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Original Article

Recombinant Truncated IpaD Protein as a Peptide Vaccine Candidate against *Shigella dysenteriae*

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Abstract

Introduction: *Shigella dysenteriae* with a low infectious dose and its strains that are resistant to antibiotics is considered a health problem all over the world. IpaD as an important protein in the *shigella* type 3 secretion systems can be a convenient target for designing recombinant subunit vaccines against the bacteria due to its immunogenic properties. The present study aimed to evaluate the immunogenicity of a recombinant protein containing immunogenic regions of IpaD as a subunit recombinant vaccine candidate against *Shigella dysenteriae*.

Materials and Methods: The gene encoding immunogenic segments of *ipaD* was sub-cloned into pET28a expression vector. The new plasmid (pET-IpaD) was transformed into *E. coli* strain Rosetta (DE3). The recombinant protein was then expressed and purified using affinity chromatography and confirmed by western blotting. The guinea pigs were then immunized with purified protein and the antibody titer and specificity of the sera were analyzed by ELISA. Finally, an animal challenge was performed using the Sereny test.

Results: According to the designed pET-IpaD plasmid, the expression of recombinant protein in *E. coli* caused the production of a recombinant protein with 22 kDa molecular weight, and the western blot technique indicated the reaction of recombinant protein with anti-histidine monoclonal antibody. The yield of the purified protein from the culture medium was estimated at about 0.57 mg/ml and the immunogenic effect of the produced protein was determined by using *in vitro* and *in vivo* studies.

Conclusions: According to the findings of the present study, it can be concluded that the recombinant produced IpaD is a perfect peptide vaccine candidate for the development of a recombinant vaccine against *Shigella dysenteriae*.

Keywords: *Shigella*, IpaD Protein, Recombinant Subunit Vaccine, Virulence Factor, Sereny Test

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Introduction

Shigella is a member of Enterobacteriaceae which is a large family of bacteria. The name *Shigella* is adopted from the Japanese microbiologist Kiyoshi Shiga who isolated this organism in 1896. *Shigella* is a gram negative, non-motile and non-capsulated bacteria and is categorized based on O somatic antigen in LPS structure into four main serotypes: A (*Shigella dysenteriae*), B (*Shigella flexneri*), C (*Shigella boydii*), and D (*Shigella sonnei*). Fifty serotypes of *Shigella* have been identified so far and relative prevalence of *Shigella* serotypes varies in different scopes of time and geography. Currently, *Shigella sonnei* consists of about 80% of the isolated *Shigella* in developing countries. In contrast, *Shigella flexneri* is the most common cause of bacillary dysentery in developed countries.[1,2] This bacterium is one of the most important foodborne and waterborne pathogens and is considered one of the health and hygiene issues around the globe. The habitat of *Shigella* species is in the large intestine of homo *sapiens* and primates and all these species cause shigellosis (dysentery or bacillary bloody diarrhea) which is known as gastroenteritis with severe abdominal pain, fever and bloody and mucoid stool. This pathogen is transmitted through individual's unclean hands (fecal-oral transmission)[3] and usually represents inappropriate environmental and personal hygiene.[4-6] Among the 50 serotypes, *Shigella dysenteriae* type 1 is severely pathogenic and can cause *Shigella* epidemic and pandemics. The prevalence of bloody diarrhea caused by *Shigella dysenteriae* type 1 is high specially in populated areas with low hygiene (e.g. nursery schools and barracks) and in critical conditions like war and natural disasters and is due to low infectious dose (less than 100 bacteria), antibiotic-resistant serotypes and production of Shiga toxin.[7,8] Bacillary dysentery is mostly common in children, as 70% of the infections are in children below 15 years old. Diarrheic diseases are the number one causes of death among the 11 causes.[9] In addition, 165 million cases of *Shigella* infections are yearly reported around the globe and

163 million of those are in developing countries which cause 1.1 million deaths with half of them being children under 5 years old. Meanwhile, cases of Typhoid caused by *Salmonella* is 6 million and cholera caused by *Vibrio cholera* is reported to be 3 million.[10,11] Due to antibiotic therapy of shigellosis and the formation of resistant serotypes and this bacteria's ability to cause severe epidemics, the preparation of a safe and effective vaccine is of great importance, although no FDA-approved vaccine has been presented in spite of the great efforts made.[12-15] Invasive factors related to type 3 excretion systems is one of the pathogenesis factors in *Shigella* and the most important factors include the proteins IpaA/B/C/D/H which are a product of *Shigella* invasive plasmid. The complex formed from the three proteins IpaB/C/D has an important role in attachment to intestinal epithelial cells (M-cells) and escaping from macrophage phagosome.[16-20]

The *ipaD* gene in *Shigella dysenteriae* contains 999 nucleotides, which encoded a 37 kDa protein called IpaD with 332 amino acids. It is located inside the operon Ipa (Invasion Plasmid Antigen) in the 31 Kb entry and in the Large Invasive Plasmid (220 kb). The IpaD protein is located at the tip of the needle of the type 3 secretion system and is essential for the invasion of bacteria into the host cell.[21-23] Also, IpaD is a multifunctional protein which controls the excretion and presentation of IpaC and IpaB into the host-bacteria space and protein carrier's penetration to the host cell.[17] IpaD has been identified as the main pathogenesis factor in *Shigella* in the immune systems of humans and monkeys. Evidence also shows that the immune response against IpaD in the host can neutralize the bacteria's virulence and invasion.[19-20] According to the investigations on the immune systems response to the important antigens of *Shigella* that prevents the entry of the bacteria to the host, this antigen is one of the hopes for the production of a vaccine for these bacteria.[24,27] In the present study, the recombinant protein consisting of the immunogenic regions of IpaD that was designed in previous investigation by bioinformatics tools,[27] synthesized, and sub-cloned into pET28 and transformed in the host *E. coli* Rosetta (DE3). Finally, purified truncated IpaD as a probable recombinant immunogenic vaccine against shigellosis was investigated using the ELISA and Sereny tests.

Materials and Methods

Cloning of the Truncated ipaD

The codon optimized synthetic gene sequence of *ipaB* and *ipaD*, was designed in a previous study, artificially.[27] In the present study, in order to obtain the truncated *ipaD* sequence, the PCR reaction was prepared using the *Pfu* enzyme (Fermentas, Lithuania) and specific primer pairs with a restriction site for *Eco*RI in the forward primer (5' GCGA GCGAATTCTCTTCTTTTTCTCCGAATAATACCAAT 3)' and a restriction site for *Xho*I in the reverse primer (5' AT ATATCTCGAGCTGGGTATAAGAAGACACGGCAT 3'). The codon optimized sequence was used as a template according to the succeeding steps: denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. For cloning, the PCR product with the length of 450 bp and pET28a vector were double digested by *Eco*RI and *Xho*I, and then, the ligation reaction was performed for 2 h at 22 °C with *T4 DNA ligase* (Fermentase, Lithuania). The product of the ligation reaction was transformed into the *E. coli* Rosetta (DE3) competent with calcium chloride using the heat shock method. Then, the screening of the obtained colonies was performed on LB agar containing 100 µg/ml of Kanamycin. Gene cloning was confirmed using the colony PCR and digestion by two restriction enzymes (*EcoRI* and *XhoI*).

Expression and Purification of IpaD

In this stage, 100 µl of overnight culture of recombinant bacteria was inoculated into the LB liquid growth medium and was aerated at 37 °C in the incubator shaker at 150 rpm. After reaching the Optical Density (OD) of 0.6 at the wavelength of 600 nm to measure the bacterial growth, the protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich, USA) with the final concentration of 1 mM and was aerated at 37 °C for 4 h in the shaker incubator at 150 rpm. Then, the cells were centrifuged for 3 min at 9000 rpm, and the cellular pallets were collected. The collected bacterial pallets from the last step was solved in the lysis buffer B (8 mM Urea, 10 mM Tris, and 100 mM Sodium dihydrogen phosphate, pH 8) for breaking the cellular wall and lysis of the cytoplasmic membrane. Next, they were put on a horizontal shaker for 30 minutes and were vortexed with glass bead for 10 minutes. The lysed cells were centrifuged at 4 °C for 20 min at 9000 rpm, and the supernatant as the soluble phase was collected for the protein purification step.

Protein purification was performed using nickel affinity resin. Before the injection of the protein-containing solution, the nickel chromatography column achieved an equilibrium with the B buffer. After the complete removal of the buffer from the column, the protein-containing solution was slowly added to the column and its output was collected in a container. In order to eliminate the proteins that were bound to the resin nonspecifically, 1.5 ml of the washing buffer C (8 mM Urea, 10 mM Tris, and 100 mM Sodium dihydrogen phosphate, pH 6.3) was added to the column and the output solution was collected separately. After the complete removal of the wash buffer C, the mentioned process was performed using the wash buffer D (8 mM Urea, 10 mM Tris, and 100 mM Sodium dihydrogen phosphate, pH 5.9), and its output solution was collected in microtubes. The isolation of the recombinant protein from the column was performed using

the elution buffer E (8 mM Urea, 10 mM Tris, and 100 mM Sodium dihydrogen phosphate, pH 4.5), and samples collected from multiple purification steps were evaluated after treatment with the buffer sample following the SDS-PAGE method. In order to expel the urea and return the protein folding, the obtained protein product was dialyzed using a dialysis sac (Cut off = 12) and the urea concentration gradient of 0-6 M. The concentration level of the isolated protein was then calculated using the Bradford method. Finally, to verify the IpaD recombinant protein production, the western blot method was used by the anti-his tag HRP-conjugate antibody (Sigma-Aldrich, USA).

Immunization Against IpaD in Guinea Pigs

For the present trial, 6-8-week-old female guinea pigs (six animals) with the approximate weight of 350 g were obtained from Razi Vaccine and Serum Research Center (Karaj, Iran) and were divided into two separate groups (control and test) with free availability to water and food and under appropriate conditions according to the ethical principles of working with animals. In the next step, 25 µg of the purified IpaD with sterile PBS was subcutaneously injected to each of the animals in the test group (4 times). Up to three booster doses were also injected S.C. with incomplete Freund's adjuvant with two weeks' intervals. It should be noted that the first round of injection was performed with complete Freund's adjuvant while the other rounds were performed with the incomplete Freund's adjuvant. Simultaneously with injections to the test group, only the sterile PBS along with an adjuvant was injected to the control animals in each step.

Also, in order to investigate the antibody titer, blood drawing from the animals was performed one week after each injection and two weeks after the final injection. For isolating the serum from the blood, they were centrifuged at 4 °C for 5 min at 1500 rpm and the serum samples were preserved at -20 °C. To quantify the antibody titer with indirect ELISA, 2 µg of antigen was bound to the bottom of the microplate with the bicarbonate-carbonate buffer (pH 9.6) and the lanes were washed with the PBST buffer (PBS with Tween-20). In the following step, the isolated serums from the four bleeding were used with a specific dilution in each lane. After washing the lanes, conjugated HPR IgG against the guinea pigs (Sigma-Aldrich, USA) was diluted with PBST to reach the dilution of 1/2000, 100 µl of which was inoculated into each lane. Then, the addition of the substrate solution (OPD, H2O2, citrate phosphate buffer) to each of the lanes was performed. Finally, the light absorption was read at 492 nm by the ELISA reader.

Challenge of Guinea Pigs Using Pathogenic Shigella flexneri

After immunizing the animals, the Sereny test was used in order to challenge and investigate the resistance of the immunized animals against infection by various serotypes of the *Shigella* bacteria.[28] To perform this challenge, the pathogenic serotype of *Shigella flexneri* approved by Imam-Hossein University was used. According to the available references, the LD_{50} dose of the pathogenic serotypes for the guinea pig was evaluated 5×10^8 $CFUml^{-1}$.[28,29] In the present study, 25 µl of the bacteria concentration containing 10 LD_{50} was prepared. To perform the challenge, the pathogenic *Shigella flexneri* was inoculated with the mentioned concentration into the eyes of the guinea pigs in control and test groups using a dropper. Then, the results were evaluated considering the existence (mild/severe) or absence of concomitant inflammation of the cornea and the conjunctiva (Keratoconjunctivitis).

Statistical Analysis

All tests were repeated three times, and the statistical analysis was performed by paired-samples *t-test* analysis using the GraphPad Prism 9.0 software (San Diego, CA). In addition, the $p<0.05$ was considered to be statistically significant.

Results

Expression Vector Construction

For designing and producing the pET-IpaD construct, the PCR reaction was prepared and performed according to the material and methods section. The presence of 450 bp bands related to the *ipaD* gene confirms the successful amplification of this sequence (Figure 1A). Also, after sub-cloning the recombinant *ipaD* gene into the pET28a expression vector, the obtained results by the colony PCR with T7 primers indicate the presence of 740 bp band related to *ipaD* gene (it should be noted that T7 primers in the pET28a vector add about 290 bp to the target gene) on 1% agarose gel (Figure 1B). As it can be seen, only colonies No. 2, 3, and 7 received recombinant constructs containing the *ipaD* gene. In addition, the enzymatic digestion of the recombinant plasmid containing the synthetic gene *ipaD* and pET28a was performed, and the 450 bp fragment (related to *ipaD*) and 5369 bp (related to pET28a) observed on 1% agarose gel showed the accuracy of the desired genetic construct (Figure 1C).

IpaD Production

After the induction of the target gene in *E. coli* Rosetta (DE3) cells using IPTG and cell lysis, the cell extract content was examined on 12% SDS-PAGE gel. As it was expected, a 22 kDa protein band related to the target protein (along with the fusion protein added to the sequence by the expression vector) was observed whereas no band was observed in the samples before the induction (Figure 2).

Protein Purification

In the purification process, after the inoculation of the sample obtained from the extraction process into the column,

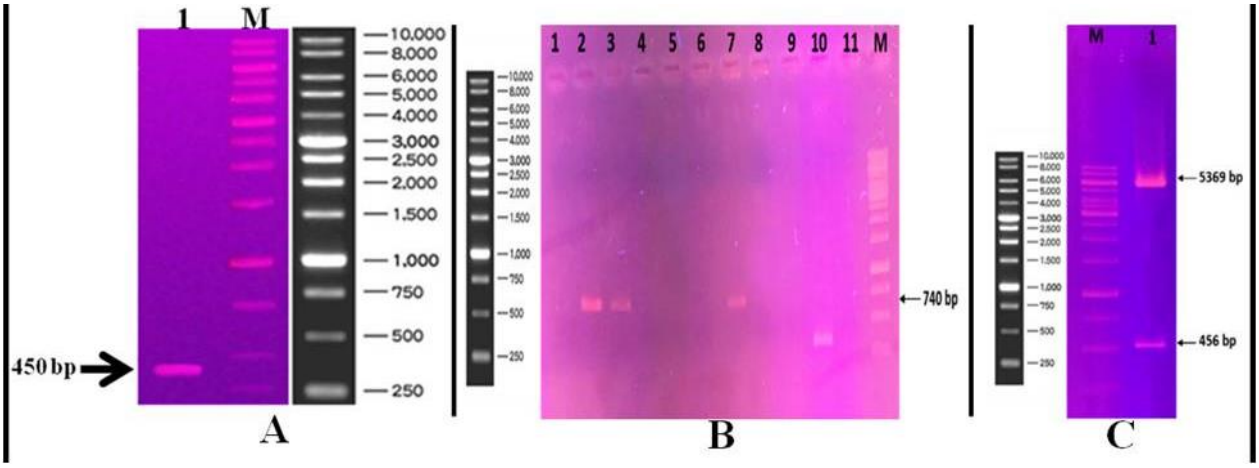

Figure 1. (A) Amplification of Gene Fragments Using *Pfu* Enzyme. Lane 1: *ipaD* gene; Lane M: 1 kb DNA ladder. (B) Colony PCR to Confirm the Presence of *ipaD* Gene in TOP10F Strain with T7 primers on 1% agarose gel. Lanes: 1-9: recombinant colonies; Lane 10: positive control (pET28); Lane 11: negative control (without template). Only colonies No. 2, 3, and 7 received the construct, Lane M: 1 kb DNA ladder. (C) The enzymatic digestion of the recombinant plasmid (pET-IpaD) using *Eco*RI and *Xho*I enzymes on 1% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: enzymatic digestion with two enzymes *Eco*RI and *Xho*I simultaneously.

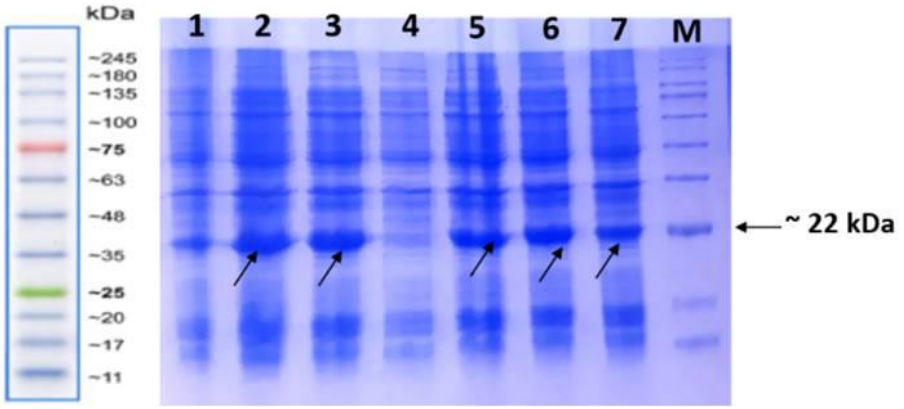

Figure 2. SDS-PAGE Analysis for the Expression of Recombinant IpaD on 12% SDS-PAGE. Lane 1: The sample before the induction; Lane 2: recombinant *E. coli* (clone 1) after four hours (lane 2) and overnight induction (lane 3). Recombinant *E. coli* (clone 2) after four hours (lane 4) and overnight induction (lane 5). Recombinant *E. coli* (clone 3) after four hours (lane 6) and overnight induction (lane 7). Lane M: protein size marker.

the purified buffers were sequentially inoculated into the column and the output buffers were collected. Examination of the output buffers revealed that the recombinant protein was higher in the wash buffer E as compared to the other wash buffers (Figure 3A). The purified protein was confirmed by the Western blotting with Anti-His tag antibody. The IpaD purified protein band was put in the right place. However, no band was seen in the control column (Figure 3B).

Serum Titration

The results of ELISA indicated that IpaD could evoke the humoral immune system and raise a high titer of IgG in the immunized guinea pigs. The serum titer reached 1:204.800 after the fourth injection. The mentioned titer was significantly higher than that of the first to third injections (p<0.0001) (Figure 4a).

Challenge of The Immunized Guinea Pigs Using Pathogenic Shigella dysenteriae

The challenge of the immunized guinea pigs was carried out using pathogenic *Shigella dysenteriae*. The results showed that all control animals lost their eye sight due to the concomitant inflammation of the cornea and conjunctiva (Keratoconjunctivitis). However, no inflammation was observed over 1-6 days in the immunized animals (Table 1, Figure 4b).

Discussion

Multiple *Shigella* serotypes, the pathogenic strains' heterogeneity, and the emerging antibiotic resistance, necessitates the broadly protective vaccine development. Different vaccine strategies have been used during several decades trying to develop an efficacious and safe *Shigella* vaccine.[30,31] The

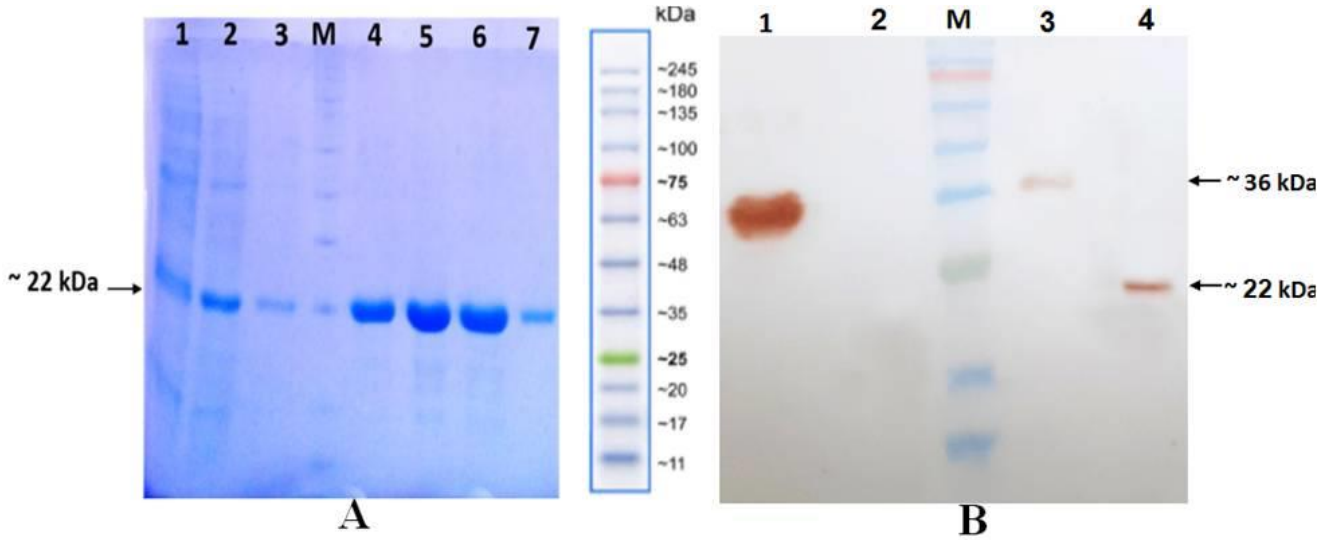

Figure 3. (A) Analysis of the Purification of Recombinant IpaD Protein Under Denaturation Conditions in Rosetta Strain on 12% SDS-PAGE. Lane 1: column output after protein loading; Lane 2: washing of column by washing buffers; Lanes 3-7: purified proteins (elutions); lane M: protein size marker. (B) Confirmation of the Purified Recombinant Proteins Using Western Blot by Anti-Histidine Antibody. Lane 1: positive control (a standard protein that fused to histidine tag, Sumo salk 1); Lane 2: negative control (recombinant bacteria before induction); Lane 3: the IpaB purified protein (positive control); Lane 4: the IpaD purified protein; Lane M: protein size marker.

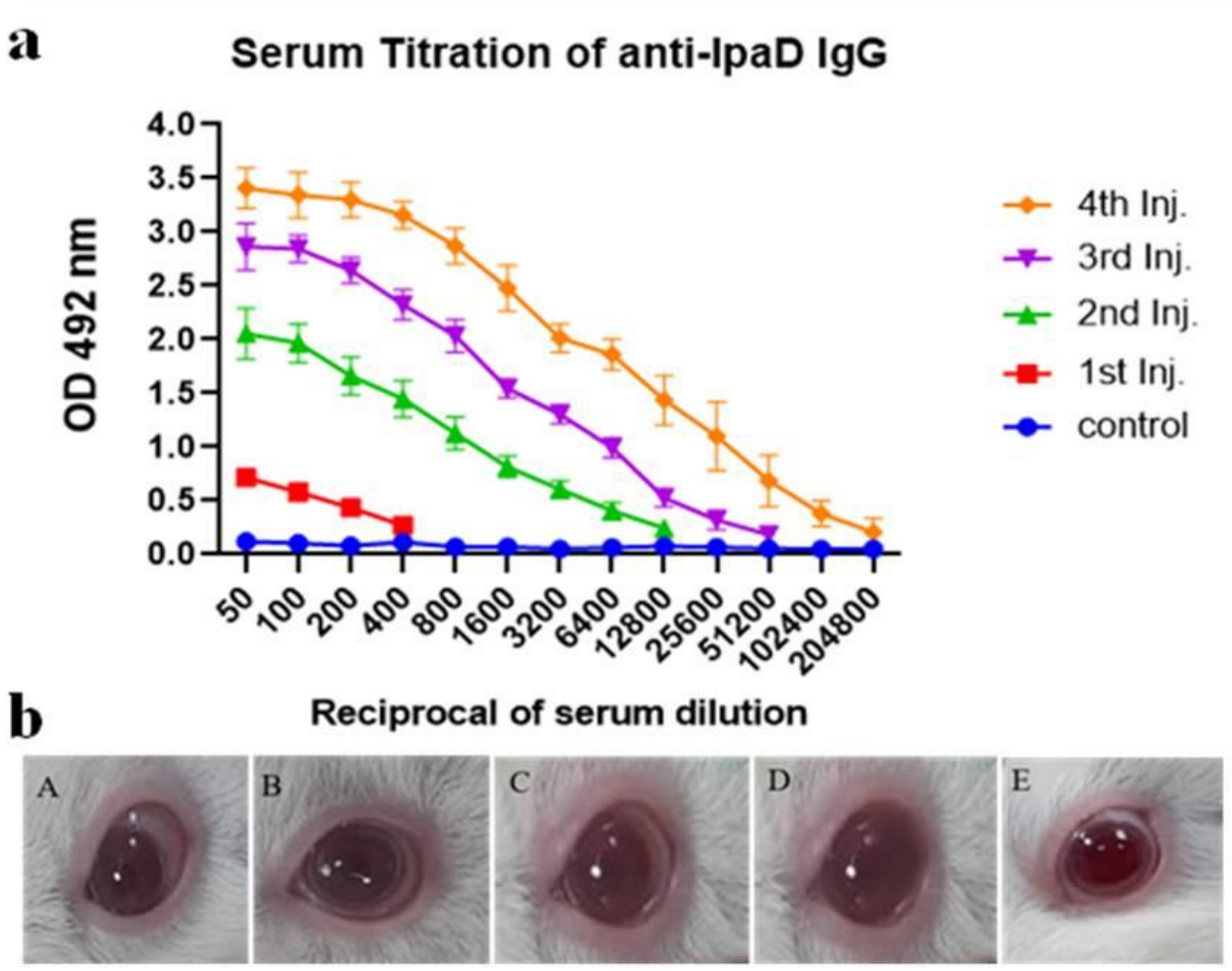

Figure 4. (a) The Serum Titration of the Guinea Pigs after each Injection Absorbed at 492 nm. (b) Schematic Representation of a Guinea Pig Control Sample Eye Following 24 (A), 48 (B), 72 hours (C), and 6 day (D), Compared with the Eyes of an Immunized Pig Following 6 Days (E).

Table 1. The Degree of Keratoconjunctival Inflammation in Eyes of the Test and Control Guinea Pigs with *S. flexneri*

Time	24 h	48 h	72 h	96 h
Control group	±	+	++	+++
Test group	–	–	–	–

The degree of keratoconjunctival inflammation in the eyes of the test and control guinea pigs with *S. flexneri* after 24, 48, 72, and 96 h (n = 5). The keratoconjunctivitis test of the guinea pigs was rated as follows: –: No disease or mild irritation, +: Mild conjunctivitis or late development and/or rapid clearing of symptoms, ++: Keratoconjunctivitis without purulence, and +++: Fully developed keratoconjunctivitis without purulence.

development strategies for the *Shigella* vaccine of the previous 50 years and the present ones comprise of two major separate categories of the live-attenuated vaccine strains as well as the inactivated candidates of *Shigella* vaccine (whole cell and subunit). Turbyfill et al. (2000) investigated the immunogenicity effects on guinea pigs and rats by isolation, detection, and characterization of the IpaB, C, and D subunits of the invasion complex proteins. Results of their study demonstrated a significant index in IgG and IgA antibodies' production in the mentioned animals against Ipa and LPS proteins.[32] Martinez et al. (2012) utilized recombinant IpaD and IpaB proteins which were produced in a totally isolated manner followed by alone or combined injection to the mice, producing a protective antibody response in the rat animal model against different *Shigella* species.[33] Shannon et al. (2013) assessed the IpaD and IpaB proteins' immunogenicity which was orally produced in mice. The systemic and mucosal immune response against *Shigella* proved the two proteins' immunogenicity.[34] Arabshahi et al. (2018) produced a recombinant chimer vaccine by fusing the *Shigella's* IpaB protein with the *Clostridium perfringens* bacteria's C-terminal region to produce a recombinant vaccine candidate that protects against *Shigella* infection.[35] Saadati et al. (2010), have produced an N-terminal fragment from IpaD (with 344 bp) that express 16.7 kDa protein. In this investigation, the presence of truncated IpaD (Ipa^{72-162}) was confirmed using the SDS-PAGE and western blot technique but the immunogenicity effect of N-terminal fragment from IpaD as a vaccine candidate has not been determined.[36] However, Hesaraki et al. (2013) indicated the immunogenicity effect of Ipa^{72-162} in guinea pigs and concluded that this protein is a suitable protective vaccine against *S. flexineri* 5a and *S. dysenteriae* type I.[37] Also, Buysse et al. (1987) used the λgt11 expression plasmid for clone *ipaB*, *ipaC*, *ipaD*, and *ipaH* genes. Recombinant phage (λgt11Sfl) expressing pWR11O-encoded polypeptide antigens were identified using rabbit antisera directed against *S. flexneri* serotype 5 (strain M9OT) invasion plasmid antigens.[38] Due to the important role of the IpaD molecule in the pathogenesis of the disease, it seems that the blockage of this molecule can prevent the development and progression of the disease. So, this study sought to express and investigate the immunogenicity of IpaD as a vaccine candidate. Our literature review showed three previous attempts for expressing IpaD as a vaccine candidate. Buysse et al., expressed intact IpaD protein (39 kDa) and Saadati et al., expressed a truncated IpaD (16.7 kDa). However, these two studies didn't report their immunogenicity study. On the other hand, in a study by Hesaraki, the effect of protein immunogenicity was investigated. However, the difference between the present with the mentioned study was the size of the recombinant IpaD immunogenic protein. Thus, we developed a new truncated IpaD contained two new immunogenic epitope regions (the residue 13-72), can play as strong immunogen and capable to induce a robust immune response for inhibit the disease.

In addition, licensed vaccine is not still available, though these attempts have been helpful in better understanding the immune response against *Shigella*, and in addition to the recent pioneering strategies produced promising vaccines.[39,40] IpaD and IpaB proteins are among effector proteins which are present at the T3SS's needle tip and play a significant role in the bacterial attachment and entry into the host cell. IpaD protein consists of 332 amino acids and is a 37 kDa protein. Moreover, IpaD is a key factor in IpaB calling on T3SS's tip. IpaD may turn to a potential antigen in the development of *Shigella* vaccine. In the present study, owing to the significance of IpaD in the bacteria entry into host cells, these proteins' immunogenic regions were considered as *Shigella* vaccine candidates. The immunogenic protein gene which is cloned in the expression vector pET28a was transformed into *E. coli* Rosetta (DE3) bacterial host. This expression vector was induced using IPTG in addition to nickel chromatography column for recombinant protein purification. The process of production and purification of the recombinant protein were done using SDS-PAGE. The purified protein, were injected to the guinea pigs with the complete (first injection), and the incomplete Freund adjuvant, then the blood samples were collected, and the antibody titers were investigated using ELISA. Finally, the Sereny test was used for animal challenge. According to the designed pET-IpaD plasmid, the expression of recombinant protein in *E. coli* caused the production of a recombinant protein with 22 kDa molecular weight and western blot technique indicated the reaction of recombinant protein with anti-histidine monoclonal antibody. The yield of purified protein from the culture medium was estimated about 0.57 mg/ml and the immunogenic effect of produced protein was determined using *in vitro* and *in vivo* studies. The purified recombinant IpaD protein was injected subcutaneously

(S.C.) along with complete Freund's adjuvant. Up to three booster doses were also injected S.C. with incomplete Freund's adjuvant with two weeks' intervals. Bleeding was done before immunization and two weeks after each booster and guinea pigs serum were collected. The antibody titer was determined by ELISA. In subcutaneous administration route, IgG assay through ELISA, showed that the level of the antibody has increased after each administration. The eye infection of the control pig and the non-infected eye of the pigs which received bacteria have also confirmed the immunization of the construct against *Shigella*. The results of the Sereny tests in guinea pigs demonstrate that subcutaneous administration of IpaD can protect in vivo against mucosal infection with bacteria.

Conclusion

The results of this study demonstrated that the produced recombinant protein has suitable immunogenicity. It can be concluded that this recombinant protein be examined to manufacture novel recombinant vaccines for *S. dysenteriae* through safety and function evaluation among the large laboratory animals as well as clinical trials.

Authors' Contributions

The authors contributed equally to this study.

Ethics Approval

For animal experimentations, adequate measures were taken to minimize pain and discomfort of the animals, and all experimental procedures were in accordance with the ethical guidelines for working with animals.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

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