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Immunization of BALB/c Mice against *Shigella sonnei* Using a Multiepitope Protein Vaccine through Intranasal and Subcutaneous Administration

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ABSTRACT

As the most common cause of bacillary dysentery or shigellosis, *Shigella sonnei* (*S nonnei*) has spread throughout the world. Invasion of the colorectal epithelial cells by this facultative intracellular bacterium occurs via various virulence factors. The increase in the resistance rate highlights the need for novel interventions, particularly increasing the urgency of the development of *Shigella* vaccines that may offer an effective solution.

A multiepitope protein vaccine (MEPV) construct previously designed using bioinformatics tools against *Shigella* species, was applied in vivo in BALB/C mice. The designed vaccine construct was expressed in a bacterial host, purified, and finally confirmed by Western blot analysis.

The immunogenicity of the purified MEPV was assessed against *S sonnei* via intranasal and subcutaneous administration routes, followed by evaluating its protective efficiency. We observed that interferon-gamma, interleukin-4, and immunoglobulin G levels were increased in all experimental groups.

Therefore, The MEPV effectively protected the mice against *S sonnei.*

Keywords: Adjuvant; Immunogenicity; *Shigella sonnei;* Vaccines

INTRODUCTION

Diarrhea is a worldwide health threat annually killing ~1.3 million individuals, particularly among children under 5 years of age (500,000 cases) as the second cause of mortality.[1] Bacillary dysentery or shigellosis is a category of bacterial diarrhea affected by the Gram-negative genus *Shigella*, mainly in developing countries.[2] The epidemiology of shigellosis is mainly related to warm seasons throughout the world and is not a zoonotic disease.[3,4] The disease is more prevalent in African and South Asian countries such as India and Pakistan, while Iran in among low rate of morbidity and

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mortality of shigellosis.[5] *Shigella sonnei* (54.1%) and *Shigella flexneri* (40.1%) have been the predominant species in Iran.[6]. In recent decades, this species has spread to middle-income countries. The symptoms of shigellosis, including diarrhea, abdominal pain, fever, and tenesmus, usually begin 1 to 2 days after contact.[7] The diagnosis is determined by the existence of red blood cells, polymorphonuclear neutrophils, and mucus in stool specimens.[8]

The recommended oral antibiotics include azithromycin and ciprofloxacin.[9] However, the evolution of multidrug-resistant *Shigella* species is a crisis.[7,10-12] In this regard, the increase in the resistance rate to antibiotics such as macrolide and fluoroquinolone highlights the need for novel interventions, especially increasing the urgency of the development of *Shigella* vaccines that may offer an effective solution.[13,14] Different platform vaccines have been developed against these pathogenic species, including using whole killed bacteria (SsWc, Sf2aWC),[5,15] live attenuated strains (FS, SmD, CVD 1208S, SC602, WRSS2, WRSS3, Truncated Shigella, ShigETEC, Ty21a typhoid vaccine expressing *Shigella* antigens),[5,16] lipopolysaccharide-protein conjugates,[17] mixtures of subunit components,[18] recombinant proteins or subunit vaccines (Shigellvak, *S sonnei* O-antigen/rEPA, S4V-EPA, Sf2a-TT15, ZF0901, $Invaplex_{AR\text{-}DETOX}$, altSolflex1–2-3, OMV Sfl2a, Ipa DB Fusion, 34kDa OmpA Sfl2a, PSSP-1),[5,19] and multiepitope protein vaccines (MEPVs). MEPVs consist of several epitopes, adjuvants, and linkers, which have received considerable attention compared to old vaccines. Their benefits mostly include high immunogenicity, safety and stability, low allergenicity, and ease of production.[20] Despite advances in recent years, no approved vaccine is accessible against shigellosis.[21]

Shigella invasion plasmid antigens (Ipas), including IpaB and IpaD, are recognized as conserved, and extremely immunogenic antigens between *Shigella* species, and their outer membrane protein (OMP) effectively elicit immune responses. *Shigella* species possess a 220 kilobase pairs virulence plasmid encoding a 30 KB Mxi-Spa secretory system (TTSS), which is principally composed of invasive plasmid antigens (i.e., IpaA, IpaB, IpaC, & IpaD) essential for the invasion to epithelial cells. The main effector or translocator proteins which are in charge of releasing bacteria into the host cell cytosol via prompting cytoskeletal rearrangements, membrane perturbing, and pathogen uptake are coded by Ipa genes.[22] Considering the nasal mucosa enriched with blood vessels results in the direct distribution of the administrated vaccine into the blood rather than the liver. Additionally, the intranasal vaccine administration has a more rapid effect than other routes.[23] In our previous research, a novel MEPV construct was designed for *Shigella* pathogenic species via bioinformatics tools.[24] The designed construct immunogenic epitopes included OmpA (3 epitopes), IpaB (7 epitopes), and IpaD (6 epitopes) containing FliC as a natural adjuvant and toll-like receptor 5 (TLR5) agonist. These epitopes elicited $CD4^+$ T immune response and interferon (IFN)-γ secretion. The designed vaccine construct also contained numerous linear and conformational B-cell epitopes. Notably, intranasal vaccination promoted the secretion of mucosal immunoglobulin (Ig) A and IgG in mice;[25] enhancing the vaccination efficacy without inducing a systemic inflammatory response or adverse effects.[26] This study was implemented to assess the immunogenicity of a novel MEPV against *S sonnei* in vivo.

MATERIALS AND METHODS

Gene Construction, Cloning, Transformation and Protein Expression

In our previous research, a novel MEPV construct was composed of highly immunogenic epitopes from *Shigella* pathogenic species antigens designed by applying a bioinformatics approach. It was optimized for *Escherichia coli* expression and cloning into the pET-14b vector with 2 restriction sites NdeI, BamHI added to the 5' and 3' ends, respectively, and then the gene was sequenced (Zist Eghtesad Mad, Iran).[24]

The synthetic genetic content of the vaccine construct was subcloned into the pET-14b vector with the 6XHis tag at the N-terminal, and the vector was transformed into the *E coli BL21 (DE3)* Plys S strain by the $CaCl_2$ method and cultured into the Luria Bertani medium (Merck, Germany) with ampicillin (Jabern Hayyan, Iran) (100 μg/mL) at 37°C until optical density (OD) 600 reached 0.6. Then, the protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) with the final concentration of 1 mM in bacterial culture and incubated for 6 h at 37°C.[27] The MEPV was harvested by bacterial cell centrifugation at 4000 rpm for 7 minutes and then assessed in the bacterial pellet by 1 2% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[28]

Purification of Vaccine Protein and Western Blot Analysis

The bacterial pellet was collected, and the expressed MEPV was purified by applying nickel chelation affinity chromatography (Ni-NTA) (Qiagen, Germany) according to the Qiagen protocol (https://www.qiagen.com). Bacterial pellet from 50 mL culture was collected and thawed at room temperature, then resuspended in 5 mL lysis buffer (100 mM NaH_2PO_4 (Merck, Germany), pH 8.0, 7 M Urea (Merck, Germany), 10 mM Tris-Hcl (Merck, Germany)) and was sonicated. The lysate cell was centrifuged at 3900 rpm for 20 minutes, then the supernatant was poured into the Ni–NTA column and washed using denaturing buffers including 8 M Urea (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea), the flow-through of the soluble fractions were collected and finally, the purified MEPV was assessed using 12% SDS-PAGE.[29]

For Western blot analysis, the purified MEPV was transferred from 12% SDS-PAGE to the PVDF membrane (Sigma, USA) by applying a transfer buffer (150 mM glycine, (Biobasic, Canada) 20 mM Tris-base (Biobasic, Canada), and 20% methanol (Merck, Germany). The membrane was incubated in the blocking buffer of 5% skim milk (Sigma, USA) at 4°C overnight and in the anti-His tag antibody (Sigma, USA) in the phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma, USA), respectively. Next, it was shaken at 37°C for 1 hour, washed with the PBS/Tween solution 3 times, incubated with shaking at 37°C for 1 hour, and finally washed 3 times with the PBS/Tween solution. The MEPV was identified by applying the substrate solution, 3,3′-diaminobenzidine (DAB) (Sigma, USA) containing 1 µL/mL H_2O_2 in a dark place.[29]

Mice

Six to eight-week-old female BALB/C mice were used in in vivo experiments. All experiments were performed with minimum suffering to animals in the experimental animal center, and equal light and darkness, food and water were given to the animals under standard laboratory conditions.[30]

Animal Immunization

Thirty-two female BALB/C mice were provided from Royan Institute, Esfahan, Iran, and were randomly assigned to 4 groups of 8. Group I was intranasally injected with 25 µL MEPV (with a concentration of 0.85 mg/mL) per mouse and Group II was intranasally injected with 25 µL PBS buffer per mouse as a control group. Furthermore, Group III was subcutaneously injected with 100 µL MEPV + alum adjuvant (80 µL of MEPV, along with 40 µL of alum's adjuvant [2:1 ratio]), and Group IV was subcutaneously injected with a mix of 100 µL (PBS + alum adjuvant) as a control group. Finally, two booster doses with a concentration of 0.85 mg/mL were injected into all mice groups on the 14th and 28th days after the primary immunization.[31]

Determination of Antibody Titers

On the 56th day after the primary immunization, the blood sample was taken from the heart of each mouse in all groups. The serum was separated for the measurement of IgG levels by the enzyme-linked immunosorbent assay (ELISA) method.[32]

Cytokines Assay

Four mice per group were euthanized on the 56th day after the administration of the primary immunization, and their spleens were removed under aseptic conditions. Mononuclear cells were separated from mice spleens via Lymphodex (Inno-train, Kronberg, Germany) and suspended in the supplemented RPMI medium (Thermo, USA) with a 10% heat-inactivated fetal bovine serum (Thermo, USA) and the antibiotic solution (5000 U of penicillin and 5 mg/mL of streptomycin) (Sigma, USA) and then plated in 96-well plates at a concentration of 1×10^5 cells/well.[31]

Next, the supernatant was stimulated by a special antigen (purified MEPV with a concentration of 10 µg/mL) for 72 hours at 37°C and 5% CO_2, followed by removing the cell culture supernatant and keeping it at -20°C until use. Ultimately, an assay of interleukin (IL)-4 and IFN-γ cytokines was performed by the ELISA method.

Challenge Organisms

For the challenge, *S sonnei* was grown on trypticase soy agar with Congo red (final concentration of 0.05%) and incubated overnight at 37°C. Overall, 10 colonies were selected and cultured in lysogeny broth (LB) and then incubated with shaking at 37°C until OD 600 reached 1.0. The bacterial culture was centrifuged at 6000 rpm for 10 minutes, and the obtained pellet was suspended in PBS. On the 56th day after the primary immunization, mice were challenged with virulent *S sonnei* via the nasal route with a lethal dosage of *S sonnei* at approximately 2.1×10^6 CFU per mouse. They included the control (mice immunized subcutaneously

and intranasally with PBS + adjuvant and only PBS, respectively) and experimental (mice immunized with MEPV and MEPV+ alum adjuvant) groups. The animals were monitored daily for 30 days after the challenge, and their survival was recorded compared to the control group mice.[33,34]

Statistical Analysis

One-way analysis of variance (ANOVA) was employed for the statistical analysis of the data. For comparisons, $p<0.05$ was considered statistically significant for all tests. The data were presented as the mean±standard deviation (SD). GraphPad Prism 9 Software (Version 5.04, GraphPad Software, San Diego, CA, USA) was used for graphical presentations.

RESULTS

Gene Construction, Cloning, Vaccine Protein Expression, Purification and Western Blot Analysis

The gene for the vaccine construct was cloned into the pET-14b expression vector (total size of 1224 base pairs) and was digested by NdeI-BamHI and the digested products were observed on agarose electrophoresis gel (Figure 1A).

The expression of the MEPV was analyzed by the SDS-PAGE method. The protein expression was obtained in the LB medium six hours after induction with the concentration of IPTG 1 mM at 37°C. The MEPV was purified through affinity chromatography with a Ni-NTA column (Figure 1B).

The purified MEPV was detected by the Western blot technique (Figure 1C).

Determination of Antibody Titers

Serum IgG displayed the highest levels in the immunized group with intranasal purified MEPV with highly significant differences (*p<0.0001*) compared to the control group (PBS). Moreover, it represented the highest levels in the immunized group subcutaneously with a mix of purified MEPV + alum adjuvant compared to the control group (PBS + alum adjuvant) with highly significant differences (*p<0.0001*). In the serum, the level of IgG was more considerable in the immunized group subcutaneously compared to the immunized group intranasally (Figure 2).

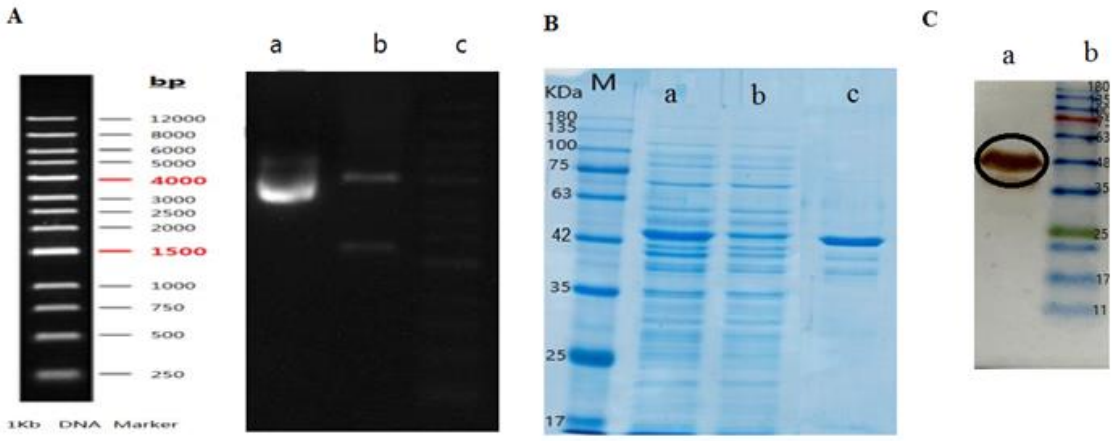

Figure 1. Cloning of the multiepitope protein vaccine (MEPV) construct gene in the pET-14b expression vector: In part (A), well (a) indicates vector, well (b) represents plasmid digested with HindIII-XbaI and well (c) depicts DNA marker. In part (B), 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of MEPV expressed in *E coli BL21 (DE3) Plys S* with 1 mM IPTG concentration at 37°C has been represented: well (M) denotes the protein marker, well (a) indicates the expressed MEPV after 6 hours of the induction, well (b) outlines transformed *E coli* before inducing expression, and well (c) depicts the purified MEPV. The samples in part (B) were derived from the same experiment with gel processed in parallel. Part (C) presents Western blot analysis of the purified MEPV where well (a) indicates the purified MEPV and well (b) represents the protein marker.

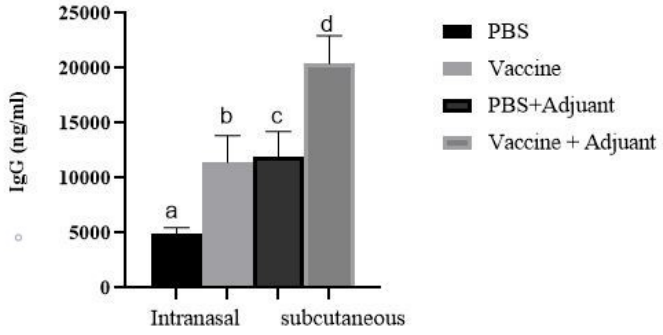

Figure 2. Determination of immunoglobulin G (IgG) antibody responses induced by the multiepitope protein vaccine (MEPV) using enzyme-linked immunosorbent assay

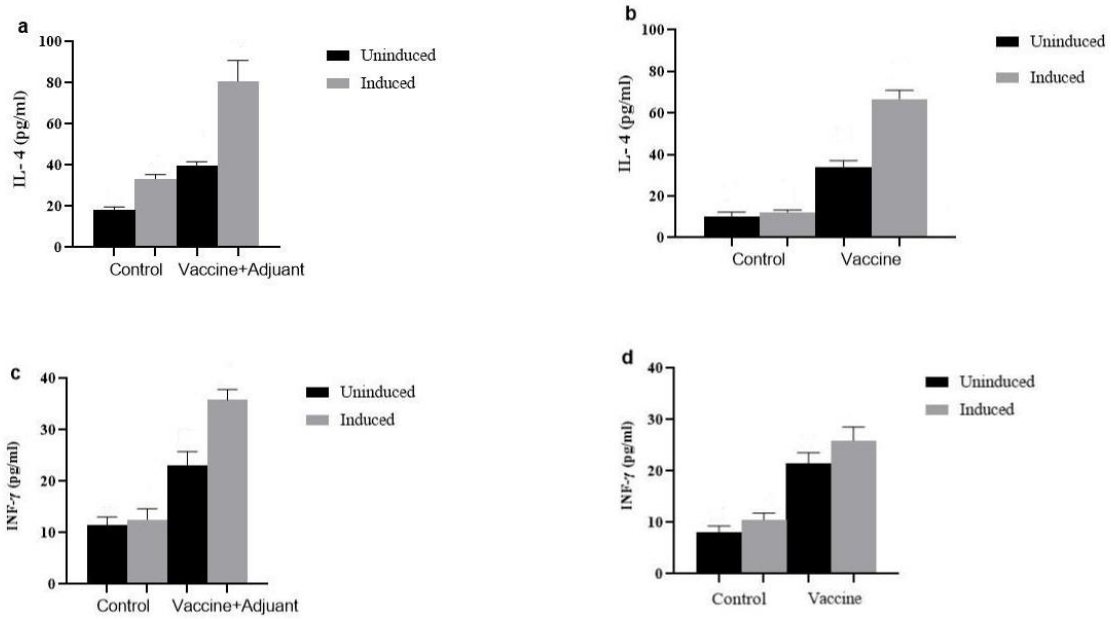

Figure 3. Cytokine production from the splenocytes of control and immunized mice, the highest levels were found in mice immunized with the multiepitope protein vaccine (MEPV) + adjuvant

Cytokines Assay

The cytokines were assessed from splenocytes cultured with the purified MEPV. The IL-4 and IFN-γ levels were considerably diverse among all experimental groups (MEPV and MEPV + alum) and significantly different ($p<0.0001$) compared to the control groups (PBS and PBS + alum). However, lower levels of IL-4 and IFN-γ were obtained in the mice group immunized with the MEPV intranasally, while the highest levels were found in mice immunized with the MEPV + alum subcutaneously (Figure 3).

The splenocytes were isolated from all the groups of mice (n=4 per group) and cultured (1×10^5 cells/well) either in the absence (uninduced) or presence of the MEPV (induced) for 72 hours in a CO_2 incubator at 37°C. (a) IL-4 (immunized subcutaneously), (b) IL-4 (immunized intranasally), (c) IFN-γ (immunized subcutaneously), and (d) IFN-γ (immunized intranasally). A significant increase in Th1-type cytokines (IFN-γ) was observed in mice receiving the MEPV. Similarly, Th2-type cytokines such as IL-4 had a significant increase in the MEPV group. The MEPV

group received 25 µL of the protein vaccine. The adjuvant group received 80 µL of protein vaccine, along with 40 µL of alum's adjuvant (2:1 ratio). The production of IL-4 and IFN-γ significantly increased in mice treated with the MEPV and MEPV + alum's adjuvant. For all tests, titer values indicated the mean±SD of 4 mice per group; $p<0.0001$ by one-way ANOVA by Tukey's multiple comparison test.

Challenge Organisms

On day 56, the vaccinated mice were challenged with *S sonnei*, and their survival was monitored for 30 days after infection. No protection was observed for mice in all control groups; however, 50% and 75% protection rates were observed in the vaccinated mice group intranasally and subcutaneously, respectively (Figure 4).

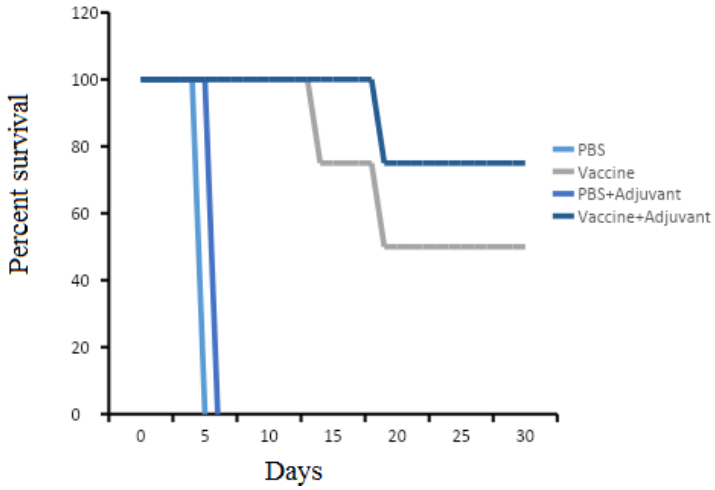

Figure 4. Immunization against *Shigella* infection. Mice were challenged on day 56 after the first vaccination. The results are obtained from the survival percentage for four mice in each group.

DISCUSSION

S sonnei is the leading cause of bloody diarrhea or shigellosis in developed countries and the second most common reason in the low- and middle-income world, particularly among children aged 1 to 4 years.[6,35-38] *S sonnei* is known as one of the leading causes of shigellosis in developing countries with an increasing rate in the Middle East, Latin America, and Asia, this bacterium has resistance against first- and second-line antibiotics, highlighting the need for improvement of vaccines.[39] Therefore, the achievement of an efficient broad-spectrum vaccine for all *Shigella* serotypes is necessary.

An effective vaccine for *Shigella* should provoke cell-mediated immunity, which has a major role in bacterial eradication. Overall, $CD4^+$ T cells promote the production of antibodies, secretion of pro-inflammatory cytokines from innate immune cells, recruitment of macrophages, and accumulation of $CD8^+$ T cells. Vaccine efficacy can be influenced by several factors such as the route of administration, adjuvant, and antigen dose.[40] Higher safety levels and stability rate, rapid production, and affordability of the vaccine are also considerably essential.[41] MEPVs against bacterial pathogens have been recently considered due to their remarkable characteristics.[42] Taking this issue into consideration in our previous research, we designed a novel MEPV for *Shigella* pathogenic strains in silico. Our approach for designing a safe and efficient vaccine for inhibiting this infection included using conserved and cross-protective antigens (i.e., IpaB, IpaD & OMPA) that could provide broad protection against multiple strains of *Shigella*. The designed vaccine construct is composed of the immunogenic epitopes of antigens, including OmpA (three epitopes), IpaB (seven epitopes), and IpaD (six epitopes) connected by appropriate peptide linkers. Additionally, the D0 and D1 flagellin domains of *Salmonella enterica* were applied as the TLR5 agonist and a mucosal adjuvant in the designed vaccine construct.[41] The protection against intracellular pathogens such as *Shigella* species needs a dominant Th1-type immunity.[43] In *Shigella* infection, several cytokines such as IL-4, in combination with pro-inflammatory Th1 and Th2 cytokines, are

stimulated by the host.[44] In this regard, the selected epitopes in our designed vaccine construct also elicited the $CD4^+$ T-cell mediated immune response and IFN-γ secretion. In other in silico and in vivo studies of MEPV candidates using various adjuvants (ctxB, LT-IIc, and flagellin domains), the provocation of immune responses has similarly included innate and acquired responses using bacterial proteins including Ipa, VirG, Omp, and IcsA.[24,45,46]

In the current research, the MEPV construct was expressed in a bacterial host and purified with Ni-NTA affinity column chromatography, and eventually detected by the Western blot method. The evaluation of immunogenicity and protection efficiency against *S sonnei* was analyzed using the purified MEPV in BALB/C mice. The vaccine was evaluated with various administration routes, including intranasal and subcutaneous administration with MEPV and MEPV + alum, respectively. A strong humoral immune response was induced after mice immunization using the two different routes of administration in all the experimental mouse groups, which was measured via the produced IgG. However, mice immunized through the intranasal route demonstrated a slower increase in the levels of IgG compared to those immunized by the subcutaneous route.

According to previous reports, many vaccines through increased levels of IgG have displayed high levels of protection in mice against *Shigella* species,[47-49] which is similar to our candidate MEPV in this research. In bacterial *Shigella* infections, several cytokines are induced in the host, including IL-4 and pro-inflammatory Th1 and Th2 cytokines.[44] Therefore, herein the levels of the secretion of IL-4 and IFN-γ were evaluated in the immunized mice. IL-4 was considerably induced in all experimental groups and demonstrated the lowest levels in mice immunized through the intranasal route compared to those immunized through the subcutaneous route.[50] Additionally, high levels of IFN-γ were observed in all experimental groups, confirming more levels in mice immunized through the subcutaneous route in comparison with those immunized via the intranasal route.

Our findings confirmed that the immunization of mice by the MEPV with a natural adjuvant (flagellin) or in combination with adjuvant alum induced both humoral and cellular immune responses in mice. Based on the report by Chao Wang et al, flagellin enhances mucosal cellular immunity.[26] Therefore, it can be applied as a natural adjuvant in the vaccine construct to stimulate an effective immune response, which is in line with our findings. Conversely, the immune response induced by MEPV + alum (subcutaneously) was higher than that of MEPV + the natural adjuvant (intranasally). Variations in the immunogenicity efficacy were attributed to the administration route. Accordingly, the designed vaccine was effective and efficient due to the induction of both cellular and humoral immune responses in mice in both administration routes. Limitations of this study mainly included a low number of studied animals, narrow experimental duration, and lack of population coverage assessment. In addition, the main challenges in the production of MEPVs include autoimmune responses or other adverse reactions, high cost, complexity, and immunization of all epitopes which have been solved to some extent by immunoinformatic studies.[51,52]

The results of this study indicated that the designed MEPV can protect mice against *S sonnei*. This study provided novel insights into the potential efficacy of the MEPV in combination with a natural adjuvant against *S sonnei.*

STATEMENT OF ETHICS

This study was approved by Fasa University of Medical Sciences (ethics code: IR.FUMS.REC. 1399.155).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable

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