OD) value of splenic cell culture supernatant after incubation in RPMI media in experimental group 1 and control group 3 and p value<0.001.

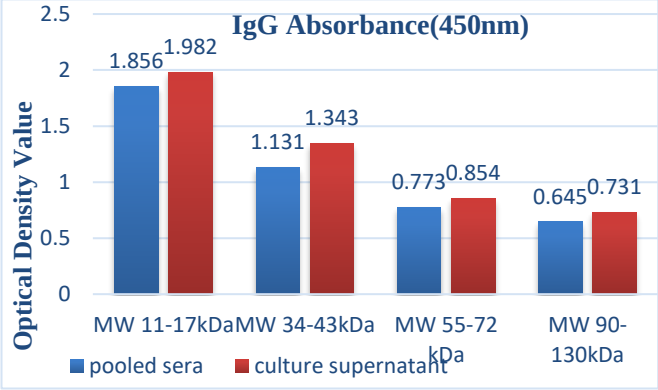

Fig. 5. OD values of the pooled sera and splenic lymphocyte culture supernatant against eluted antigens of different molecular weights separated by SDS-PAGE.

4. Discussion

E. coli usually exists as a normal flora in the human gut. However, some strains are dangerously pathogenic, leading to the highest AMR-associated mortality.[6] In recent years, the occurrence of E. coli bacteria with high antibiotic resistance, resulting in multidrug-resistant infections, has increased significantly.[17] Vaccination is an essential approach for controlling antimicrobial resistance (AMR) that involves administering substances containing antigens to boost immune responses against pathogenic organisms. After 14 days of the challenge, 100% of the immunized mice survived; however, all the mice in group 2 died, as their serum IgG levels were insufficient. The survival of all vaccinated mice may be due to the development of protective antibodies following vaccination. This is also supported by the fact that serum and splenic cell culture supernatant of immunized mice had high levels of IgG antibodies. The death of all mice in group 2 following the lethal challenge could be attributed to a lack of protective antibodies because they were not immunized. This was further supported by the presence of low IgG levels in both serum and splenic cell culture supernatant. In one study, it was reported that a formalin-killed whole-cell combination E. coli vaccine candidate exhibited 100% survival when challenged with living E. coli, compared to unimmunized mice.[18] In another study, immunization with a subunit E. coli vaccine resulted in considerably higher levels of IgG in the immunized mice group compared to the control group.[19] Splenic cell culture supernatant of an immunized group of mice showed a higher level of IgG ($p< 0.001$) from unimmunized group 3 mice in the present study. This is explained by the significant generation of antibodies by memory B cells and sensitized B lymphocytes in the spleen of vaccinated mice after they were activated by sonicated antigens of E. coli in the splenic cell culture medium. A study revealed that the experimental mice group showed significantly higher titers of IgG antibodies in both blood and spleen.[20]

In this study, the amount of IgG antibodies in immunized mice was increased after each inoculation ($p< 0.001$), which represents the efficacy of the immune system to induce the B cell molecules and lead to an increase in the titer of the IgG antibodies. These serum antibodies may be essential for activating the classical complement pathway of infection, resulting in the effective destruction of the bacteria. The current study found that the OD values of IgG antibodies in experimental mice sera were highest after the third inoculation and were slightly decreased after the lethal challenge. This might be because some of the antibodies of immunized mice were used to kill the live bacteria after the challenge. Immunized mice were challenged intraperitoneally with live E. coli. Specific IgG antibodies developed against antigens bound to the bacteria, resulting in complement activation via the classical pathway, which could cause bacterial lysis. Another possible mechanism might be phagocytosis, which happens when IgG attaches to the bacterial antigen (opsonization). Results of ELISA in this study showed that the OD value of the protein bands ranging from 11 to 17kDa was higher than the OD values of other protein bands separated by SDS-PAGE. From these findings, it can be assumed that E. coli protein bands ranging from 11 to 17kDa are more antigenic and produce more antibodies. A study demonstrated that an E. coli protein induced the highest immune response with a molecular weight of 16.2kDa.[21] So, according to the findings of this study, protein bands ranging from 11 to 17kDa could be used as a potential vaccine against E. coli. Vaccination is the most cost-effective and beneficial way to prevent infectious diseases, especially for vulnerable groups like children, the elderly, and those with compromised immune systems, thereby reducing the risk of serious infections that may require antibiotic treatment. A vaccine against E. coli should be successful in reducing sepsis cases and sepsis-related mortality globally and help lower the incidence of UTIs and recurrent UTIs. This study introduces a novel approach for developing a vaccine using formalin-inactivated E. coli of varying molecular weights against urinary tract infections (UTI), recurrent UTI, sepsis, and meningitis, particularly those caused by drug-resistant organisms. In this study, however, the antibody response against individual antigens (Ag) ranging from 11 to 17 kDa was not evaluated.

5. Conclusion

This study found that immunization with formalin-inactivated E. coli resulted in higher titers of protective IgG antibodies in serum and splenic cell culture supernatant, as evidenced by a 100% survival rate in immunized mice compared to the control group. The current study found that a specific molecular weight protein band (11-17kDa) eluted by SDS-PAGE had a higher OD value interacting with pooled mice sera and splenic cell culture supernatant of the vaccinated group than other bands. From these findings, it may be concluded that proteins of 11–17kDa may help in the development of a vaccine that provides long-term protection against E. coli infection in the future.

Conflict of Interest

The authors declared that there is no conflict of interest.

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