Biologicals 41 (2013) 269–274

Contents lists available at SciVerse ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Molecular cloning and biologically active production of IpaD N-terminal region

Mahdi Hesaraki [a], Mojtaba Saadati [b,*], Hossein Honari [b], Gholamreza Olad [b], Mohammad Heiat [c], Fatemeh Malaei [b], Reza Ranjbar [c]

[a] Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[b] Department of Biology, Faculty of Basic Sciences, Imam Hossein University (IHU), Tehran, Iran
[c] Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form
19 March 2013
Accepted 22 March 2013

Keywords:
Shigella dysenteriae
IpaD
Sereny test
Protectivity

ABSTRACT

Shigella is known as pathogenic intestinal bacteria in high dispersion and pathogenic bacteria due to invasive plasmid antigen (Ipa). So far, a number of Ipa proteins have been studied to introduce a new candidate vaccine. Here, for the first time, we examined whether the N-terminal region of IpaD$^{72-162}$ could be a proper candidate for *Shigella* vaccine. Initially, the DNA sequence coding N-terminal region was isolated by PCR from *Shigella dysenteriae* type I and cloned into pET-28a expression vector. Then, the heterologous protein was expressed, optimized and purified by affinity Ni–NTA column. Western blot analysis using, His-tag and IpaD$^{72-162}$ polyclonal antibodies, confirmed the purity and specificity of the recombinant protein, respectively. Subsequently, the high immunogenicity of the antigen was shown by ELISA. The results of the sereny test in Guinea pigs showed that IpaD$^{72-162}$ provides a protective system against *Shigella flexneri* 5a and *S. dysenteriae* type I.

1. Introduction

Shigella spp are gram-negative rod-shaped pathogenic bacteria from the Enterobacterial family. In worldwide, shigellosis causes one million deaths annually [1]. *Shigella* is a robust microorganism in invading the intestinal epithelium of hosts so that 10–100 bacteria are sufficient to cause shigellosis [2,3]. Research shows that each year throughout the world about 164.7 million cases got infected with this bacteria among them 1.1 million people are estimated to die from the infection [1]. *Shigella* traverses the epithelial barrier through specialized membranous epithelial cells, called microfold cells (M cells). A set of large 220 kb plasmid coded proteins which are secreted through type III secretion system (TTSS) help *Shigella* spp uptake into M cells [4]. The invasion plasmid antigen (Ipa) proteins released by the TTSS which are necessary for the invasiveness of *Shigella* [5]. Expression of several proteins encoded by the large 220 kb plasmid (IpaB, IpaC, IpaD, Spa/Mxi proteins, and VirG or IcsA) is required for the complete virulence phenotype of *Shigella* spp [6,7]. The role of Ipa proteins in internalization and the immunomodulatory capacity of LPS may be crucial for the effective immunogenicity of Invaplex [8].

In IpaD deletion mutants, the IpaB–IpaD complex is not present, resulting in higher than normal levels of IpaB and IpaC being secreted into the surrounding medium [9]. Structural and sequencing analysis of IpaD has considerable similarities in all the *Shigella* spp [10]. The similarities of IpaD between *S. dysenteriae* type I (GenBank accession number: NC_007607) with *Shigella flexneri* (NC_004851), *Shigella boydii* (NC_010660) and *Shigella sonnei* (NC_007385) are 94%, 98% and 95%, respectively.

Whereas IpaD plays an essential role in *Shigella* spp pathogenesis, it could be considered as a potential candidate vaccine and it seems more researches on the antigenicity of IpaD should be recruited. On the other hand, Stensrud indicated that N-terminal region of IpaD (Leu75–Glu162) binds directly to the bile salt deoxycholate and this interaction is critical for triggering the pathogenesis process by IpaD. Moreover, they reported that any interference in the function of this region may limit the function of IpaD and subsequently blocking of the attaching process and (or) invasion of the bacterium to the target cells [11]. In this study, we investigated the immunogenicity of recombinant N-terminal portion of IpaD from *S. dysenteriae* type I and its protective efficacy against *S. dysenteriae* and *S. flexneri*.

* Corresponding author. Department of Biology Sciences, Faculty of Science, Imam Hossein University, P.O. Box 19395-4644, Tehran, Iran. Tel.: +98 2177104 934; fax: +98 2177104 935.
E-mail address: saadati1_m@yahoo.com (M. Saadati).

1045-1056/$36.00 © 2013 Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.
http://dx.doi.org/10.1016/j.biologicals.2013.03.002

2. Material method

2.1. Gene isolation

S. dysenteriae type I genome was used as a genomic template for gene isolation, to do this; standard cloning techniques were used to construct the recombinant plasmids (Sambrook and Russell, 2001). Genomic DNA was isolated from *S. dysenteriae* type I by using CTAB method [12]. A 344 bp fragment covering the N terminal gene sequence of *ipaD* (nucleotides 163–483 from NC 007607.1) was directly amplified from the genomic DNA by PCR. The primer sequences used for PCR were as: *ipaD* forward: 5′-T CAT **GAA TTC** AGA ACA ACA AAT CAG-3′ and *ipaD* reverse: 5′-T CTT **AAG CTT** TTA AGT ATA TGA ACT AAC G-3′ having the respective *Eco*RI and *Hind*III restriction sites (bold-underlined sequences). The PCR was initiated by a hot start at 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C, and a final extension step at 72 °C for 5 min.

2.2. Expression vector construction

The PCR products were cloned into PGEM-T Easy vector according manufacture's instructions. After confirmation of subcloning accuracy via PCR and restriction enzymes digestion, plasmids were purified by proliferative kit (QIAGEN). The desired fragments and pET-28a (+) vector were digested by *Eco*RI and *Hind*III enzymes and purified by gel extraction kit (Bioneer) from agarose gel. Subsequently, gel purified fragments were inserted into the pET-28a to construct pET-28a/IpaD$^{72-162}$ expression vector. The construct was prepared so that appends and N-terminal polyhistidine to the protein. The construct was confirmed by digestion and then transformed into *Escherichia coli*-BL21DE3 plysS (Invitrogen) for heterologous expression.

2.3. Protein expression

A single clone of the *E. coli* cells carrying expression vectors was inoculated into 10 ml of LB (Lauria Bertaini) medium containing 80 μg/ml kanamycin at 37 °C and under shaking at 180 rpm overnight. The next day, LB medium containing 20 μg/ml kanamycin was vigorously shaked at 37 °C until cells reached log phase ($OD_{600} \sim 0.5$). The expression of recombinant protein was induced by 1 mM isopropyl-β-thiogalactopyranoside (IPTG) for 5 h. No addition of inducer was used as a negative control experiment. Two samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R 250 (Thermo scientific). For optimization of protein expression various parameters were examined, including incubation of the medium at different temperatures (15, 25, 30 and 37 °C), induction with IPTG concentrations ranging from 0.1 to 1 mM and also to determine the best post-induction time for harvesting the cells (in 6–24 h induction), the progress of expression was followed by taking samples of the culture at different time points after induction [13]. All samples analyzed with ImageJ 1.46r (NIH, USA) software for determination of the percentage of recombinant in total protein.

2.4. Recombinant protein purification and confirmation

To express recombinant protein, 5 ml of *E. coli* BL21 harboring pET28a/IpaD$^{72-162}$ was cultured overnight, then 2 ml of it was inoculated into 150 ml LB containing 20 μg/ml kanamycin. The cultures were incubated at 37 °C by shaking for 2–3 h, until the OD_{600nm} reached about 0.5. The culture containing IPTG at final concentrations of 0.7 mM was prepared. After incubation at 37 °C for 3 h cells were harvested by centrifugation (5000 rpm for 10 min at 4 °C) and separated from medium. Cells were subjected to six cycles of 15 s sonication, and an interval of 1 min was allowed between the cycles. The cell lyses was clarified by centrifugation at 14,000 rpm for 10 min at 4 °C, to test whether 17 kDa truncated IpaD was present in the supernatant (soluble in the bacterium cytoplasm) or in the pellet (most likely in inclusion bodies).

The cell pellets were dissolved in lysis buffer (100 mM NaH_2PO_4, 10 mM Tric cl, 8 M urea, pH 8) and were then kept in 4 °C for overnight. Suspension was then centrifuged in 14,000 rpm at 4 °C for 30 min and supernatant was separated and filtered through a 0.48 μm syringe filter [14]. Filtrate was applied onto a Ni–NTA column resin (QIAGEN) and after washing the column with 10 ml of a washing buffer (100 mM NaH_2PO_4, 10 mM Tric cl, 8 M urea, pH 6.3) the purified protein was separated from the column with 1 ml elution buffer (100 mM NaH_2PO_4, 10 mM Tric cl, 8 M urea, pH 4.5). The concentration of the purified protein was estimated with Bradford method [15]. The purified protein was dialyzed for overnight at 4 °C in against 4 L phosphate buffer saline (pH 7.2) to remove traces of urea. The absorbance of the protein solution after dialysis was determined at 280 nm.

2.5. Western blot analysis

100 ng of His-tag purified recombinant proteins were loaded in 12% polyacrylamide gel. After gel electrophoresis, proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell BioScience, Dassel, Germany). Non-specific interaction sites were blocked by incubating the membrane for 1 h in PBS (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 5% non-fat dried milk (Carl Roth, Karlsruhe, Germany). The membrane was then incubated with rabbit anti-His-tag polyclonal antibody against IpaD$^{72-162}$ at 1:10,000 dilution in PBS containing 0.1%-Tween 20 (PBST), followed by a second incubation step with horseradish peroxidase-conjugated anti-mouse total IgG from rabbit (Sigma–Aldrich). The band detection was performed using enhanced chemiluminescence (ECL) and exposed with X-ray film [12,16,17].

2.6. Immunization and polyclonal antibody production

Male Hartley Guinea pigs of approximately 300–350 g weight were divided into two groups (8 guinea pigs per each test and control group) for immunization and challenge. Test animals were compared with control animals to confirm the virulence of the challenge organisms and their ability to producing polyclonal antibody. In the test group, guinea pigs were immunized intraperitoneally with 30 μg purified protein with Freund's complete adjuvant on day 0 and with Freund's incomplete *adjuvant* on days 14, and 28 and then were bled from the lateral vein of ear on days 0, 14, 28. Control groups were injected with equal volume of PBS. On day 42 the titration of antibody was high, so that we started challenge at the same day and 2 weeks after challenging antibody titration was tested again. Before immunization and bleeding, all guinea pigs were anesthetized with a mixture of 40 mg/kg ketamine and 4 mg/kg xylazine. Two weeks after the final immunization (day 42), guinea pigs were challenged intraocularly with *S. flexneri* 5a and *S. dysenteriae* type I (6.0×10^8 CFU/eye), subsequently the occurrence of disease was scored daily. Guinea pigs in group test, divided in two parts so that one group was challenged with *S. flexneri* 5a and another one with *S. dysenteriae* type I as well control groups .The degree of inflammation and keratoconjunctivitis were scored as previously described. The eyes with complete or partial protection at 2–4 days were considered protected [18]. All steps of using animals were based on the Local Ethics Review Committee protocol.

2.7. ELISA

ELISA test was used to measure the stimulation levels of immune response in animals. At first, extracted antibody of animals

was purified by protein G fast flow column (Thermo scientific), then serially was diluted 1:2500 in PBST (PBS containing 0.1% Tween 20) and used in ELISA. Polystyrene microtiter plates were coated with purified recombinant protein (2 μg) and carbonate/bicarbonate sodium buffer (0.1 M, pH 9.5) and were then incubated overnight at 4 °C. After washing three times with PBST, 1 μl antibody of each animal (purified with protein G column) was added on the coated antigen and then incubated for 1 h in 37 °C. Afterwards, samples were washed and the HRP conjugated polyclonal rabbit anti-guinea pig immunoglobulins (DAKO) were added. After incubation for 1 h in 37 °C and subsequently washing three times, the o-phenylenediamine (OPD, Sigma) and H_2O_2 were added as chromogenic substrates of HRP. The reaction mixture was again incubated for 10 min in dark place and then was stopped by the addition of H_2SO_4 (0.5 M). The optic density (OD) of the reaction mixture was measured at 492 nm with conventional ELISA reader device. The OD values were obtained by subtracting the OD of the blank (control animal serum) from that of each sample [19,20]. Anti-IpaD$^{72-162}$ titer values are estimated to be the last dilution in which the OD of test sample is significantly higher than that of the control sample.

3. Results

3.1. Production and purification of recombinant IpaD$^{72-162}$

ipaD N-terminal region encoding sequence was amplified by PCR (Fig. 1A) and after cloning into pET-28a expression vector, sequence and correct insertion were confirmed (Fig. 1B). The cells carrying the expression construct (Fig. 2) were induced by IPTG and expression of IpaD$^{72-162}$ was observed as judged by the presence of a band around 17 kDa in SDS-PAGE analysis (Fig. 3A). For further evaluation of recombinant IpaD$^{72-162}$ production Western blot analysis was performed using anti-His-tag antibody (Fig. 3B). By comparing the results, the best induction condition to reach optimized protein expression was estimated. We examined the high level of expression could be achieved with 0.7 mM IPTG and 3 h of induction at 37 °C (Table 1).

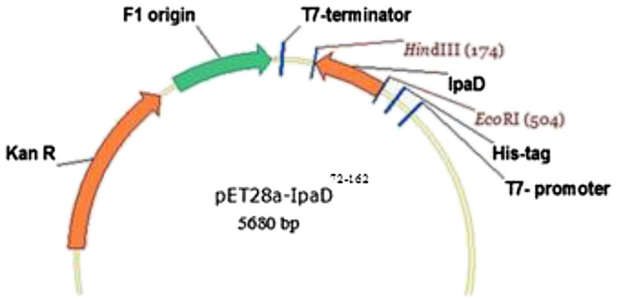

Fig. 2. Schematic map of pET-28a/r*ipaD* vector. IpaD is expressed under the control of the T7-promoter and terminator.

Protein expression under optimized condition was done and pellet was separated with centrifugation, final concentration of the protein after dialyze was 0.7 μg/μl. Following this SDS-PAGE and Western blot applied to confirm the heterologous protein expression. 6xHis taq antibody was subjected to western blot analysis (Fig. 3).

3.2. Immunogenicity of the IpaD$^{72-162}$ in guinea pigs

Guinea pigs were injected three times with 30 μg of recombinant protein in two weeks intervals. After the first injection, the production of IgG antibodies was rapidly increased and elevated gradually after two boosters. ELISA analysis showed that the anti-IpaD$^{72-162}$ IgG production had been saturated two weeks after intraperitoneal administration of three doses of the truncated IpaD

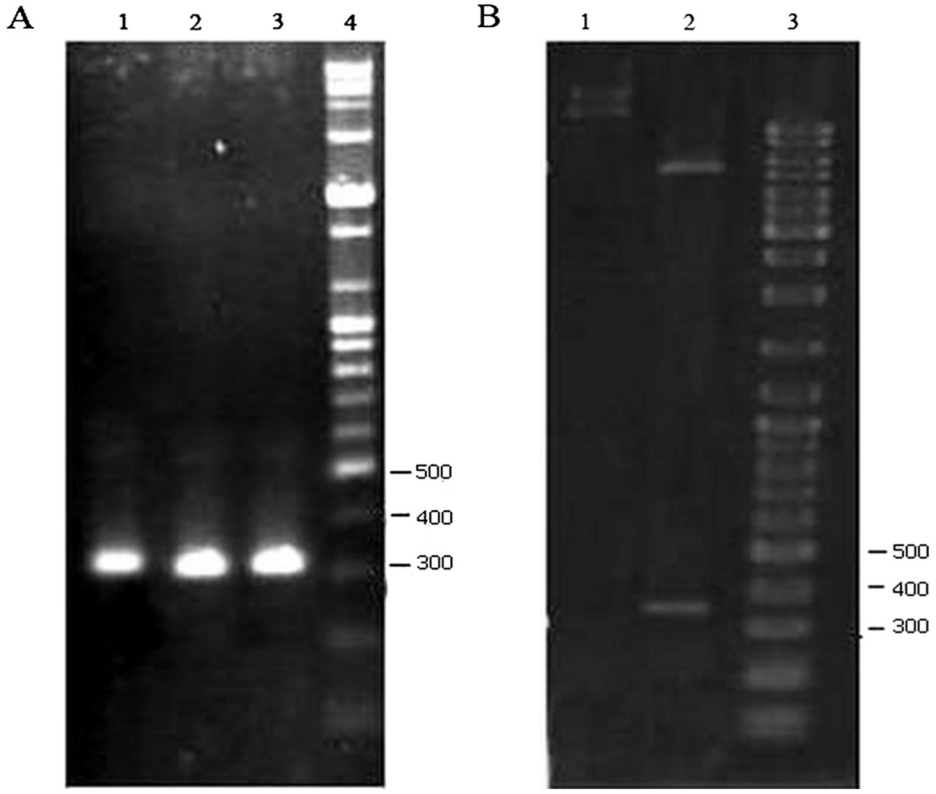

Fig. 1. Analysis of PCR products on agarose gel electrophoresis. A) Lanes 1–3: products of the N-terminal region of *ipaD*. Lane 4: DNA ladder marker (Fermentas). B) Lane 1: pET-28a/*ipaD*. Lane 2: recombinant plasmid digested by *Hind*III and *Eco*RI. Lane 3: DNA ladder marker (Fermentas).

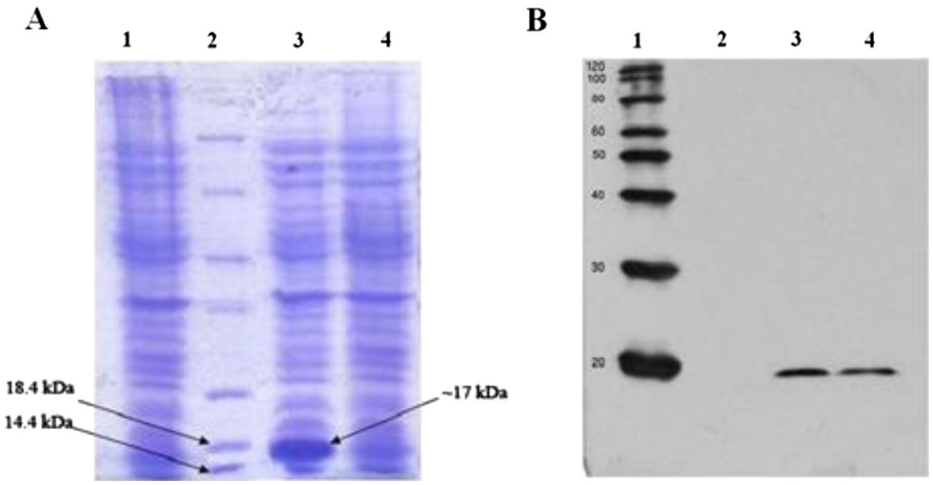

Fig. 3. A) SDS-PAGE analysis of initial expression recombinant IpaD$^{72-162}$. Lane 1: *E. coli* BL21 crude lysate. Lane 2: protein ladder (Fermentas sm0431). Lane 3: *E. coli* BL21 crude lysate transformed with pET-28a/r*ipaD* after induction. Lane 4: *E. coli* BL21 crude lysate transformed with pET-28a/r*ipaD* without induction. B) Western blot analysis of expressed IpaD using His-tag specific antibody. Lane 1: marker (MagicMark™ XP Western protein standard, Invitrogen). Lane 2: *E. coli* BL21 crude lysate without pET-28a/*ipaD*. Lane 3: *E. coli* BL21 crude lysate transformed with pET-28a/*ipaD* after induction. Lane 4: Purified heterologous protein (10 ng).

Table 1
Protein expression optimization in different induction time points and concentration of IPTG.

Temperature	Induction time	Percentage of recombinant in total protein with different IPTG concentrations:		
		0.5 mM IPTG	0.7 mM IPTG	1 mM IPTG
25	12	24.0	24.7	24.9
30	10	24.3	25.4	25.5
37	6	19.9	20.2	20.1
	1	–	19.7	–
	2	–	24.7	–
	3	26.0	27.2[a]	27.1
	4	–	21.1	–
	5	–	20.9	–

[a] The optimum concentration of heterologous protein was achieved at 37 °C, 0.7 mM IPTG and 3 h induction.

(Table 2). To further evaluate the specificity of the immune response, obtained anti-sera were analyzed by Western blotting against recombinant IpaD$^{72-162}$. As shown in Fig. 4 a single band was detected using either purified or unpurified samples with expected molecular weight of IpaD$^{72-162}$.

3.3. Protectively analysis

For sereny test analysis, immunized guinea pigs were challenged intraocular with virulent *S. flexneri* 5a or *S. dysenteriae* type I (6.0×10^8 CFU/eye). All of guinea pigs were protected from severe infection in comparison with control guinea pigs which were injected with PBS. As can be seen in Fig. 5, mild irritation and scarlet around the sclera were obvious after 48 h in test animals, while keratoconjunctivitis was observed in all of the animals in control groups. Two weeks after the challenge test, all guinea pigs were bled. The titer of induced IgG antibody against truncated IpaD had a significant increase which implicated the protective role of recombinant protein on the guinea pigs against *Shigella* spp (Table 2).

Table 2
Titration of anti-recombinant protein, IpaD$^{72-162}$ antisera.

Time of blood collection (days after the first injection)	Anti IpaD$^{72-162}$ titer
14	1600
28	3200
42	6400
56[a]	12,800

[a] Blood samples from animals which had been infected with *Shigella* spp after bleeding on day 42.

4. Discussion

In this study, we tried to introduce a new recombinant protein for improving *Shigella* vaccination. Ipa proteins which are expressed on bacterial surface with the help of a TTSS as a chaperone are potential targets for this purpose. Comparing with C-terminal region of IpaD which contains hydrophilic areas, the N-terminal region is considerably more immunogenic and more likely

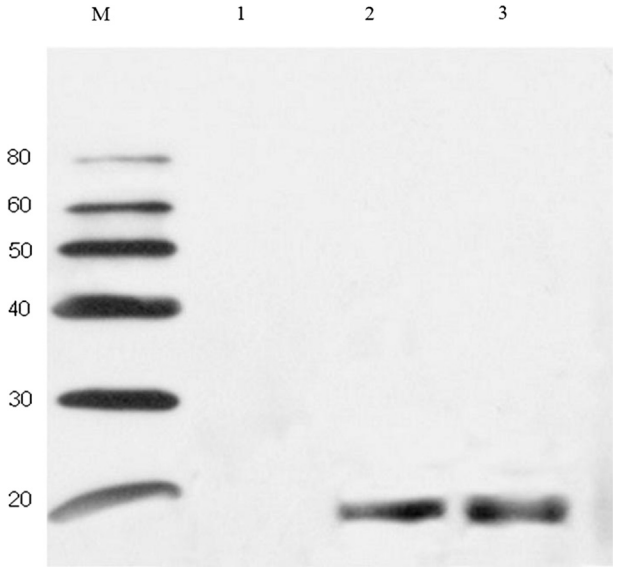

Fig. 4. Western blot analysis of IpaD$^{72-162}$ polyclonal antibody against recombinant protein. Lane M: protein marker (MagicMark™ XP Western Protein standard, Invitrogen). Lane 1: *E. coli* BL21 crude. Lane 2: *E. coli* BL21 cell lysate transformed with pET-28a/r*ipaD*. Lane 3: purified and injected heterologous protein (10 ng).

Fig. 5. Development of experimental keratoconjunctivitis. Guinea pig's eyes, two days after infection. The bacterial cell suspension (6.0×10^8 CFU/eye) was dropped into the conjunctiva sacs of male Hartley guinea pigs and the animals were under surveillance for two consecutive days. Control animal injected with PBS and challenged with *S. dysenteriae* type I (A) or *S. flexneri* 5a (C). Immunized animals with $IpaD^{72-162}$ and challenged with *S. dysenteriae* type I (B) or *S. flexneri* 5a (D).

to be exposed on the surface of *Shigella* cells [9]. Thus we focused on N-terminal portion of IpaD ($IpaD^{72-162}$) as a key player in *Shigella* spp infection. Menard et al. demonstrated that IpaD in company with a complex of IpaC/B is necessary to begin *Shigella* entry into epithelial cells while IpaA is not important for the first infection phase. They also showed that mutation in *ipaD* inhibits *S. flexneri* uptake by Hella cells [21]. In agreement with these data, our results showed that immunity against IpaD is capable of shigellosis inhibition. Martinez-Becerra et al. protected mice from *Shigella* spp with administration of IpaB/IpaD and with a double mutant heat-labile toxin (dmLT) but not with IpaD alone or even IpaB/IpaD without dmLT [22]. However, we demonstrated such protection only with $IpaD^{72-162}$. In previous reports on *Shigella* vaccination with Ipa family proteins either native purified protein or whole chain recombinant form were used [8,9,18,22–25]. In contrast we were able to evoke a protective immune response with $IpaD^{72-162}$ which contains only 90 amino acids from N-terminal domain of IpaD. This 90 region is formed a coiled–coiled structure which is responsible for deoxycholate binding [11,26]. It has been known that this interaction is critical for formation of Ipa complex on the bacterium surface [27]. Consistent with these facts, we showed that directing the immune response to this region can efficiently inhibit *Shigella* infection. In agreement with our results, recently several immunogenic epitopes have been mapped in the N-terminal region of IpaD in contrast with less immunogenic C-terminal part [9].

Although Lys72–Gln162 region from IpaD of *S. dysenteriae* type I is not identical to that of *S. flexneri*, we identified that $IpaD^{72-162}$ can raise a protective immunity against *S. flexneri* as a distant relative of *S. dysenteriae* type I among *Shigella* spp. In conclusion, this study introduces $IpaD^{72-162}$ as a new candidate vaccine which potentially can be protective against different species of *Shigella*.

Ethical approval

This work was approved by the, Imam Hossein University Ethics Review Committee (approval number 2010-07-0327).

Ethical approval: This work was approved by the, Imam Hossein University Ethics Review Committee (approval number 2010-07-0327).

Conflict of interest

No conflict of interest to declare.

References

[1] World Health Organization. Global burden of disease (GBD) 2002 estimates. Geneva, Switzerland: World Health Organization; 2004.
[2] Sk Niyogi. Shigellosis. Journal of Microbiology 2005;43:133–43.
[3] Clemens J, Kotloff K, Kay B. Generic protocol to estimate the burden of *Shigella diarrhoea* and dysenteric mortality. Geneva: World Health Organization; 1999.
[4] Schoeder GN, Hilbi H. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. Clinical Microbiology 2008;21:134–56.
[5] Barta ML, Guragain M, Adam P, Dickenson NE, Patil M, Geisbrecht BV, et al. Identification of the bile salt binding site on IpaD from *Shigella flexneri* and the influence of ligand binding on IpaD structure. Proteins 2012;80:935–45.
[6] Man A, Prieto G, Nicoletti C. Improving M-cell-mediated transport across mucosal barriers. Journal of Immunology 2004;113:15–22.
[7] Sansonetti PJ. Rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*: the yin and yang of innate immunity. Canadian Journal of Infectious Diseases & Medical Microbiology 2006;17:117–9.
[8] Kaminski RW, Turbyfill K, Oaks EV. Mucosal adjuvant properties of the Shigella invasin complex. Journal Infection & Immunity 2006;74:2856–66.
[9] Turbyfill K, Mertz J, Mallett CP, Oaks EV. Identification of epitope and surface-exposed domains of *Shigella flexneri* invasion plasmid antigen D (IpaD). Journal Infection & Immunity 1998;66:1999–2006.
[10] Espina M, Ausar F. Conformational stability and differential structural analysis of LcrV, PcrV, BipD, and SipD from type III secretion systems. Journal Protein Science 2007;16:704–14.
[11] Stensrud KF, Aam P, La Mar C, Olivec A, Lushington G, 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. Journal of Biological Chemistry 2008;283:18646–54.
[12] Edwards S, Johnstone C, Thompson C. A simple and rapid method for the preparation of genomic plant DNA for PCR analysis. Nucleic Acids Research 1991;19:1349.
[13] Psakis G, Nitschkowski S, Holz C, Kreb D, Maestre M, Polaczek J, et al. Expression screening of integral membrane proteins from *Helicobacter pylori* 26695. Protein Science 2007;16:2667–76.

[14] Palmer I, Wingfield PT. Preparation and extraction of insoluble (inclusion-body) proteins from *Escherichia coli*. In: Coligan J, Mayland B, Dunn BM, editors. Current Protocols in Protein Science. John Wiley & Sons, Inc; 1995. p. 1–5.
[15] Bradford MM. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Journal Analytical Biochemistry 1976;72:248–54.
[16] De Marzo N, Jovine L, Rizzotti P, Boschetto P, Miotto D, Saetta M, et al. Modification of serum proteins in guinea pigs immunized and challenged with toluene diisocyanate. Scandinavian Journal of Work, Environment & Health 2000;26:153–60.
[17] Aguer C, Foretz M, Lantier L, Hebrard S, Viollet B, Mercier J, et al. Increased FAT/CD36 cycling and lipid accumulation in myotubes derived from obese type 2 diabetic patients. PLOS one. 2011;6:e28981.
[18] Turbyfill K, Kaminski RW, Oaks EV. Immunogenicity and efficacy of highly purified invasion complex vaccine from *Shigella flexneri* 2a. Journal Vaccine 2008;26:1353–64.
[19] Ji F, Li H, Xu J, Shi J. Enzyme-linked immunosorbent-assay for deoxynivalenol (DON) toxins3:968–78.
[20] Basiri M, Mousavi SL, Basiri H, Rasooli I. An epitopic approach to designing and characterization of a multiple antigenic polypeptide against Botulinum neurotoxins A and E. World Journal of Microbiology and Biotechnology 2010;26:1659–66.
[21] Menard R, Parson C, Gounon P, Sansonetti P, Dehio C. The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. Journal of Microbiology 1996;93:1254–8.
[22] Martinez-Becerra F, Kissmann J, Diaz-McNair J, Choudhari S, Quick A, Mellado-Sanchez G, et al. Broadly protective Shigella vaccine based on type III secretion apparatus proteins. Infection & Immunity 2012;80:1222–31.
[23] Turbyfill K, Hartman AB, Oaks EV. Isolation and characterization of a *Shigella flexneri* invasin complex subunit vaccine. Journal Infection & Immunity 2000;68:6624–32.
[24] Oaks EV, Turbyfill K. Development and evaluation of a *Shigella flexneri* 2a and *S. sonnei* bivalent invasin complex (invaplex) vaccine. Journal Vaccine 2006;24:2290–301.
[25] Tribble D, Kaminski R, Cantrell J, Nelson M, Porter C, Baqar S, et al. Safety and immunogenicity of a *Shigella flexneri* 2a invaplex 50 intranasal vaccine in adult volunteers. Journal Vaccine 2010;28:6076–85.
[26] Espina M, Olivec A, Kenjale R, Moore DS, Ausar F, Kaminski RW, et al. IpaD localizes to the tip of the type III secretion system needle of *Shigella flexneri*. Journal Infection & Immunity 2006;74:4391–400.
[27] Olive AJ, Kenjale R, Moore DS, Picking WL, Picking WD. Bile salts stimulate recruitment of IpaB to the *Shigella flexneri* surface, where it colocalizes with IpaD at the tip of the type III secretion needle. Journal Infection & Immunity 2007;75:2626–9.