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Oral immunization of *Escherichia albertii* strain DM104 induces protective immunity against *Shigella dysenteriae* type 4 in mouse model

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RESEARCH ARTICLE



ABSTRACT

The recent rise of antibiotic resistance and lack of an effective vaccine make the scenario of shigellosis alarming in developing countries like Bangladesh. In recent years, our group reported the vaccine efficacy of a non-pathogenic *Escherichia albertii* strain DM104 in different animal models, where an ocularly administered vaccine in the guinea pig eye model against *Shigella dysenteriae* type 4 challenge showed high protective efficacy and also induced a high titer of serum IgG against *S. dysenteriae* type 4 whole cell lysate (WCL) and LPS. In this study, we report further evaluation of the non-invasive and non-toxic environmental strain DM104 as a vaccine candidate against *S. dysenteriae* type 4 in mice model. Oral immunization of live DM104 bacterial strain demonstrated better protective immunity in mice model by showing 90% protection in mice against live *S. dysenteriae* type 4 lethal dose challenge and by inducing effective humoral and mucosal immune responses.

KEYWORDS

Shigella dysenteriae, *Escherichia albertii*, shigellosis, protective efficacy, vaccine

INTRODUCTION

Shigellosis, a severe, intensely inflammatory gastrointestinal disease caused by *Shigella dysenteriae* is an important cause of morbidity and mortality not only in developing countries, but also in the developed countries. In the United States, 10,382 and 10,898 cumulative cases of shigellosis were reported in 2014 and 2015 (just through August of 2015), respectively [1]. Only 10–100 cells are sufficient to cause the infection that is readily transmitted by direct fecal-oral contact [2]. Despite the progress made in recent years, no licensed vaccine which is safe, efficacious and cost-effective, is currently available to prevent Shigellosis [3].

A number of *Shigella*-like bacteria from freshwater environments in Bangladesh those are serologically cross-reactive with different *Shigella* spp. have been isolated [4, 5]. One of these strains, DM104 was phylogenetically identified as *Escherichia albertii* and showed a similar lipopolysaccharide (LPS) profile to that of *S. dysenteriae* type 4 [6]. The *E. albertii* DM104 isolate was found to be non-invasive, and did not produce any entero- or cytotoxins, as well as showing negative results in the mouse lethal activity assay [7]. As an ocularly administered vaccine in the guinea pig eye model against *S. dysenteriae* type 4 challenge, the non-pathogenic DM104 strain showed high protective efficacy and also induced a high titer of serum IgG against *S. dysenteriae* type 4 whole cell lysate (WCL) and LPS. Protective efficacy of different routes of immunizations such as intranasal, oral, and intrarectal routes were also determined and compared by challenging immunized guinea pigs against live *S. dysenteriae* and by measuring both the serum IgG and mucosal IgA antibody titers [8]. In respect of

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antibody response and protective efficacy, intranasal immunization was found to show promising results. The immunogenicity and protective efficacy of DM104 strain using intranasal and intraperitoneal routes of immunizations were also determined and compared in mouse model against live *S. dysenteriae* type 4 challenge [9]. In the present study, we evaluated the immunogenicity and protective efficacy of the DM104 strain in orally immunized mice model against *S. dysenteriae* type 4 challenge and found that the DM104 bacterial strain provided 90% protection and also induced effective mucosal as well as humoral immune response in mice and proved to be a better route of immunization.

MATERIALS AND METHODS

Bacterial strains

The *E. albertii* DM104 strain was originally isolated from river water and the 16S rRNA gene sequence (GenBank accession number, JQ996386) identified it as *E. albertii* [6]. The DM104 isolate along with *S. dysenteriae* type 4 strain used in this study, were all obtained from the stock culture of the Department of Microbiology, University of Dhaka.

Animals and ethical issues

Swiss albino mice were obtained from the Animal Resources Branch of International Center for Diarrhoeal Diseases Research, Bangladesh (icddr,b) and maintained in the Department of Microbiology, University of Dhaka, under proper hygienic conditions. All animal experiments were performed following the guidelines set by the Ethical Review Committee for Animal Experimentation of the Faculty of Biological Sciences, University of Dhaka. The work adheres to the USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, and the “ILAR Guide for the Care and Use of Laboratory Animals”.

Preparation of bacterial whole cell lysate (WCL)

One mL of a 6 h culture of *S. dysenteriae* type 4 in Brain Heart Infusion broth (BHIB) was inoculated into 200 mL of BHIB and incubated further for overnight. The cultures were centrifuged at 8,000 rpm for 10 min at 4 °C and the cell pellet was sonicated at 30 Hz for 5 min on ice and centrifuged at 10,000 rpm for 10 min [10]. Supernatant containing the WCL was filtered through a 0.45 µm pore size membrane filter and stored at –20 °C.

Immunization of the animals

A total of 20 mice were included for immunization and each mouse was immunized through oral route with 200 µL of live DM104 strain (approximately 1.2×10^9 CFU/mL) suspended in 5% bicarbonate, using a feeding needle. All the mice received identical vaccine doses, four times at 48 h intervals and one booster dose on day 42 after the 4th dose

[11]. Same number of mice treated similarly with phosphate buffered saline (PBS) only, were used as control group.

Collection of sera before bacterial challenge experiment

Two immunized mice were taken and lateral tail vein bleeds were performed 72 h prior to bacterial challenge experiment for collection of sera. Sera samples were kept at –20 °C and later assayed for both IgG and IgA antibody responses against sonicated WCL of the *S. dysenteriae* type 4 strain using ELISA [12]. Wells were coated with *S. dysenteriae* type 4 WCL (1 µg/well) and incubated overnight at 4 °C. Serum IgG and IgA antibody titers were determined by using peroxidase-labeled goat anti-mice IgG (Sigma, USA) and IgA (Innovative Research, USA) diluted at 1:2,000 and 1:1,000 in blocking buffer respectively, and incubated for 2 h at room temperature. O-phenylenediamine dihydrochloride (OPD) was used as substrate and absorbance was measured at 450 nm with an ELISA reader (Bio-Rad, USA). Sera from mice treated with PBS only, were used as control. All the samples were assayed in triplicate. End point titers were determined for each serum by taking the reciprocal of the dilution at which the average OD₄₅₀ value was greater than the mean plus 10 standard deviation of values from negative control sera samples or 0.1; whichever was greater [12].

Bacterial challenge experiment

Ten mice from both DM104 immunized and control groups were intraperitoneally challenged with a dose of 100 µL containing 1.5×10^8 CFU/mL of live *S. dysenteriae* type 4, four weeks after the last dose of immunization. All challenged mice were observed daily for any death for up to 20 days and the remaining 8 immunized mice were taken for other experiments as described below.

Recovery of bacteria from tissues and organs of immunized mice

Two mice from the immunized group were taken for the recovery of bacteria experiment. Briefly, mice were infected intraperitoneally with a dose (100 µL of 1.5×10^8 CFU/mL) of live *S. dysenteriae* type 4, four weeks after the last dose of immunization and then sacrificed on day eight post-infection by cervical dislocation for collection of organs. The abdominal cavity was aseptically opened and spleen, liver and kidney were removed, homogenized separately and serially diluted in PBS to determine the number of CFU. Aliquots of 100 µL homogenates were plated onto MacConkey agar in duplicates. The plates were incubated at 37 °C for bacterial count (Oliveira et al., 2010). Similarly, two mice from the control mice group were taken for control experiment.

Cell proliferation assay

For the cell proliferation assay [13] two mice from each group were sacrificed four weeks after the last dose of immunization and the spleen cells were separated aseptically, suspended in Dulbecco's Modified Eagle's Medium



(DMEM) and were assayed in terms of their antigen-specific proliferative response by using quick cell proliferation assay kit II (Bio Vision, USA). The absorbances of the treated and untreated samples were measured using a microtiter plate reader at 480 nm.

Cytokine detection in culture supernatants of spleen cells

In a similar way, two more mice from each group were sacrificed four weeks after the last dose of immunization and the spleen cells were separated aseptically, suspended in DMEM medium supplemented with 5% FBS, penicillin G and streptomycin. After stimulation of the spleen cells with *S. dysenteriae* type 4 whole cell lysate, the cells were assayed for the production of IFN- γ and IL-4, at different time intervals by dot blot technique [13, 14]. Three μ l of spleen cell culture supernatant collected at different time intervals were adsorbed onto nitrocellulose membrane. Membranes were blocked with 3% skim milk in PBS and incubated with the primary monoclonal goat anti-mice IL-4 or goat anti-mice IFN- γ antibodies (Sigma, USA). The secondary HRP-conjugated anti-goat IgG antibody (Sigma, USA) was diluted in 3% skim milk (1:10,000) and incubated for 2 h at room temperature. The color of the dot was developed by adding 0.05% DAB (3, 3'-diaminobenzidine) (Sigma, USA) and 0.015% H₂O₂ in 0.01 M PBS at pH 7.2.

Detection of nitric oxide (NO) in the organ homogenates

For the detection of nitric oxide (NO) in different organ homogenates, two mice from each group were sacrificed four weeks after the last dose of immunization and lung, liver and spleen were aseptically taken out and separately homogenized in 1 mL PBS using a tissue homogenizer. The homogenized samples were centrifuged at 5,000 \times g for 10 min and the supernatants were assayed for nitric oxide [13]. Nitric oxide, quantified by the accumulation of nitrite in the supernatants, were measured using Nitric Oxide Colorimetric Assay Kit (Biovision, USA).

Statistical analysis

Mean \pm SEM or mean \pm SD were determined and ELISA OD titers were compared by Student's *t* test. A statistical comparison of protection data was determined using a Fisher's exact *t* test. A *P*-value of <0.05 were considered to be statistically significant. An unpaired Student's *t* test was used to compare the mean value of CFU of bacteria recovered from infected tissues.

RESULTS

Immune responses and protection following oral immunization

In this experiment, mice were given four doses of 200 μ L of live DM104 suspension containing 1.2×10^9 CFU/mL in

buffered (5% bicarbonate) suspension at 48 h intervals, followed by a booster dose 42 days after the 4th dose. When challenged intraperitoneally with live 100 μ L of 1.5×10^8 CFU/mL of *S. dysenteriae* type 4, four weeks after the last dose, 9 out of 10 DM104 immunized mice survived, when observed for 20 days, showing 90% protection. On the other hand, all the control mice treated with PBS only, died within 8 days after live *S. dysenteriae* type 4 challenge (Fig. 1). Sera collected from mice orally immunized with five doses of live DM104 strain (as described above) also induced increased level of IgG and IgA against *S. dysenteriae* type 4 WCL (Fig. 2), when compared with sera collected from the control mice (*P* < 0.05).

Recovery and estimation of live bacteria from tissues and organs

To check the presence of live *S. dysenteriae* type 4 bacteria in the homogenized spleen, liver or kidney samples of the live DM104 strain immunized mice, the homogenized samples collected on day eight post-infection, were separately plated

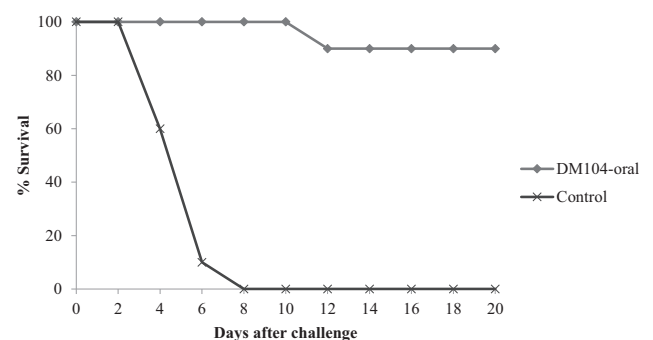


Fig. 1. Protective efficacy of DM104 strain in mice model. Mice (*n* = 10) were immunized with live DM104 bacteria, through oral route and were challenged with 1.5×10^8 CFU/mL of live *S. dysenteriae* type 4. Orally immunized mice showed 90% protection while almost all control mice died within 8 days of challenge

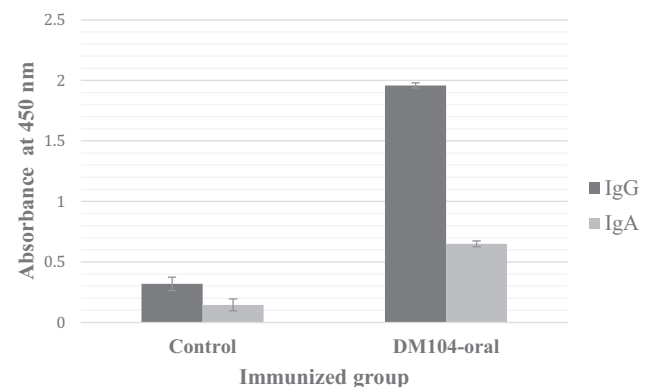


Fig. 2. Immunogenicity of the DM104 in mice model. Increased level (mean \pm S.D.) of IgG and IgA were found in blood sera of all live DM104 immunized mice through oral route. On the other hand, the level of antibodies in control mice without any immunization showed lower level

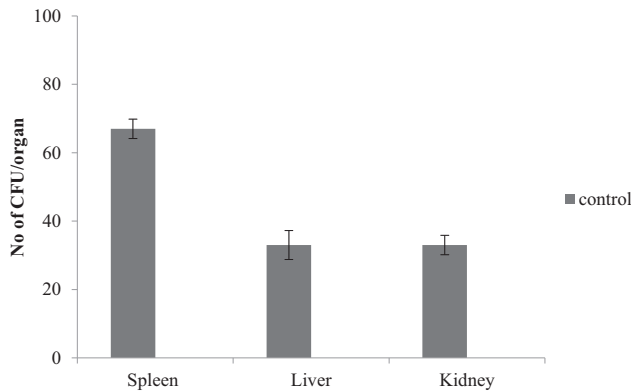


Fig. 3. Survival and dissemination of bacteria in spleen, liver and kidney of control and immunized mice. Data shown represent the mean number of CFU calculated per organ which was 67 ± 3 , 33 ± 5 and 33 ± 3 CFU/mL in spleen, liver and kidney respectively, of control mice collected on day eight post-infection. Organs collected from orally immunized mice did not show any bacterial growth. SDs are shown

on Mac Conkey agar. However, none of the homogenized samples were found to contain any live *S. dysenteriae* type 4 bacteria. On the other hand, homogenized spleen, liver and kidney samples collected from the control mice were found to contain 67 ± 3 , 33 ± 5 or 33 ± 3 CFU/mL of *S. dysenteriae* type 4 on the Mac Conkey agar, respectively (Fig. 3).

Cell proliferation assay

To investigate whether oral immunization with live DM104 strain could induce cell-mediated immunity against *S. dysenteriae* type 4, spleen cells from immunized and control mice were assayed in terms of their antigen-specific proliferative response. Spleen cells were harvested four weeks after the last dose of immunization and cultured in the DMEM medium for 24 and 48 h to measure cell proliferation. WCL preparation of *S. dysenteriae* type 4 induced higher proliferation of spleen cells collected from the immunized mice in comparison to the control mice (Fig. 4).

IFN- γ and IL-4 analysis by dot blot technique

The culture supernatants of *S. dysenteriae* type 4 whole cell lysate stimulated spleen cells were collected at different time intervals of 0, 12, 24, 36 and 48h and were analyzed for the production of IFN- γ or IL-4 by dot blot technique. Spleen cells of live DM104 immunized group of mice produced higher level of IFN- γ in comparison to controls (Fig. 5a). On the other hand, no IL-4 was detected in the spleen cell supernatant of the DM104 immunized mice (Fig. 5b).

Determination of nitric oxide (NO) concentration

Nitric oxide, quantified by the accumulation of nitrite in the supernatants of homogenized lung, liver and spleen collected from the immunized and control mice were measured using Nitric Oxide Colorimetric Assay Kit (Biovision, USA). Significantly higher NO levels were detected in the organs of immunized mice compared to the control group (Fig. 6).

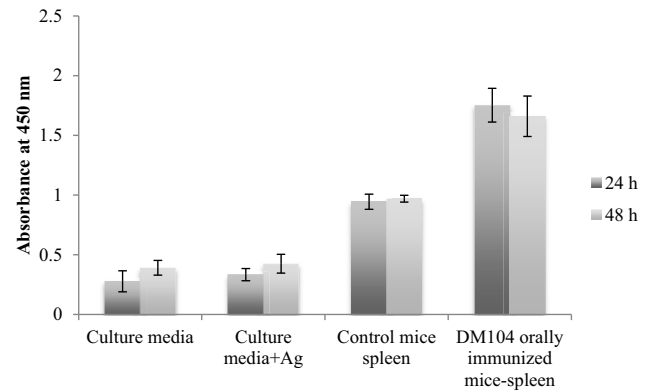


Fig. 4. Spleen cell proliferative response. Spleen cells were harvested from orally immunized (DM104) and control mice (PBS) four weeks after the last dose of immunization and cultured in the presence of medium for 24 and 48 h to measure cell proliferation. The whole cell lysate of *S. dysenteriae* type 4 induced proliferation of spleen cells in higher amount in immunized mice compared to the control mice in both 24 and 48 h cultures. Each sample was assayed in triplicate. Data represent the mean \pm SD from three mice

DISCUSSION

The protective immunity provided by the *E. albertii* strain DM104 in guinea pig model [7, 8] and mouse model [9] challenged with *S. dysenteriae* type 4 was previously reported by our group. In the present study, vaccination of mouse model with *E. albertii* strain DM104 conferred protection against *S. dysenteriae* type 4 infection. This had been demonstrated in orally immunized mice that, when challenged with *S. dysenteriae* type 4, the immunized mice showed higher survival rates and higher bacterial clearance when compared with the control mice. In the present work we also report that the immune response developed by the vaccinated mice with the *E. albertii* strain DM104 is featured by both humoral and cell-mediated immunity.

In a potency assessment with immunized mice via the oral route, the live cells of DM104 induced high serum IgG and IgA antibody titers against WCL of *S. dysenteriae* type 4. While all control animals died following challenge, almost 90% protection was obtained following oral immunization in the vaccinated group (Fig. 1). The IgG and IgA titers of DM104 immunized sera were quite high which clearly demonstrated the immunogenicity of the vaccine candidate (Fig. 2).

Eight days after infection with *S. dysenteriae* type 4, control mice showed bacterial burden in the spleen, liver and kidney, whereas none of the homogenized spleen, liver or kidney samples of DM104 immunized mice, were found to contain any live *S. dysenteriae* type 4 bacteria (Fig. 3). These results confirm that immunization with live *E. albertii* strain DM104 confers resistance to *S. dysenteriae* type 4 infection.

The specific cellular immune response developed after vaccination with DM104 was revealed by the proliferation of the spleen cells of immunized mice in response to the *in*

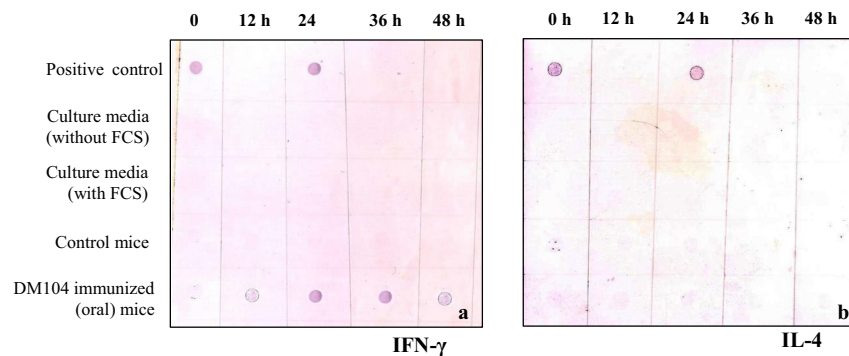


Fig. 5. Cytokine (IFN- γ and IL-4) analysis by dot blot immunoassay. Spleen cells from immunized and control mice, aseptically removed four weeks after the last dose of immunization were cultured. The culture supernatants of *S. dysenteriae* type 4 WCL stimulated spleen cells were collected at different time intervals of 0, 12, 24, 36 and 48 h and were analyzed for cytokine, IFN- γ or IL-4 by dot blot immunoassay. Cells produced higher level of IFN- γ in comparison to controls (a). On the other hand, no IL-4 was detected in the spleen cell supernatant of the DM104 immunized mice (b)

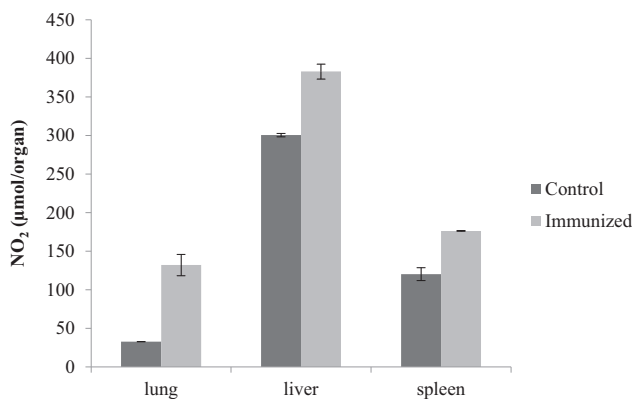


Fig. 6. Nitrite production in the organs of mice. Mice were orally immunized with 4 doses at 48 h interval and a boost 42 days after the 4th dose with DM104. Four weeks after the last dose of immunization, the lung, liver and spleen were harvested and homogenized for nitric oxide detection. The nitrite levels were measured using Nitric Oxide Colorimetric Assay Kit. Significantly higher NO levels were detected in the organs of immunized mice compared with the control group. Data represent the mean \pm SD from three mice

in vitro stimulation with *S. dysenteriae* type 4 WCL antigen (Fig. 4). Similar findings were also reported by others, where they have found that vaccination with distinct antigens carried by attenuated *Salmonella enterica* serovar Typhimurium is associated with antigen-stimulated lymphoproliferative response [15, 16].

Spleen cells from immunized and control mice, aseptically removed four weeks after the last dose of immunization, were cultured. The culture supernatants of *S. dysenteriae* type 4 WCL stimulated spleen cells were collected at different time intervals and were analyzed for cytokine, IFN- γ or IL-4 by dot blot immunoassay. IL-4, an essential cytokine for generation of Th2 responses [17], was not detected in the spleen cell supernatant of the DM104 immunized mice (Fig. 5). On the other hand, spleen cells produced higher levels of IFN- γ in comparison to control spleen cell. Thus, the result depicted that *S. dysenteriae* type

4 WCL stimulated the production of Th1 cytokine and activated the development of Th1 immunity after immunization. Thus, the secretion of high levels of IFN- γ , which is the signature cytokine of Th1 cells, may have activated phagocytic cells to kill microbes by stimulating the generation of reactive oxygen species and reactive nitrogen intermediates. IFN- γ plays a critical role in promoting the clearance of intracellular shigellae [18].

This has been supported by the nitric oxide (NO) experiment, where the level of NO was measured in the lung, liver and spleen of DM104 immunized mice prior to the challenge with *S. dysenteriae* type 4. Significantly higher NO levels were detected in the organs of immunized mice in comparison to the control group (Fig. 6).

In all of our studies, an extensive characterization was carried out with *E. albertii* vaccine strain, DM104. High protective activity in guinea pig and mice models induced by the DM104 strain along with its non-pathogenic properties strongly suggests that the *Shigella*-cross reacting *E. albertii* DM104 strain could be a promising vaccine candidate against shigellosis. However further studies are necessary to evaluate whether of immunization procedure was able to induce a long-term protection against *S. dysenteriae* type 4.

Conflict of interest: The authors have no conflict of interest to declare.

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