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Original Article

تأثیر پروتین نوترکیب IpaB در ایجاد پاسخ‌های ایمنی علیه شیگلا دیسانتری در خوکچه هندی

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چکیده

سابقه و هدف: شیگلوزیس بیماری عفونی ناشی از شیگلا دیسانتری است و سالانه جان میلیون‌ها نفر را در سراسر جهان تهدید می‌کند. توسعه سویه‌های مقاوم به آنتی‌بیوتیک، واکسن‌ها را به عنوان اولویت اصلی سازمان بهداشت جهانی علیه بیماری قرار داده است. پروتین IpaB که بخشی از سیستم ترشحی نوع III (T3SS) در شیگلا است، می‌تواند پاسخ ایمنی مطلوبی در برابر باکتری ایجاد کند. هدف از این مطالعه ارزیابی ایمنی‌زایی پروتین نوترکیب حاوی نواحی ایمونوژن IpaB به عنوان گزینه واکسن زیر واحد نوترکیب علیه شیگلا دیسانتری است.

مواد و روش‌ها: ژن کدکننده پروتین ایمونوژن در وکتور بیانی pET28a همسانه‌سازی و به میزبان باکتریایی E.coil سویه Rosetta (DE3) ترانسفورم شد و توسط IPTG القا گردید. پروتین خالص با استفاده از ستون کروماتوگرافی نیکل به دست آمد و برای ایمن‌زایی به خوکچه هندی تزریق شد. تیتر آنتی‌بادی تولید شده با استفاده از روش ELISA غیرمستقیم ارزیابی شد و در نهایت آزمون چالش حیوانی با آزمایش سرنی در خوکچه هندی انجام شد.

یافته‌ها: باند ۳۶ کیلو دالتون به عنوان پروتین IpaB در SDS-PAGE مشاهده شد که با وسترن بلات تایید شد. آنالیزهای ایمونولوژیکی تولید تیتر بالایی از آنتی‌بادی اختصاصی علیه IpaB (1:102400) را در خوکچه هندی واکسینه شده نشان داد. عدم وجود هرگونه کراتوکنژنکتیویت در خوکچه‌های هندی واکسینه شده در تست Sereny نشان دهنده سطح بالایی از محافظت در برابر شیگلا دیسانتری است.

نتیجه‌گیری: یافته‌های این تحقیق نشان می‌دهد که پروتین نوترکیب تولید شده یک گزینه مناسب به عنوان واکسن برای مهار و درمان در برابر شیگلا دیسانتری است.

واژگان کلیدی: شیگلا، پروتین IpaB، واکسن زیرواحد نوترکیب، فاکتور بیماری‌زایی، آزمایش سرنی.

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Original Article

The effect of recombinant IpaB protein on the develoment of immune responses against *Shigella dysenteriae* in Guinea Pig

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Abstract

Background & Objectives: Shigellosis is an infectious disease caused by *Shigella dysenteriae* and threatens the lives of millions of people worldwide annually. IpaB protein, which is part of the *Shigella* type III secretion system (T3SS), it can elicit a favorable immune response against the bacterium. The aim of this study is experimental evaluation of the immunogenicity of a recombinant protein containing immunogenic regions of IpaB as a subunit recombinant vaccine candidate against *Shigella dysenteriae.*

Material & Methods: The gene encoding immunogenic protein that cloned into pET28a expression vector was transformed in the bacterial host *E.coil* strain Rosetta (DE3) and induced by IPTG. The purified protein was achieved using nickel chromatography column and injected into guinea pigs for immunization. The produced antibody titer was assessed by indirect ELISA assay and finally animal challenge was performed using the Sereny test in guinea pigs.

Results: A 36 KDa band as IpaB protein was observed in SDS-PAGE which was confirmed by western blot. The immunological analyses showed production of high titer of specific anti-IpaB antibody (1:102,400) in immunized Guinea pig. The absence of any keratoconjunctival inflammation in immunized guinea pigs in Sereny test indicated high level protection against virulent *Shigella dysentriea.*

Conclusion: The results showed that IpaB can elicit high titer of antibody and protection against *Shigella dysentriae* in Guinea pigs.

Keywords: Shigella, IpaB protein, Recombinant Subunit Vaccine, Virulence Factor, Sereny test.

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Introduction

Shigella species are a Gram-negative, facultative anaerobic, nonmotile, nonspore forming, and rod-shaped Eubacteria bacteria and usually classified into four serogroups (or Species): group A (*S. dysenteriae* wtih 12 serotypes), group B (*S. flexneri* wtih 6 serotypes), group C (*S. boydii* wtih 18 serotypes), and group D (*S. sonnei* with one serotype) based on the biochemical properties and group-specific O antigens within the outer portion of the semipermeable membrane. The

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relative prevalence of the Shigella serotypes varies over time and geography. The resulting infection by this organism called shigellosis, also known as bacillary dysentery or Marlow syndrome, is most typically associated with diarrhea and other gastrointestinal symptoms in humans (1). Among the four species listed, *S.dysenteriae* causes pandemic dysentery worldwide. Shigellosis is a severe diarrheal disease associated with high morbidity and mortality rates, particularly in the developing world. It is also responsible for long-term effects on cognitive and physical development in children (2). According to the United States Centers for Disease Control (CDC) and Prevention, Shigella is estimated to cause 80-165 million cases worldwide every year, resulting in 0.6 million deaths, particularly in young children (3).The bacterium can be transmitted from person to person through fecal-oral contact or through contaminated fomites. Ingestion of as few as 10 Shigella can cause illness in adult volunteers (4).

IpaB protein is the most important virulence factor of Shigella which encoded by *ipa*B gene located within an operon called Ipa (Invasion Plasmid Antigen) in the region entry and on the large invasive plasmid (220kb) (5). The *ipa*B gene in the *S. dysenteriae* contains 1743 nucleotides, which encoded the 62 KDa protein called IpaB and contains 580 amino acids that are located in the cytoplasm of infected macrophages (6,7).The IpaB protein is located at the tip of the needle of type III secretion system and is essential for the invasion of the bacterium into the host cell. IpaB is sufficient to induce apoptosis in macrophages and works by directly binding to the essential component of the cell death machinery, IL-1b converting enzymes (8). This protein is one of the most important pathogenic factors in Shigella that have been identified as a major antigen by the human and monkey immune systems (9,10). Observations show that, immune responses in the host against *Ipa*B can counteract virulence and bacterial invasion (11). Due to the increased drug resistance of different strains of Shigella, researchers are seeking a vaccination strategy to prevent shigellosis (12).

Many methods have been developed for the vaccine against Shigella in recent decades, including live attenuated vaccines (13-16), killed whole-cell vaccines (17,18), DNA vaccine, conjugated vaccine, Subunit vaccine, invaplex vaccine and delivery of Shigella lipopolysaccharide to carriers such as proteasomes (19-20). All of these methods have disadvantages, for example in the attenuated live vaccine, the remaining pathogen in the vaccine strains has limited the use of this method (21). DNA vaccines are non-live and non-replicative and so, cannot be converted to the pathogen type unlike the live vaccine. The hazard of integration of bacterial gene into the recipient's chromosomal DNA with the resulting risk of insertional mutagenesis or spreading of antibiotics resistance genes, are exist among the risks that WHO and FDA list for the use of DNA vaccines. Conjugated, subunit, and recombinant vaccines can be used for all people including those with immune system weakness and chronic health problems that is an advantage for a vaccine. A limitation of the mentioned vaccines is that they might be injected in several doses in order to make immunity against the pathogen. No FDA-approved vaccine against Shigella has been prepared so far, but efforts to develop an effective vaccine are still ongoing (22).

Subunit recombinant vaccines have been very much considered due to cost savings, safety, and long-time immune induction, as well as

multi-subunit vaccines rather than single units, which provide more complete response (23). In this experimental study, we investigated the immunogenicity and protective effect of the new recombinant protein that designed in form of in silico as new vaccine candidate and contained immunogenic subunits of the *Ipa*B protein from *S. dysenteriae* using in-vitro and in-vivo assays (24).

Material and Methods

1. Bacteria, Plasmid and Chemicals: The chemical and biological materials were obtained from the following sources and all chemical materials were of the highest grades available: pET28 vector from Novagen; pET-B4D from Malek-ashtar University of Technology (24); *E. coli* strain Roseta (DE3), *Taq* and *Pfu* DNA polymerase, *Eco*RI and *Xho*I restriction enzymes, T4 DNA ligase, protein and DNA ladder from Fermentase, isopropyl β-D-1-thiogalactopyranoside (IPTG), anti- his tag antibody and Kanamycin from Roche; agarose, acrylamide and bisacrylamid, from Sigma, sodium dodecyl sulfate (SDS), Diaminobenzidine (DAB), 2-Mercaptoethanol, ethylenediamine tetraacetic acid (EDTA), NaCl, CaCl2, Urea, Triton X100, Phenylmethanesulfonyl fluoride (PMSF), Tris, bovine serum albumin (BSA), boric acid, Luria Bertani, from Merck.

2. Cloning of the ipaB gene: The amplification of the truncated *ipa*B gene was performed using the pET-B4D gene construct, as the template, that was synthesized by the Biomatik Co. (Canada) (24), and with the forward (5' TACTATGAATTCCTGCCGCTGGCCAAAA T 3') and reverse primer (5' TCATA-TCTCGAGTTTGCGTTCCATTTCGGTTTTG 3') that were designed by Oligo 7.60, and has a restriction site for *Eco*RI and *Xho*I in forward and reverse primers, respectively. Also the PCR reaction carried out with *pfu* enzyme according to the succeeding steps were followed: denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Double enzymatic digestion was done to prepare the pET28a vector as well as the 837 bp *ipa*B gene segment attained from PCR for cloning (25). Next, the T4 DNA ligase, was used to perform the ligation reaction for 2 hours at 22°C. Then, the heat shock method was employed to transform the product of the ligation reaction (new plasmid that named pET-*Ipa*B) into the *E. coli* strain Rosetta (DE3) competent with calcium chloride (26). Subsequently, LB agar containing 100 µg/ml of Kanamycin was used to screen the obtained colonies. The PCR as well as digestion by *Xho*I and *Eco*RI restriction enzymes were used to confirm gene cloning (27).

3. Protein expressing and purification: For protein expression, after transformed of the pET-IpaB plasmids into competent *E. coli* Roseta (DE3), a single colony of the fusion protein was cultured and incubated at 37°C for overnight and in the following day, 1 ml of the overnight culture was used to inoculate 250 ml of LB/kanamycin. Then, the aeration in the incubator shaker was done at 37°C at 150 rpm. To measure the bacterial growth, first the optical density (OD) of 0.6 was reached at the wavelength of 600 nm. Then, the induction of the protein expression was performed using isopropyl β-D-1-thiogalactopyranoside (IPTG) with the ultimate 1mM concentration, and the aeration was done in the shaker incubator at 37°C for 4 hours at 150 rpm. Subsequently, the centrifugation of the cells was performed for 5 minutes at 9000 rpm, Then, the lysis buffer

(B buffer, 8 mM Urea, 10 mM Tris, pH 8, and 100 mM Sodium dihydrogen phosphate) was employed to solve the collected bacterial pallets from the last step to break the cell wall as well as the lysis of the cytoplasmic membrane. Afterwards, the collected bacterial pallets were placed on a horizontal shaker for 30 minutes and then vortexed for 10 minutes with glass bead. Centrifugation of the lysed cells was performed at 4°C for 20 minutes at 9000 rpm. Then, the collection of supernatant as the soluble phase was performed to be used in the purification step of the recombinant protein (28).

The protein purification was carried out using nickel affinity resin. For this, the nickel chromatography column equilibrated with the B buffer (pH=8) prior to injecting the protein-containing solution. When the buffer was completely removed from the column, the protein-containing solution to the column was added, and then the collection of its output in a container was performed. For eliminating the proteins which were nonspecifically bound to the resin, 1.5 mL of washing buffer C (pH=6.3) was added into the column. In follow, separated proteins were collected and when the wash buffer C was completely removed, the wash buffer D with pH = 5.9 was used to repeat the mentioned process. Then, the microtubes were used to collect the output solution. The elution buffer E (pH=4.5) was used to isolate the recombinant protein from the column. The SDS-PAGE method was employed to evaluate the samples that were collected from multiple steps of purification following the treatment with the buffer sample (29). The urea concentration gradient (0-6 M) as well as dialysis sac (Cut off=12) was used to dialyze the obtained protein product and return the protein folding and expel the urea. Also, the western blot method with the anti-his tag HRP-conjugate antibody was used to confirm the IpaB recombinant protein. Finally, the concentration of the purified protein determined and calculated by the Bradford method (30,31).

4. Immunization of guinea pigs with IpaB: To perform the current trial, first six female guinea pigs that were 6–8 weeks old and about 350 g weight were attained from Razi Vaccine and Serum Research Center (Karaj, Iran). The guinea pigs were allocated into a control group and a test group, and were kept under proper condition with free access to food and water, based on the ethical principles of working with animals. Affinity purified recombinant IpaB protein (25 µg) was injected subcutaneously (SC) along with complete Freund's adjuvant. Up to 3 booster doses were also injected SC with incomplete Freund's adjuvant with 2 weeks' intervals. Bleeding was done before immunization and two weeks after each booster and guinea pigs serum were collected. The control group was received only adjuvant with PBS.

ELISA plates (NUNC, Denmark) were coated with 100µl (2 µg) per well of *Ipa*B dissolved in bicarbonate buffer and incubated at 37°C (2 h). BSA was used as negative control. The wells were washed four times with PBST and blocked by skim milk solution 2% (w/v) in PBST. The wells were loaded with serially diluted of the sera (100 µl per well) and the wells were then incubated with 1:2000 dilution of conjugated HPR IgG against the guinea pigs (Sigma-Aldrich, USA) in PBST. Each step completed with 1h incubation at 37ºC and 4 times washing with PBST. Finally, the plates were developed with 100 µL per well of OPD (Sigma-Aldrich, USA) substrates in citrate-phosphate buffer (pH 5) for 20 min at

room temperature in dark. After stopping the reaction with sulfuric acid (2.5 M), the plate was read at 492 nm with an ELISA reader (Dynex, USA) (31).

5. The challenge of guinea pigs: Following the immunization of the animals, the resistance of the immunized animals against infection by Shigella serotypes was challenged and investigated using the Sereny test (32). The pathogenic serotype of *Shigella flexneri* that was approved by Imam-Hossein University (Tehran, Iran), was used for performance of the challenge. As the pertinent references indicate, the LD50 dose of the pathogenic serotype was calculated to be 5×108 CFU×ml-1 for the each guinea pigs (33). 25 µl of the bacterial concentration that contained 10 LD50 was prepared in the current study. The inoculation of the pathogenic *Shigella flexneri* with the already-prepared concentration into guinea pigs' eyes in the test and control groups was performed with the use of a dropper to do the current challenge. Finally, the absence or the presence (mild/severe) of concomitant inflammation of the cornea and the conjunctiva (Keratoconjunctivitis) was recorded.

6. Statistical Analysis: All tests were repeated in three times and the statistical analysis was performed using a paired-samples t-test analysis using the GraphPad Prism 9.0 software (San Diego, CA). $P<0.05$ was considered to be statistically significant.

Results

1. Synthetic IpaB: According to previous study (24), synthetic sequence was designed from the wild-type sequence of *ipa*B and *ipa*D mRNA, with the insertion of *Nde*I (5/ end) and *Xho*I (3/ end) sites, subsequently. The sequence in our study was cloned into pET21a (the Biomatik Co., Canada) and named pET-B4D. In this synthetic sequence with 1395 bp molecular size, six-histidine codon CAC, was designed before stop codon TAA for one-step purification by immobilized metal-ion chromatography. The findings provided by colony PCR revealed that all of the examined clones were positive and obtained the anticipated construct. It was possible to observe the 1568-bp band on 1% agarose gel. Moreover, the addition of 173 bp to the desired gene after PCR with T7 primers was taken into consideration. The enzymatic digestion was conducted on recombinant plasmids with *Nde*I and *Xho*I enzymes. Lastly, the presence of the recombinant sequence that had the length of 1568 bp in the vector was confirmed by the enzymatic section. Moreover, 1395-bp and 5443-bp bands were present on the agarose gel following the digestion. As the following figure demonstrates, successful gene amplification can be confirmed considering the existence of 837-bp bands that are related to *ipa*B gene.

Also, the findings provided by PCR colony with T7 primers indicate that the 1127-bp band, which is linked with the *ipa*B gene, is present on 1% agarose gel (Fig. 1A). As the mentioned figure shows the recombinant construct that includes the *ipa*B gene is received by only colonies No. 6, 7, and 11. Consider that 290 bp is added to the target gene by T7 primers in the pET28a vector. Furthermore, the enzymatic digestion of the recombinant plasmid that includes the synthetic gene *ipa*B and pET28a was conducted. The correctness of the expected genetic construct was confirmed by observing the 5369-bp (related to pET28a) and the 837-bp fragment (related to *ipa*B) on 1% agarose gel (Fig. 1B).

2. Expression and purification of IpaB: Examination of the cell extract content was performed on 12% SDS-PAGE gel following

the use of IPTG and cell lysis to induce the target gene in *E. coli* Rosetta (DE3) cells. Although no band was present in the samples prior to the induction, a 36 kDa protein band that was linked to the target protein and the fusion protein that was added to the sequence using the expression vector were observed as it was anticipated (Fig. 2).

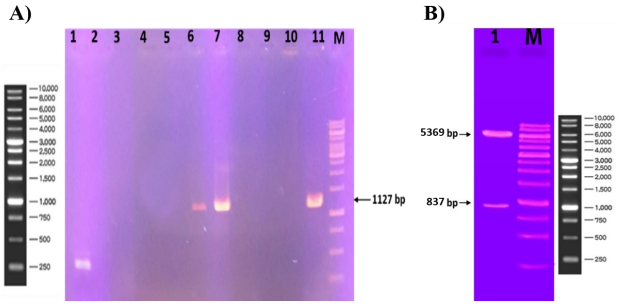

Fig 1: (A) Confirming the existence of *ipa*B gene in TOP10F strain following colony PCR with T7 primers on 1% agarose gel. Lane 1: positive control (pET28), lane 2: negative control (without template), lane 3-11: Recombinant colonies. The construct was received by only colonies No. 6, 7, and 11, lane M: 1kb DNA ladder. (B) The use of double enzymatic digestion employing *Xho*I and *Eco*RI enzymes on 1% agarose gel to confirm the recombinant plasmid (pET-IpaB) and its transformation into a host bacterium. Lane M: 1kb DNA ladder, lane 1: concurrent enzymatic digestion with two enzymes *Xho*I and *Eco*RI.

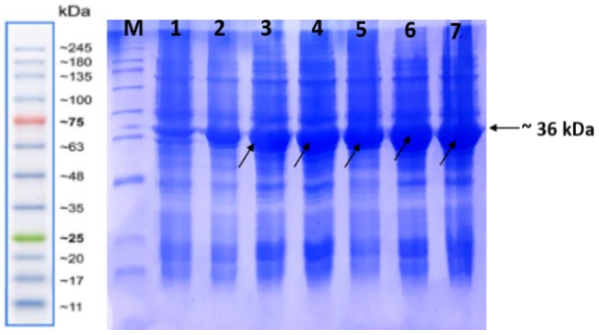

Fig 2: Analyzing the recombinant protein IpaB expression in *E.coil* Rosetta strain on 12% SDS-PAGE gel. Lane 1: recombinant *E.coli* before induction. Recombinant *E.coli* (clone 1) after four hours (lane 2) and overnight induction (lane 3). Recombinant *E.coli* (clone 2) after four hours (lane 4) and overnight induction (lane 5). Recombinant *E.coli* (clone 3) after four hours (lane 6) and overnight induction (lane 7). Lane M: protein size marker.

For the protein purification, the sequential inoculation of the purified buffers in the column and collection of output buffers were performed following the inoculation of the sample attained from the extraction process into the column. The recombinant protein was found to be higher in buffer E in comparison with the other wash buffers following the evaluation of the output buffers (Fig. 3-A). Western blotting as well as anti-his tag were employed to confirm the protein. The IpaB purified protein band was positioned in the correct place. No band was present within the control column (Fig. 3-B).

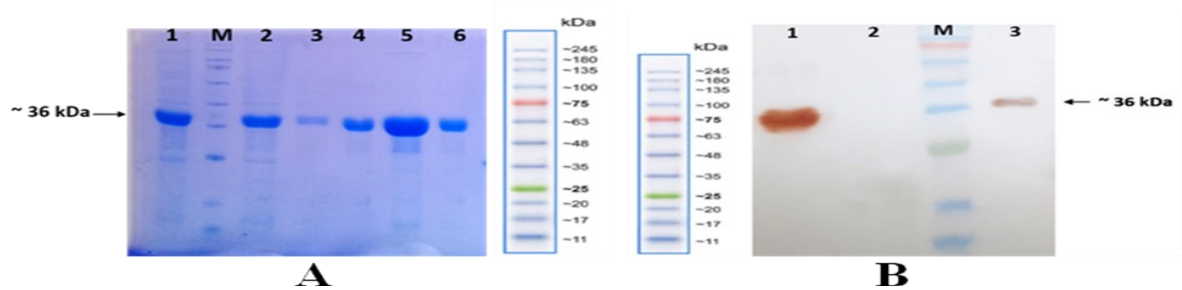

Fig 3: (A) Analyzing the IpaB protein purification in Rosetta strain on 12% SDS-PAGE gel under denaturation conditions. Lane 1: column output following the protein loading, lane 2: the column washing with washing buffers, lane 3-6: purified protein (elutions), lane M: protein size marker. (B) Confirming the purified recombinant IpaB proteins within the Rosetta strain using Western blot technique and 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 *ipa*B purified protein, lane M: protein size marker.

3. The immunogenicity effect of IpaB: The findings of ELISA revealed that the humoral immune system could be evoked and a high titer of IgG could be raised in the immunized guinea pigs by IpaB. As shown in Fig. 4, after one injection and 3 boosters, all guinea pigs were hyper immunized and anti-IpaB IgG titer received up to 1:102,400.

As expected, after first injection, anti-IpaB IgG was raised little and after each booster the antibody titer was increased. The antibody titer after one booster was received to 1:6400 and by second booster the anti IpaB IgG titer was grown up and reaches to 1:51,200. Finally, last booster highly evoked immune response and anti-IpaB IgG was received upto 1:102,400 which indicated a hyper immunization in Guinea pigs. As expected, the control group was not showed any anti-IpaB IgG.

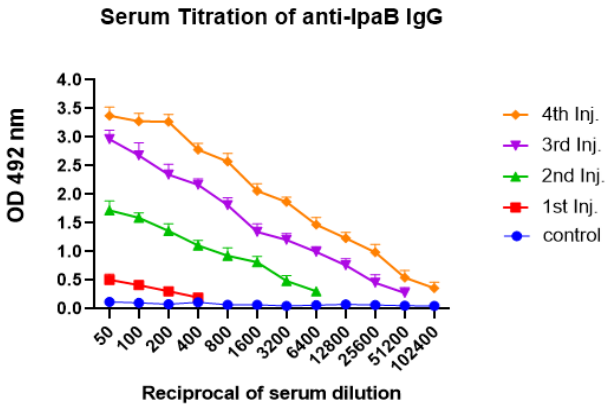

Fig 4: The guinea pigs' serum titration absorbed at 492 nm following each injection.

4. The challenge of guinea pigs: The findings indicated all the control group loss their eye sight which caused by simultaneous inflammations occurred in the cornea and conjunctiva (Keratoconjunctivitis). However, it should be mentioned that no signs of keratoconjunctivitis were observed in immunized animals over 1-6 days (Fig. 5 and table 1).

Table 1: The keratoconjunctival inflammation degree in the eyes of guinea pigs in the test and control groups with *S. flexneri.*

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

The keratoconjunctival inflammation degree in the eyes of guinea pigs in the test and control groups with *S. flexneri* following 24, 48, 72, and 96 h (n = 5). The keratoconjunctivitis test of the guinea pig 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.

Discussion

Shigella spp .are food and waterborne pathogens that cause severe diarrheal and dysenteric disease associated with high morbidity and mortality. The disease caused by this bacterium is called shigellosis. Shigella

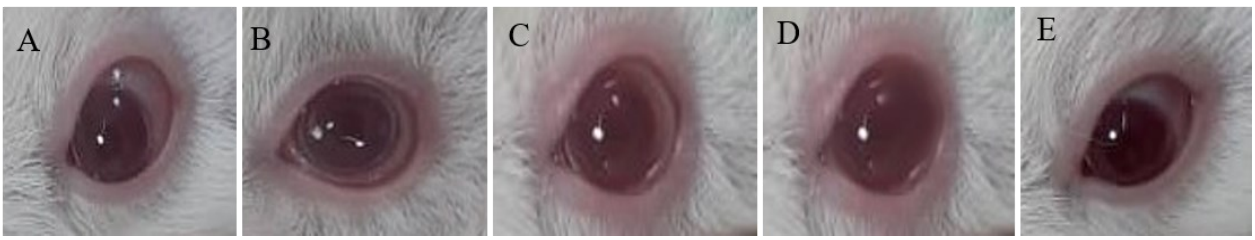

Fig 5: A guinea pig of control group following 24 (A), 48 (B), 72 hours (C), and 6 day (D), compared with the eyes of an immunized pig following 6 days (E).

bacteria, after entering the human intestine, can be trapped by M cells and transcytosed and delivered to the immune cells of Peyer's patches. The bacteria can also invade the adjacent epithelial cells. After being swallowed by the macrophage cell, this bacterium causes the release of interleukin-1 and interleukin-18. The main pathogenicity of Shigella is due to its invasiveness to neighboring cells and reproduction in these cells. As a result of infection, colonic cells are damaged and exhibit impaired absorption of water and nutrients, leading to watery diarrhea accompanied by blood and mucus in stools. These bacteria cause pathogenicity by various methods including toxic and invasive factors. The genes required for invasion are located on a 31kb conserved region called the entry site that is carried by the large virulence plasmid (34). This region encodes the components needed for the assembly and function of a T3SS and many effector proteins, which, together with IpaB, IpaC, and IpaD, allows the direct translocation of effector proteins from the bacterial cytoplasm into the host cell. The existence of multiple Shigella serotypes and the heterogenic distribution of pathogenic strains, as well as emerging antibiotic resistance, require the development of a broadly protective vaccine. Diverse vaccine strategies have been utilized over several decades in an attempt to develop a safe and efficacious Shigella vaccine. The Shigella vaccine development strategies of the last 50 years and the current ones include the two main distinct categories of live-attenuated vaccine strains and inactivated Shigella vaccine candidates (subunit and whole cell) (35). Turbyfill et al (2000) examined the effects of immunogenicity on rat and guinea pigs by isolating, detecting, and characterizing the following subunits of the invasion complex proteins (IpaB, C, D). The results showed a significant index in the production of IgA and IgG antibodies in these animals against the proteins Ipa and LPS (36). Martinez et al (2012) used recombinant IpaB and IpaD proteins produced in a completely isolated manner and injected alone and in combination to mice, which produced a protective antibody response in an animal model (rat) against different species of Shigella (37). Shannon et al. (2013) evaluated the immunogenicity of the IpaB and IpaD proteins produced orally in mice. The mucosal and systemic immune response against Shigella confirmed the immunogenicity of the two proteins (38). Arabshahi et al. (2018) designed a recombinant chimeric vaccine fusing the IpaB protein of Shigella with the C-terminal region of the *Clostridium perfringens* bacteria (39). Although a licensed vaccine is not available yet, these attempts have helped in better understanding the immune response to Shigella, and together with recent innovative strategies led to promising vaccines (40). Among effector proteins, IpaB and IpaD proteins are located at the needle tip of the T3SS and play an important role in the attachment and entry of bacteria into the host cell. IpaB is a 62 KDa Protein with 580 amino acids which is an essential virulence factor for the control of T3SS, help bacteria to escape the phagocytic cells (phagosome escape), and macrophage apoptosis. IpaB can become a potential antigen for Shigella vaccine development. In this study, due to the importance of IpaB in the entry of bacteria into host cells, immunogenic regions of these proteins have been considered as vaccine candidate against shigella. The immunogenic protein gene was cloned in pET28a expression

vector and transformed into the bacterial host *E.coli* Rosetta (DE3). The expression vector was induced by IPTG and nickel chromatography column was used to purify the recombinant protein. The recombinant protein production and purification process were performed using SDS-PAGE. The purified protein in four times, first with complete Freund's adjuvant and then with incomplete Freund's adjuvant, were injected into the Guinea Pigs, and then the blood samples were taken from the Guinea Pigs and their antibody titers were measured by ELISA. Eventually, animal challenge was performed by Sereny test. In subcutaneous administration route, IgG assay through ELISA, showed that the level of the antibody has been increased after each administration. The eye infection of the control group and the non-infected eye of the guinea pigs which were received bacteria have also confirmed the immunization of the construct against Shigella. The results of the Sereny test in guinea pigs demonstrate that subcutaneous administration of IpaB can protect in vivo against mucosal infection with bacteria.

Conclusion

This protein as immunogen can trigger immune response exceedingly and inhibited keratoconjunctivitis in Sereny test. The results obtained in this work indicate the protective efficiency of IpaB protein against *shigella dysanteriae* invasion.

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Conflict of interest

The authors declare no conflicts of interest.

References

1. Oany AR, Pervin T, Mia M, Hosein M, Shahnaij M, Mahmud S, Kibria KMK. Vaccinomics Approach for Designing Potential Peptide Vaccine by Targeting spp. Serine Protease Autotransporter Subfamily Protein SigA. J Immunol Res.2017; 2017:6412353.
2. Walker RI. Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries. Vaccine. 2005; 23(26):3369 –85.
3. Livio S et al Shigella isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis. 2014; 59(7):933–41.
4. DuPont HL, Levine MM, Hornick RB, Formal SB.. Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis. 1989;159(6):1126 –8.
5. Ruhlman T, Ahangari R, Devine A, Samsam M, Daniell H. Expression of cholera toxin B–proinsulin fusion protein in lettuce and tobacco chloroplasts–oral administration protects againstdevelopment of insulitis in non-obese diabetic mice. Plant biotechnol j. 2007;5(4): 495-510.

6. Stensrud KF, Adam PR, La Mar CD, Olive AJ, Lushington GH, Sudharsan R, et al. Deoxycholate interacts with IpaD of *Shigella flexneri* in inducing the recruitment of IpaB to the type III secretion apparatus needle tip. J Biol Chem. 2008;283(27):18646-54.
7. Buysse JM, Stover CK, Oaks EV, Venkatesan M and Kopecko JD. Molecular Cloning of Invasion Plasmid Antigen (ipa) Genes from *Shigella flexneri*: Analysis of ipa Gene Products and Genetic Mapping. J bacteriol. 1987; 169(6)2561-9.
8. Guichon A, Hersh D, Smith MR and Zychlinsky AS. Structure-Function Analysis of the *Shigella* Virulence Factor IpaB. J bacteriol. 2001;183(4):1269-76.
9. Honari H, Amlashi I, Minaei ME, Safaei S. Immunogenicity in guinea pigs by IpaD –STxB recombinant protein. J Arak Med Uni. 2013; 16(73): 83-93.[In Persian].
10. Zhang L, Wang Y, Olive AJ, Smith ND, Picking WD, De Guzman RN, et al. Identification of the MxiH needle protein residues responsible for anchoring invasion plasmid antigen D to the type III secretion needle tip. J Bio Chem. 2007;282(44):32144-51.
11. Venkatesan MM, Buysse JM, Kopecko DJ. Characterization of invasion plasmid antigen genes (ipaBCD) from Shigella flexneri. Proc Natl Acad Sci U S A.1988;85(23):9317- 21.
12. Orrett FA.Prevalence of *Shigella* serogroups and their antimicrobial resistance pattern in southern Trinidad. j Health Popul Nutr. 2008;26(4):456-62.
13. Barnoy S, Jeong KI, Helm RF, Suvarnapunya AE, Ranallo RT ,Tzipori S , Venkatesan M M. Characterization of WRSs2 and WRSs3, new second-generation virG(icsA)-based *Shigella sonnei* vaccine candidates with the potential for reduced reactogenicity. Vaccine. 2010; 28 (6):1642–54.
14. Katz DE, Coster TS, Wolf MK, Fernando C , Trespalacios FC, Cohen D, Robins G, Hartman AB , Venkatesan MM , Taylor DN , Hale T L . Two studies evaluating the safety and immunogenicity of a live, attenuated *Shigella flexneri* 2a vaccine (SC602) and excretion of vaccine organisms in North American volunteers. Infect Immun. 2004; 72(2):923–30.
15. Kotloff KL, Pasetti M F , Barry E M , Nataro J P ,Wasserman S S, Sztein M B , Picking W D , Levine M M. Deletion in the *Shigella* enterotoxin genes further attenuates *Shigella flexneri* 2a bearing guanine auxotrophy in a phase 1 trial of CVD 1204 and CVD 1208. J Infect Dis. 2004;190:1745–54.
16. Phalipon A and Sansonetti PJ. Live Attenuated *Shiglla flexneri* Mutants as Vaccine Candidates Against Shigellosis and Vectors for Antigen Delivery. Biologicals. 1995; 23(2):125-134.
17. Walker RI. Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries. Vaccine; 2005; 23(26): 3369-85.
18. McKenzie R, Walker RI , Nabors GS , Van De Verg L L , Carpenter C , Gomes G , Forbes E , Tian J H , Yang H H , Pace J L , Jackson W J , Bourgeois A L. Safety and immunogenicity of an oral, inactivated, whole-cell vaccine for *Shigella sonnei*: preclinical studies and a Phase I trial.Vaccine.2005; 24:(18):3735-45.

19. Ravenscrot N, Haeuptle MA, Kowarik M, Fernandes FC, Carranza P, Brunner A, Steffen M, Wetter M,Keller S, Ruch C, Wacker M. Purification and characterization of a Shigella conjugate vaccine, produced by glycoengineering Escherichia coli. Glycobiology. 2016;26 (1):51-62.

20. Kaminski RW, Oaks EV. Inactivated and subunit vaccines to prevent shigellosis. Expert Rev Vaccines. 2009;8(12):1693-704.

21. Turbyfill KR, Kaminski RW, Oaks EV. Immunogenicity and efficacy of highly purified invasin complex vaccine from *Shigella flexneri* 2a. Vaccine.2008 ;26(10):1353-64.

22. Farhani, I.; nezafat, N.; Mahmoodi, SH.; Designing a novel multi-epitop peptide vaccine against pathogenic *Shigella* spp based immunoinformatics approaches. Int J Pept Res Ther. 2018;25(3).

23. Turbyfill KR, Hartman AB, Oaks EV.Isolation and characterization of *Shigella flexneri* invasin complex subunit vaccine. Infect Immun. 2000;68(12):6624-32.

24. Arianzad SA, Zeinoddini M, Haddadi A, Nazarian SH, Hasansajedi R. *In Silico* design of chimeric and immunogenic protein-containing IpaB and IpaD as a vaccine candidate against *Shigella dysenteriae*. Cuurent Proteomics.2020; 17(4):333-41.

25. Khademi F, Yousefi-Avarvand A, Derakhshan M, Zahra Meshkat Z, Mohsen Tafaghodi M, Ghazvini K, Ehsan Aryan E, Mojtaba Sankian M.. Mycobacterium tuberculosis HspX/ EsxS Fusion Protein: Gene Cloning, Protein Expression, and Purification in Escherichia coli. Rep Biochem Mol Biol. 2017;6(1): 15–21.

26. Sambrook J, Russell DW. Preparation and transformation of competent E. coli using calcium chloride. Cold Spring Harbor Protocols. 2006 Jun 1;2006(1):pdb-rot3932.

27. Gaikwad SS, Lee HJ, Kim JY, Choi KS. Expression and serological application of recombinant epitope-repeat protein carrying an immunodominant epitope of Newcastle disease virus nucleoprotein. Clinical and experimental vaccine research. 2019 Jan;8(1):27.

28. Sambrook J. Russell DW, Maniatis T. Molecular Cloning, A Laboratory manual. 3 Ed. New York: Cold Spring Harbor Laboratory Press; 2001.

29. Schägger H. Tricine–sds-page. Nature protocols. 2006 Jun;1(1):16.

30. Mahmood T, Yang P. Western Blot: Technique, Theory, and Trouble Shooting. N Am J Med Sci. 2012 Sep; 4(9): 429–434.

31. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72, 248–254.

32. Panda CS, Riley LW, Kumari SN, Khanna KK, Prakash K.Comparison of alkaline phosphatase-conjugated oligonucleotide DNA probe with the Sereny test for identification of *Shigella* strains. J Clin Microbiol. 1990;28(9);2122-4.

33. Jneid B, Rouaix A, _Feraudet-Tarisse C, Simon S. SipD and IpaD induce a cross-protection against *Shigella* and *Salmonella* infections. PLoS Negl Trop Dis. 2020; 28:14(5): e0008326.

34. Yang SC, Hung CF, Aljuffali IA, Fang JY.The roles of the virulence factor IpaB in *Shigella* spp: in the escapefrom immune cells and invasion of epithelial cells. Microbiol Res. 2015;181:43–51.

35. Barry E, Pasetti M, Sztein M, Fasano A, Kotloff K, Levine M. Progress and pitfalls in *Shigella* vaccine research. Nat Rev Gastroenterol Hepatol. 2013;10(4): 245–255.

36. Turbyfill KR., Hartman AB, Oaks EV. Isolation and characterization of a *Shigella flexneri* invasin complex subunit vaccine. Infect Immun. 2000;68(12): 6624–32.

37. Martinez-Beccera FJ, Chen X, Dikenson NE, Choudhari SP, Harrison K, Clements JD, Picking WD. IpaB of *Shigella* spp. And its potential as a pan- shigella vaccine.Infect Immun.2013;81 (12):4470-77.

38. Shannon J, Hein SJ, Diaz-McNair J, Andar AU, Drachenberg CB, Vandeverg L, Walker R, Picking WL,Pasetti MF. Intradermal Delivery of *Shigella* IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and System Immunity, and Protection across Serotype. J Immun. 2014; 192(4):1630-40.

39. Arabshahi S, Nayeri Fasaei B, Derakhshande A, Novinrooz A. *In silico* desing of a novel chimeric *Shigella* IpaB fused to C terminal of *Clostridum perfringens* entrotoxin as a vaccine candidate. Bioengineered. 2018;9(1):170-77.

40. Camacho A, Irache J, Gamazo C. Recent progress towards development of *Shigella* vaccine. Expert Rev Vaccines.2013;12:43–55.