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Immunogenic Potency of a Chimeric Protein Comprising InvH and IpaD against *Salmonella* and *Shigella* spp

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ABSTRACT

Shigella and *Salmonella* cause serious problems in many subjects, including young children and the elderly, especially in developing countries. Chimeric proteins carrying immunogens increase immune response. In-silico tools are applied to design vaccine candidates. Invasion plasmid antigens D *(ipaD)* gene is one of the *Shigella* virulence factors. The N-terminal region of the *IpaD* plays a significant role in invading the host cell. Invasion protein H (*invH)* gene plays important role in bacterial adherence and entry into epithelial cells. A recombinant chimeric construct, containing IpaD and InvH was designed and used as a vaccine candidate against *Shigella* and *Salmonella enteritidis*.

After bioinformatics assessments, the construct was designed, synthesized, and expressed in *E.coli*. Chimeric protein, IpaD, and InvH were purified with Ni-NTA chromatography. Purified proteins were confirmed with western blotting and then were injected into separate mice groups. The antibody titer was estimated with an enzyme-linked immunosorbent assay (ELISA). Mice were challenged with 10, 100, and 1000 LD50 of *Salmonella,* and the sereny test was performed for *Shigella.*

The Codon adaptation index of the chimeric gene was increased to 0.84. Validation results showed that 97.9 % of residues lie in the favored or additional allowed region of the Ramachandran plot. A significant antibody rise was observed in all test groups. The immunized mice with chimer and InvH could tolerate 100 LD50 of *Salmonella.* In the sereny test, the application of bacteria treated with immunized mice sera of both antigens showed no infection in Guinea pigs' eyes.

The recombinant protein could protect animal models against *Salmonella* and *Shigella* and therefore can be considered as a suitable vaccine candidate against these two pathogens.

Keywords: InvH protein; IpaD protein; Recombinant proteins*; Salmonella; Shigella*

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INTRODUCTION

Despite significant improvements in food, sanitation, and health conditions, diarrhea is still a

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common and high prevalence of human disease.[1] Diarrhea and vomiting are the second globally leading cause of death. About 25 million intestinal infections occur annually leading to illness and death in children under 5 years old and the elderly.[2] Pathogens causing diarrhea include bacteria, viruses, and parasites and among them, bacteria such as *Salmonella* and *Shigella* are often associated with food poisoning.[1] Shigellosis and Salmonellosis are caused by *Shigella* and *Salmonella* species, respectively.[2] *Shigella* is the cause of infectious and dysentery diarrhea and is, therefore, a serious global public health challenge. Among the intestinal bacteria, *Shigella* acquire great importance with a very low ID50 where the entry of even 100 organisms into the body is sufficient to cause disease.[3]Although *Shigella* has no known animal sources, there is no effective vaccine available, and oral vaccines provide poor immune response .[2]

Salmonella is a Gram-negative, non-spore-forming, facultative anaerobic bacteria[4] The most common form of *Salmonella* infection is food-borne acute enterocolitis and gastroenteritis. Typhoid, paratyphoid, and enteric fever with 25 million new infections and over 20,000 deaths annually are serious problems for global health.[5]

Few antigens of *Shigella* can cause diseases in humans and most of these antigens are carried by a 220 kbp plasmid. The key invasion factor of *Shigella* is Invasion plasmid antigens D (IpaD), a 37 kDa protein playing a very important role in the invasion to host cells through its N and C terminal regions. Bile salts are other important factors for bacterial invasion, and the interaction between bile salts, especially deoxycholate, with IpaD play an important role in providing the position for other proteins to locate on the cell membrane of the bacterium. These conditions allow the bacteria to connect to the host cells and attack them. Antibody recognizing the N-terminal of the IpaD is capable to stop the ability of bacteria in creating holes in red blood cells. Anti-IpaD antibodies could prevent *Shigella* from entering the host cell.[6]

Some pathogenic bacteria secrets and delivers the effector proteins into the eukaryote host cells through type III secretion system. . This system in *Salmonella enterica* serovar Typhimurium contains 20 proteins. A subset of these components which is called the needle complex contains Invasion protein G (InvG), PhoP-repressed genes K (PrgK), and PhoP-repressed genes H (PrgH). InvG is a member of the secretin family of outer membrane exporter proteins.[7] InvH is necessary for multimeric assembly and insertion of InvG into the bacterial outer membrane.[7, 8] A loss of function due to the mutation in the *invH* gene resulted in a significant reduction in the number of needle complexes on the surface of the bacterium, observed by electron microscopy in cells given an osmotic shock. InvH is not only essential for the needle complex assembly but also increases the formation of the complex via its stabilizing activity on InvG.[7] *S. typhimurium*, *Salmonella gallinarum*, *Salmonella typhi*, and *Salmonella enteritidis* with a mutation in invH show reduced invasion ability on the Henle-407 cells.

The decrease of invasiveness due to mutation was different for various serotypes where Salmonella typhi and Salmonella enteritidis showed the lowest decrease while the host-adapted strains of *S. typhi* and *S. gallinarum* were highly affected.[9]

Chimeric constructs can present more than one antigen at a time and thereby could play important roles in immunization.[10] They contain many subunits with diverse epitopes and linker sequences with adjuvant properties which can increase the immunogenic efficacy of recombinant proteins.

Bioinformatics approaches ultimately offer more rapid advances through preclinical vaccine studies and comparative structural and immunological analysis of antigens could lead to the judicious selection of a combination of immunogens for a multisubunit chimeric vaccine.[11]

The two antigens selected in this study (InvH and IpaD) play a major role in the pathogenesis of these two bacteria and several studies have been performed to show their immunogenicity.[12-14] Therefore it was worthful to design a chimeric protein harboring these two antigens and study its immunogenic potency against the two major bacterial pathogens of *Shigella* and *Salmonella.*

MATERIALS AND METHODS

Insilico Design of the Construct

The sequence encoding 107 amino acids of the ipaD N-terminal region and the InvH sequence were obtained from NCBI to design the chimeric construct.

The similarity and percentage of the similarity of the 2 protein sequences were found via BLAST-P (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A suitable linker sequence, (EAAAK), is inserted between the 2

genes to maintain the structure of each protein individually.[15] The 3D structure of the chimeric protein is investigated via I-TASSER software. The three-dimensional models in PDB format were studied by Accelrys Discovery Studio Visualizer 2.5.5 software. The quality of proposed models was determined by uploading 3D structures in PDB format into ProSA-web, frequently employed in protein structure validation.[16] The quality of the resulting stereochemistry of structure was validated by Ramachandran plot in RAMPAGE software.[17] After the initial bioinformatics cassette design, the sequence was analyzed for its GC content and restriction sites. Using the Gene Optimizer software, the sequence was codon-optimized for the expression in *E.coli.*

The study of the preservation of amino acid sequences and RNA stability was done via Blast-X software and RNA fold program respectively.

The ProtParam program was used to determine the molecular weight of protein, the composition and charge content of its amino acids, and the half-life and instability index of the protein.

Also, the conformational B cell epitope (CBTOPE) and BCEpred bioinformatics software were used to predict the humoral immunity induction by the chimeric protein.[18] The primers needed for amplification of *the ipaD* gene were designed and checked by Oligo Analyzer software.

Cloning, Expression, and Purification of the Recombinant Proteins

The chimeric sequence was synthesized on the pET28a vector by the Biomatik Company (Canada). The plasmid was transformed into *E.coli* B121 (DE3) cells and cultured on an LB agar plate containing 70 μg/mL kanamycin.

The sequence encoding 107 amino acids of the ipaD N-terminal region was amplified from the genome of *Shigella sonnei;* using gene-specific primers. The PCR product was digested with *BamHI* & *HindIII* enzymes (Fermentase), ligated to the pET28a with T4 DNA ligase, and transformed into *E.coli* B121 (DE3) cells.

The clone for recombinant InvH was from our previous works at Shahed University.[19] All 3 clones were cultured in LB Broth at 37°C till the OD_{600} reached 0.6-0.7 and then induced with Isopropyl β- d-1-thiogalactopyranoside (IPTG) with a final concentration of 1 mM for 4-5 h. Cells were harvested, lysed and protein expressions were analyzed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Inclusion bodies were dissolved in phosphate buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl,) containing 8M urea.

Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography was used for protein purification. Each column was equilibrated with binding buffer and loaded with approximately 500 μg of protein. Columns were washed 5 times with a 20 mM concentration of imidazole to remove nonspecific binders and the recombinant proteins were eluted with a 250 mM concentration of imidazole. All collected fractions were analyzed on the SDS-PAGE and protein concentration was determined by Bradford assay.

Western Blot Analysis

The purified recombinant proteins were electrophoresed on SDS-PAGE and transformed onto the nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol). The membrane was blocked with the blocking buffer (PBST + 5% Skimmed Milk Powder) for 15 h at 4°c. The membrane was washed with PBST (137 mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4 + 0.05% Tween) 3 times and was added with mice anti-His-tag IgG in PBST (1:10000) and incubated for 1h at 37°c. The membrane was washed with PBST and a chromogenic substrate, DAB (3, 3′-diaminobenzidine), was added. The reaction was stopped by washing the membrane with distilled water.[20]

Animal Immunization

The 5-week old female BALB/c mice (20-25gr) purchased from Razi Institute, IRAN, were divided into 4 groups each containing 5 mice. The groups were named IpaD, InvH, chimer, and control. Mice were kept in the animal care facility of Shahed University under standard and ventilated conditions. The principles in the Guide for the Care and Use of Laboratory Animals were followed and all animal experiments were conducted in compliance with the Welfare Act and regulations related to experiments involving animals.[21] The animal care rule was ethically certified by Shahed University with the ethical code of 6001.

Groups IpaD and InvH were subcutaneously injected with 100 μL (20 μg) of recombinant proteins mixed with complete Freund's Adjuvants (CFA, Razi institute) on day 0 and with 3 boosters on days 15, 30, and 45 using

incomplete Freund's Adjuvants (IFA, Razi institute). Mice in the chimeric group were injected 3 times and the control group received PBS with adjuvant. Blood samples were collected from the eye corners of mice on days 29, 44, and 59 after the first injection, and the sera were collected and stored at -20°C.

Antibody Response

Antibody response was determined via Indirect Enzyme-linked Immunosorbent Assay (ELISA).

The wells of the ELISA plate were coated with 5 µg of each recombinant protein in 100 µL of coating buffer and incubated at 4°C overnight. Wells were blocked with a blocking buffer for 1h at 37°C. A serial dilution (1:100 to 1:12800 in PBST) of serum antibody was prepared and added to adequated wells and the plate was incubated at 37°C for 2 h. Anti-mouse IgG HRP conjugate (1/2000 dilution in PBST) was added to each well and incubated at 37°C for 1.5 h. After washing, 100 µL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 10 minutes in a dark place. Wells were washed after each incubation. The reaction was stopped by 3N H_2SO_4 and the absorbance was measured at $O.D_{450}$.

Post Immunization Animals Challenges

For *Salmonella typhimurium*, 10^3 to 10^8 colony-forming units (CFU) of the bacteria were used and based on the number of deaths in different groups, the LD50 was determined. Animals were challenged with 10^4 to 10^7 CFU of the bacteria through intraperitoneal injection and the survival rates against bacteria were monitored for 96 h.

For *Shigella sonnei*, sereny (keratoconjunctivitis) test was carried out.[22, 23] Based on the previous studies, 10^6, 10^7, and 10^8 CFU of bacteria were inoculated to the 3 eyes of 2 guinea pigs, and the infection dose was estimated.[24] After determining the ID50, the same dose of bacteria was dissolved in 50 µL physiologic serum, mixed with 50 µL of immunized mice sera of chimeric and IpaD groups, and allowed to stand for 20 minutes. The mixtures were applied to the eyes of guinea pigs and the possible infection in the eyes was monitored for 3 days.

Statistical Analysis

The results of ELISA were analyzed using Graph Pad Prism 8 software. Mean comparison of groups was analyzed by Duncan's multiple range tests to determine the significance of differences in the experimental groups. Mean values ± standard error of the mean (SEM) are from five independent replicates. . Statistical significance is indicated by *** ($p \leq 0.001$), ** ($p \leq 0.01$), * ($p \leq 0.05$), and ns (no significant).

RESULTS

Bioinformatics Analysis

Required sequences were obtained from NCBI and the schematic diagram of the construct was designed with DOG1.0 software.[25] The prediction of the 3D structure was carried out by I-TASSER and the C-score of the model was within the permitted range of -5 to 2, which is a confident rate to determine the quality of the predicted structures by this software. The z-score of the input structure was -3/73, standing within the range of scores typically found for native proteins of similar size. The Ramachandran plot quality assessment analysis showed most residues within>90% (favored+allowed) regions. The analysis revealed that 93.8% of amino acid residues from modeled structure generated by I-TASSER were incorporated in the favored regions and 4.1% of residues were in allowed regions of the plot.

Gene optimization showed an increase in the Codon Adaptation Index (CAI) from 0.68 to 0.84 which is the indication for a good expression. The chimeric gene showed no fuzzy node or long loops at the 5' end (start of translation). The minimum energy of this structure was calculated to be -434.05 kcal/moL; indicating sufficient mRNA stability to express in the *E.coli* system.

The linear and discontinuous epitopes are predicted to belong to both proteins making the chimeric structure (Table 1 and 2).

Table 1. Conformational B-cell epitopes from full-length proteins; using conformational B cell epitope (CBTOPE) server. CBTOPE has been developed for predicting the B-cell epitope from its amino acid sequence.

Amino acid	position	probability scale	Amino acid	position	probability scale
PV	2-3	4	DA	191-192	4
E	11	4	EL	194-195	4
N	15	4	SA	198-199	4
CQSLPYVP	22-29	4	D	206	4
N	35	4	V	239	4
E	92	4			
L	178	4			
S	182	4			
K	190	4			

Table 2. B-cell epitopes from full-length proteins; using BCPred (BCPred +AAP). Antigenicity of full-length proteins, as well as all B-cell epitopes, were calculated; using VaxiJen.

AAP Predictions	Amino acid Positions	BCPred scores	VaxiJen scores	BCPred Predictions	Amino acid Positions	BCPred scores	VaxiJen scores
PVQQPGAQKEQL ANANSIDE	2	1	0.6054	KPVQQPGAQKE QLANANSID	1	0.997	0.6462
KKEYPINKDARE LLHSAPEE	183	1	0.1455	FQEHPQYMRSKE DEEQLMTE	73	0.981	0.5706
LEAAAKEAAAKE AAAKRTTN	121	1	1.1918	SLSNQNADNSAS KNSAISSS	37	0.964	1.1512
RELWDKIAKSIN NINEQYLK	214	0.999	0.0485	RELWDKIAKSIN NINEQYLK	214	0.962	0.0485
QEHPQYMRSKED EEQLMTEF	74	0.063	0.6521	DARELLHSAPEE AELDGYQM	191	0.917	0.5157

Expression and Purification of the Recombinant Proteins

The expression of each clone was analyzed on SDS-PAGE and the proteins with 16.7 kDa for IpaD, 19 kDa for InvH, and 31 kDa for chimeric construct appeared on the gel. Recombinant proteins were purified by Ni-NTA affinity chromatography using different concentrations of imidazole and then verified with western blotting (Figure 1).

Determination of Serum IgG Titer

The evaluation of the IgG antibody titers from sera samples was performed with Indirect-ELISA. In the InvH and IpaD groups, the antibody titer was significantly raised compared to the control groups after 2nd blood sampling. Significant differences were also observed between all 3 boosters in the InvH group. In contrast to InvH and IpaD groups, in the chimeric group, all three blood sampling showed significant differences compare to the controls, and antibody rise continued till day 59 (Figure 2).

Post Immunization Animals Challenges

The estimated LD50 dose of *Salmonella typhimurium* was 10^4 CFU of bacteria. The result of the challenge with immunized mice injected with 10^5, $10^{6,}$ and 10^7 CFU of bacteria showed that mice immunized with InvH and the chimeric group were resistant up to 100 LD50 and stayed alive for more than 96 h when administered with 10^6 CFU. Whereas all mice of the control group died within 24 h. None of the immunized mice groups could resist 10^7 CFU and die after 24 h (Figure 3).

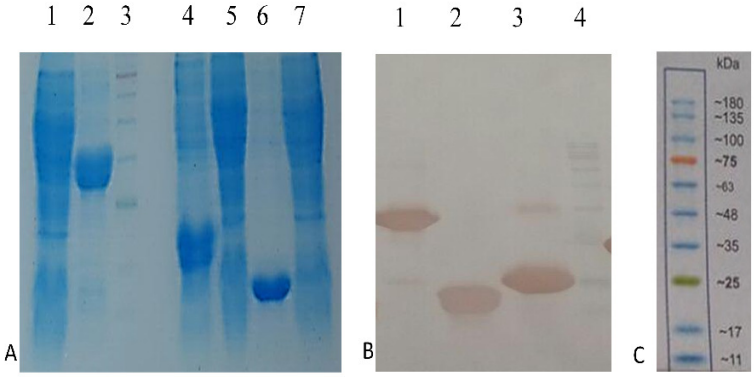

Figure 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) analysis of protein expressions: lane 1, 5, 7 pET28a induced with IPTG as control, lane 2 Chimer induced with IPTG (31 kDa protein). Lane 3 Molecular weight marker. Lane 4 InvH clone induced with Isopropyl β- d-1-thiogalactopyranoside (IPTG) (19 kDa). Lane 6 IpaD clone induced with IPTG (16.7 kDa). (B) Western Blot analysis of the 3 proteins, lane 1: Chimeric protein, lane 2: IpaD, lane 3: InvH. Lane4: Molecular weight marker. (C) Protein Ladder (Tris-Glycine 4~20%)

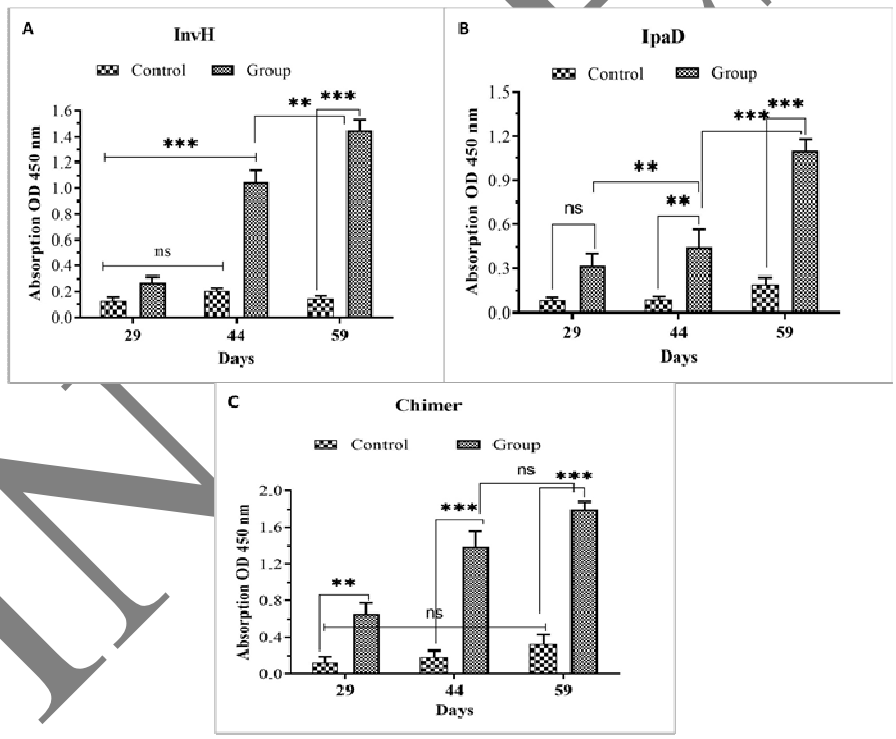

Figure 2. Mean comparison of three immunized mice groups on protein ELISA results. A) InvH B) IpaD and C) chimeric recombinant proteins. Purified recombinant proteins were used as antigens. Mean values ± standard error of the mean (SEM) are from five independent replicates. Asterisks show significant difference between control and test groups using Duncan's multiple range tests values. Statistical significance is indicated by * (P ≤ 0.001), ** (P ≤ 0.01), * (P ≤ 0.05), and ns (no significant).**

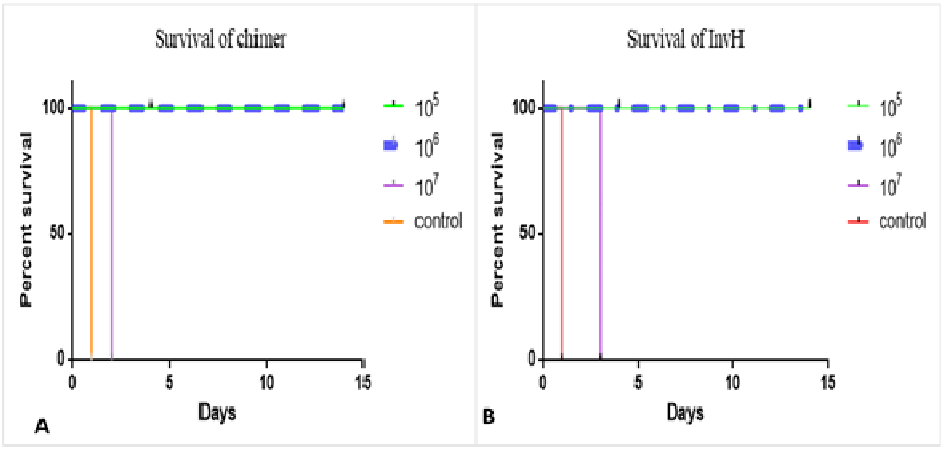

Figure 3. Survival curves for A) Recombinant chimeric protein and B) InvH immunized mice injected with 10^5, 10^6, and 10^7 colony-forming unit (CFU) of bacteria.

The infection dose of *Shigella sonnei* was determined 10^8 CFU and the result of the challenge showed no infection in the guinea pig's eye after 3 days while the eye of the control pig showed full purulent keratoconjunctivitis (Figure 4).

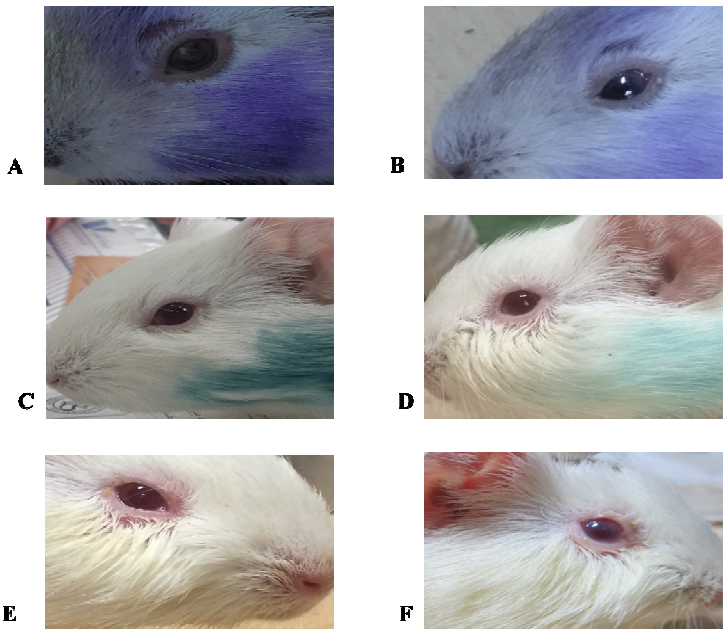

Figure 4. Sereny test with *Shigella* in guinea pigs. A, C, and E: guinea pigs eye before inoculation. B: guinea pig eye 3 days after the inoculation of *Shigella* mixed with chimeric immunized mice serum, there is no sign of infection. D: guinea pig eye 3 days after the inoculation of *Shigella* mixed with IpaD immunized mice serum, there is no sign of infection. F: Purulent infection of the eye in control guinea pig 3 days after *Shigella* inoculation into the eye.

DISCUSSION

Among various diarrhea-causing pathogens, *E.coli, Shigella,* and *Salmonella* are the major causes of endemic and epidemic diarrhea around the world.[26]

Shigella is transmitted via the fecal-oral route, and humans can be infected by consuming infected foods, plants, water, and milk. Moreover, the infection dose of this bacteria is low and there is no recently effective vaccine available against *Shigella.*[27]

The same is true for *salmonella*, a proper vaccine against *salmonella* has not yet been developed. Therefore the risk of human salmonellas is outbreaks and other problems encountered with these 2 pathogens necessitates attempting to produce an appropriate vaccine against these two pathogenic agents.[28]

IpaD plays a significant role in the antigenicity of the *Shigella* and is a key factor in the bacterial invasion in the host epithelial cells.[6]

Cell surface antigens are the ideal vaccine candidates due to their roles in interactions between the pathogen and the host.[28] InvH as an invasive agent to the epithelial cell could be a proper candidate for immunization.[9]

B-Cell and T-Cell epitopes predicted by numerous software approved the ability of InvH to induce both humoral and cellular immune systems remarkably.[12]

Since the portal of entry and the mechanism of invasion of the two pathogens are somewhat similar, therefore we thought that a chimeric construct carrying two immunogenic proteins, one from *Shigella* and the other from Salmonella may offer the possibility to elicit an effective humoral immune response against these two pathogens.

Chimeric proteins, because of their different subunits and versatile epitopes, can increase the immunogenicity of recombinant proteins and can induce a widespread cellular and humoral immunogenic response.[29] A chimeric construct that contains several pathogens (subunits) from different etiological agents can provide immunogenicity against them at the same time and it is prominently supported by the earlier studies;[30-32] so they can play an important role in vaccine design.

A previous study shows that the amino-terminal half of IpaD is exposed on the surface of *shigellae*. Accordingly, in this study, 107 amino acids from the N-terminal of IpaD were selected.[6]

InvH and IpaD were separated from each other via EAAAK (Glu, Ala, and Lys) linker. This linker because of its salt bridge related to the glutamic and lysine amino acid can cause neighboring domains not to merge by creating a stable helix structure.[33]

We have previously used the EAAAK linker to separate the structure of the main subunits of the CFA / I, CS2, and CS3 protein and the heat-sensitive toxin binding subunit (LTB).[34] Amani et al also used $(EAAAK)_4$ to separate the EspA, Intimin, and Tir protein from each other.[35]

Bioinformatic analysis revealed that the characteristics of our chimeric construct have structural features similar to the native one. The result of the Ramachandran plot, the minimum energy of the structure, and all the other assessment approved the appropriate design of the structure.

A CAI of 0.83 instead of 0.68 in prior, demonstrates that the optimized genes can have a good expression in *E. coli*.; increasing the antibody titer significantly and also the continuous rise of antibody titer until day 59 of the chimer indicates the sustainability of the vaccine effect and hence its efficacy.

Our immunized mice could tolerate 10 and 100 LD50 and stayed alive for 96 h, indicating the proper efficacy of these recombinant proteins. Concerning the presence of invH in all *Salmonella* strains, the recombinant chimeric protein containing InvH can be used for protective measures for *Salmonella* strains.

The eye infection of the control pig and the non-infected eye of the pigs which were received bacteria treated with immunized mice sera have also confirmed the immunization of the construct against *Shigella*.

The results of the chimeric proteins challenges in both models were very close and perhaps even better than the results of the challenges of each of the individual recombinant proteins, IpaD, and InvH. The reason can be what we have already aforementioned for the chimeric proteins acting as better immunogens. Therefore, it is much more beneficial to use a recombinant chimeric protein that can play the role of two immunized agents at the same time compared to the separate use of each of them.

In conclusion, the challenge experiments show that chimeric protein harboring both InvH and IpaD subunits can provide heterologous protection and hence can be used as a bivalent immunogen candidate against Salmonella and Shigella as the two major diarrhea producing bacteria. The recombinant protein used here

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contains a fusion tag at its N-terminal and is better to be removed in clinical trials.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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