Immunoprotectivity of *Salmonella enterica* serovar Enteritidis virulence protein, InvH, against *Salmonella typhi*

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ARTICLE INFO	ABSTRACT
	Objective(s)*:** Typhoid fever is a dreadful disease of a major threat to public health in developing countries. Vaccination with bacterial immunodominant components such as surface proteins may prove as a potent alternative to live attenuated vaccines. InvH, an important part of needle complex in type three secretion system (TTSS) plays important role in efficient bacterial adherence and entry into epithelial cells. ***Materials and Methods: In this work we used a 15 kDa recombinant InvH protein of *Salmonella enteric* serovar Enteritidis to provoke antibody production in mouse. The mice were immunized by recombinant InvH and challenged with *Salmonella typhi.* Histopathology of spleen and liver were studied. ***Results:*** The immunized mice showed a significant rise of antibody after the second booster. The immunization induced protection against high doses of *S. typhi*. The bacterial challenge with sera showed significant protection against challenge dose of 2×10^9 CFU. Immunized sera reacted with *S. typhi* markedly. Immunoreaction of bacterially infected sera and InvH protein was significantly higher than the control group. Bacterial loads of *S. typhi* in spleen was more than liver. Decreased bacterial load was evident in immunized mice after 7 days. Histological examination of the liver showed the immunized mice liver remained unaffected. ***Conclusion:*** Efficacy of the virulence protein, InvH, in inhibition of this phenomenon by active immunization was shown here. It may be concluded that InvH, as an antigen, can develop protection against *S. typhi* infections. InvH may be exploited in protective measures as well as a diagnostic tool in *Salmonella* infections.

Introduction

Typhoid fever, caused by *Salmonella typhi* remains a dreadful disease and a major threat to public health in many developing countries around the world. Globally, incidence is estimated at over 20 million cases and more than 200,000 deaths in the year, in developing countries where this infection is endemic (1-3). Unlike other *Salmonella enteric* serovars, *S. typhi* can only infect human from contaminated food or water by entering gastrointestinal tract through overcoming the high osmolarity stress of the human small intestine. Intestinal invasion caused by reaching the distal ileum and penetrating the specialized intestinal epithelial M cells of Peyer's patches. Quinolones and third generation cephalosporins are often used against multi-drug resistant infections (4-7). The Widal test is a serological diagnosis test based on the detection of *S. typhi* in blood (2, 5). The major invasive factors of *S. typhi* are the secretion of invasion-related proteins, flagella and Vi capsular antigen (4, 8-10). Many Gram-negative bacteria such as *Yersinia*, *Salmonella*, *Erwinia*, and *Pseudomonas* use type III secretion systems (TTSS), specialized organelles to translocate effectors proteins from the bacterial cytoplasm into the host-cell cytoplasm (11, 12). *Salmonella* pathogenicity islands, SPI-1 and SPI-2, code several structures of TTSS exposed to the host immune system making them potential vaccine candidates. InvH, an outer membrane lipoprotein, is an important part of TTSS in *Salmonella* strains (11-13). InvH is required for the

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pathogenicity of *Salmonella* strains, lysis of macrophages, secretion of several proteins like Sip, secretory and inflammatory immune responses and localization of several part of TTSS (InvG, PrgH, PrgK) (14-16). In this study we evaluated mice immunization with InvH protein in order to find as to whether it could mount a significant humoral immune response as measured by serum IgG titres and also to see if immunization could protect mice aganist *S. typhi*.

Material and Methods
Bacteria and growth cultures
S. typhi (PTCC 1609) was grown in Luria-Bertani (LB) broth or on nutrient agar (Merck, Germany) culture medium at 37°C.

Expression and purification of InvH protein
The PCR primers 5′-AAGAATTCAAACCGGTACAGCAACCTG-3′ and 5′-AAAAGCTTTTATAAGGATTGCAGTCTTTCATG-3 were used to amplify the *invH* gene of *S. typhi*. The PCR product was digested with *EcoR* I and *Hind* III and cloned into pET28a (+) vector. InvH-pET28a construct was transformed into *Escherichia coli* BL21 (DE3) and expression was induced with 1 mM IPTG. The bacterial cells were centrifuged and harvested. Sonication was employed to lyse the pellet suspended in buffer (100 mM NaH_2PO_4, 10 m MTris. Cl, 8 M urea). Centrifugation at 12000×g for 30 min resulted in separation of the supernatant which was then analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant InvH supernatant was purified by Ni-NTA chromatography according to the supplier's (Qiagen) directions.

Western blotting
SDS-PAGE in 12% gel was used to separate the protein samples which were then transferred to nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris–base, 0.037% SDS, and 20% methanol). Ten ml blocking buffer (5% skim milk in PBST [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 0.05% (v/v) Tween-20]) was used to incubate the membrane at room temperature under constant shaking for 1 hr. The membrane was washed 3 times with PBS-T and incubated again in a 1:1000 dilution of mice anti-His-tag -conjugated HRP in PBS-T for 1 hr at room temperature. The membrane washed with PBS-T. To visualize the membrane, 3,3′-diaminobenzidine for 1 hr at room temperature was used.

Animal husbandry
Four to six week old male BALB/c mice weighing about 16–22 g were procured from the Razi Institute, Tehran, Iran. The animals were maintained in standard and well-aerated conditions in the animal care facility at Shahed University. Animal Welfare Act and regulations related to experiments were followed. The criteria pertaining to Care and Use of Laboratory Animals were followed (17).

Immunization of mice
Groups of 10 male five to six-week-old BALB/c mice were immunized intraperitoneally (IP). The mice were procured from Razi Institute, Tehran, Iran. On days 0, 15, 30 and 45, 10 µg of the recombinant protein was injected subcutaneously to two groups of five mice each per mouseusing Freund's complete adjuvant at the first dose and incompleteadjuvant in the subsequent doses.

Determination of LD$_{50}$
LD$_{50}$ was determined for unimmunized by IP administration of live *S. typhi* ranging from 3×10^5 to 3×10^9 CFU.

Determination of antibody titers
The sera were collected 15 days after each immunizations. Unimmunized mice sera were used as control. 96-well microtiter plates coated with 5 mg purified recombinant InvH protein were incubated at 4°C overnight. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST). 100 µl/well of mouse serum serial dilutions from 1:400 to 1:51200, were then added to the wells and blocked with 5% skimmed milk. After washing three times with PBST, the plates were incubated at 37°C/1 hr with 100 µl per well of horseradish peroxidase conjugated (HRP-conjugated) secondary antibody already diluted to 1:1000 with PBST. The wells of the plates were then washed three times with PBST. A 100 µl of tetramethylbenzidine (TMB) solution were added to each well and incubated for 20–30 min at 37°C in dark. The reaction was stopped by addition of 2 M H_2SO_4. In order to measure stability of the antibody titer, samples were collected after 2 weeks, 1-4 months after immunization or bacterial inoculation.

Challenge studies
Seven days after the final booster dose, the immunized and control mice were challenged IP with *S. typhi*. The mice were observed daily for morbidity and mortality for 30 days. The unimmunized mice were challenged with 10^9 CFU *S. typhi* incubated with different doses of immunized mice sera for 30 min at 37°C.

Histopathological examination
Liver and spleen samples were aseptically collected from both test and control mice groups challenged with *S. typhi*. The samples were weighed and homogenized in 1 ml of PBS. Formalin was used to fix the tissue samples. 6-mm-thick sections were cut from paraffin embedded tissues. The sections

were stained for light microscopy with hematoxylin and eosin (HE).

Whole cell ELISA

S. typhi was cultured in LB broth until an optical density equivalent to 5×10^8 bacteria per ml was achieved. The cells were then washed three times in phosphate-buffered saline (0.05 M, pH 7.4) and suspended to a final concentration of 2.6×10^8 bacteria per ml in carbonate coating buffer (0.05 M, pH 9.6). Each well received 100 µl of the appropriate bacterial suspension in carbonate coating buffer. The wells were added with serial dilutions of sera ranging from 1:400 to 1:6400. 100 µl of anti-mouse IgG conjugated with HRP (Sigma, USA) as secondary antibody diluted to 1:3000 was added to each well. The immunoreaction was detected by adding OPD for 30 min and the reaction was stopped with H_2SO_4 (2 M).

Cross-reactions

Five micrograms per well of the recombinant protein was used to coat the surface of a 96-well microtiter plates and incubated at 4℃ overnight. Serial dilutions from 1:200 to 1:3200 of mice antisera against *S. typhi*, and uninfected mice were added to the wells. 100 µl of the secondary antibody diluted to 1:3000 was added to each well. The immunoreaction was detected as in whole-bacterial cell ELISA.

Enumeration of S. typhi in spleen and liver

The immunized and unimmunized mice were inoculated IP with 3×10^7 CFU *S. typhi*. Liver and spleen were aseptically collected after 3, 5, 7 and 9 days. Samples were homogenized and incubated in 1ml selenite cysteine broth and were subsequently plated on SS agar plates.

Statistical analysis

All the experiments were carried out in triplicate. The data are expressed as mean ±standard deviation (SD). *P-values* were calculated by Student's t test to determine the significance of differences in the experimental groups. *P-values* of <0.05 were considered as significant.

Results

Western blotting

Western blot using anti-His-tag antibodies confirmed expression of the recombinant protein (Figure 1).

Serological responses

The antibody titers of sera from immunized mice showed a significant ($P < 0.001$) rise after the second booster as compared to the control group injected with adjuvant (Figure 2). The immunized and control
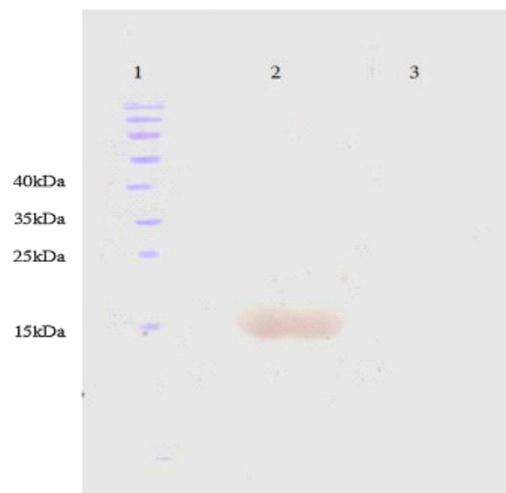

Figure 1. Western blot analysis of recombinant InvH. Lane 1: Protein weight markers; Lane 2: Purified recombinant InvH; Lane 3: Uninduced bacterial cell lysate

mice inoculated with *S. typhi* showed stable antibody titer (Figure 3). *P-values* were $P < 0.001$ and $P < 0.05$ for mice immunized with InvH protein and mice inoculated with *S. typhi* respectively (Figure 3).

Determination of LD₅₀ and animal challenge with S. typhi

LD_{50} was determined for unimmunized and immunized mice as 3×10^8 and 5×10^{11} CFU respectively by IP route for *S. typhi* (Figure 4). The immunization with the recombinant protein induced protection against high doses of *S. typhi*. The bacterial challenge with sera showed significant protection against challenge dose of 2×10^9 CFU (Figure 4).

Whole cell ELISA

Immunized sera reacted with *S. typhi* significantly ($P<0.001$). Antibody responses for immunized sera or inoculated with *S. typhi* were significant as compared to control group (Figure 5).
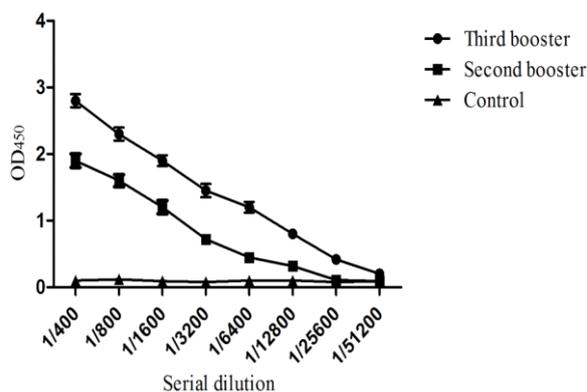

Figure 2. Indirect ELISA of serumfrom immunized mice with InvH protein and control group, the result shown significant difference (*P-value* <0.001)

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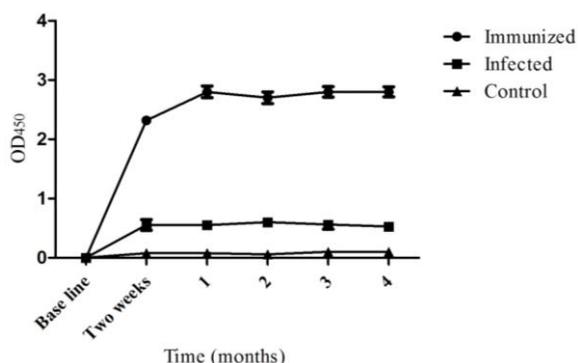Immunoprotectivity of S. enteritidis InvH against S. typhi

Figure 3. Stability of antibody titer after immunization of mice with InvH or inoculation with *Salmonella typhi*. The results show stability of antibody titer for 4 months after immunization

Figure 4. Survival rate analysis of mice after challenge with *Salmonella typhi*. LD_{50} was indicated for unimmunized and immunized mice as 3×10^8 and 5×10^{11} bacteria respectively

Cross reaction test

Immunoreaction of bacterially infected sera and InvH protein was significantly (*P*<0.05) higher than the control group (Figure 6).

Bacterial uptake in liver and spleen

Bacterial loads of *S. typhi* in spleen were more than liver. Decreased bacterial load was evidence in immunized mice after 7 days (Figure 7).

Histopathology

Figure 8 shows micrographs of liver sections from negative control (A), infected mice (B) and immunized mice (C) inoculated with *S. typhi*. The immunized mice liver remained unaffected.

Discussion

Increasing antibiotic resistance in *S. typhi* is a major therapeutic concern in developing countries. That is why the majority of research work is focused on developing an effective vaccine. Researches have largely been restricted to OMPs, VI capsular polysaccharide antigen and live attenuated strains of S*. typhi* (2, 6, 8, 14, 15). The efficacy of *Salmonella* pathogenicity island-1 (SPI-1) proteins was evaluated as vaccine candidates for protection
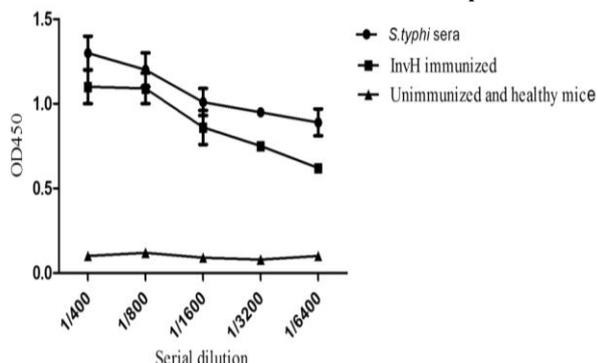
against *S. enterica* serovar Enteritidis oral challenge. The results demonstrated that SPI-1 type III secretion system proteins elicit antigen specific IgG antibody responses in chickens. Vaccination of hens with SPI-1 proteins using a seeder model of infection did not affect the levels of *S. enterica* serovar Enteritidis in the cecal contents or internal organs of their progeny. It was therefore deduced that co-occurrence of SPI-1 with other proteins may be used as vaccines for protection against colonization by *S. enterica* serovar Enteritidis in poultry (18). InvH is required for efficient assembly of the needle complex in type III secretion system following bacterial invasion to epithelial cells and disease spreading (13). In this study, we showed that InvH recombinant protein is highly immunogenic imparting complete protection against lethal dose of *S. typhi*. After the second booster, antibody titer was increased significantly (*P* <0.001) as compared to the control and was stable for at least 4 months. The findings suggest that protective immunogenicity of this protein increased the infection resistance of mice with *S. typhi* up to 10^3 LD_{50}. In addition, whole cell ELISA and bacterial neutralization tests with immunized sera indicated that produced antibody in immunized mice has the ability of attaching to InvH

Figure 5. Whole bacterial cell ELISA with sera from mice infected by *Salmonella typhi*, immunized mice with InvH and unimmunized and healthy mice. The results was shown significant different among mice infected by *S. typhi*, immunized mice and unimmunized and healthy mice (*P-value*<0.001)
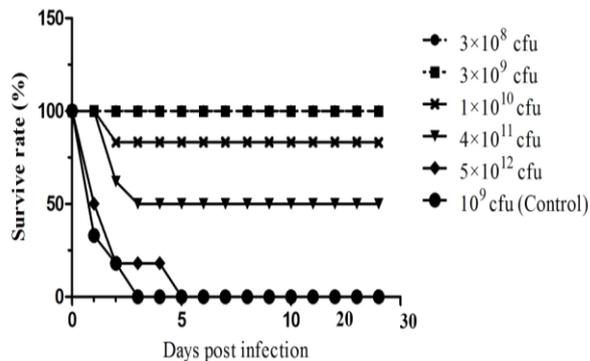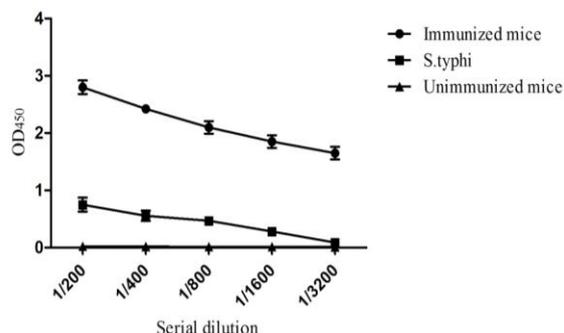

Figure 6. Cross-reactions with immunized mice sera and sera from mice infected with *Salmonella typhi* and InvH protein. The results was shown significant different among immunized, infected mice and control group (*P-value*<0.05)
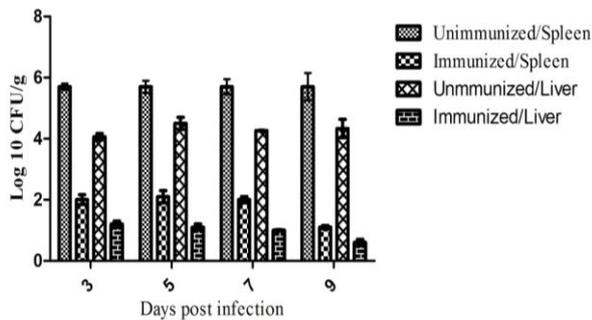
Figure 7. Presence of *Salmonella typhi* in liver and spleen of immunized and unimmunized mice. Mice were immunized with InvH protein, *P-value* was <0.001 compared with unimmunized mice

protein on bacterial surface inhibiting bacterial pathogenicity and infection. The tested mice survived with lethal infection of *S. typhi*. Phylogenetic relationship between serovars using multilocus sequence typing (MLST) was investigated. The serovars typhimurium and enteritidis were chosen as they are responsible for 75% of human infections in Europe. No differences in content of pathogenicity related genes between serovars with various host preferences and virulence towards humans were observed. It was speculated that other serovars can sustain their pathogenicity lacking one or two of these genes, whereas lack of many virulence genes would result in reduced virulence (19). In the present study the cross reaction test indicated that InvH protein reacted with the sera of infected mice with *S. typhi*. This phenomenon could be also considered as a candidate for diagnostic purposes. *S. enterica* serovar Enteritidis mutants with deletions in *phoP*, *fliC* or *phoP fliC* were tested for their virulence and their ability to induce parameters of the innate and adaptive immunity in addition to their potential for serological differentiation between immunized, non-immunized and infected chickens. The double *phoP fliC* deletion mutant was sufficiently attenuated but not diminished in its capability to inhibit the caecal colonisation and systemic invasion of homologous *S. enterica* serovar Enteritidis shortly after administration of the vaccine strain to very young chicks. Immunisation with the attenuated Δ*phoP fliC* mutant resulted in protective effects which were only slightly and insignificantly lower than after "immunisation" with a wild-type strain of *Salmonella.* This indicates a potential to induce in older chickens a strong adaptive immune response and protection against *Salmonella* exposure. The deletion in *fliC* brought about a significant differentiation between immunised and infected chickens using a commercially available ELISA kit. The double *phoP fliC* deletion mutant of *S. enterica* serovar Enteritidis might be a potential and promising live *Salmonella* vaccine candidate with novel characteristics for use in poultry (20). To construct a
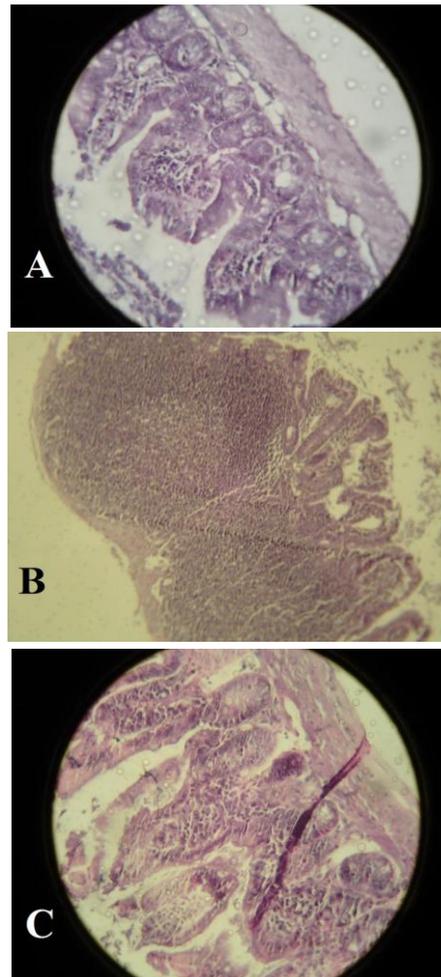

Figure 8. Micrograph (×400) showing liver sections from healthy, infected control and immunized mice challenged with *Salmonella typhi*
A. Normal liver showing normal histology and lobular pattern.
B. Infected liver: focal necrosis and accumulation of inflammatory cells (mononuclear and polymorphonuclear cells) are seen in the liver parenchymal skirt of liver lobules.
C. Liver from immunized mice challenged with *S. typhi* showing normal histology and lobular pattern

novel live *S. enterica* serovar Enteritidis vaccine candidate, *S. enterica* serovar Enteritidis was genetically engineered using the allelic exchange method to delete two virulence genes, *lon* and *cpxR*. Deletion of *lon* gene is essential to impair *Salmonella* replication and avoid overwhelming host systemic disease. Deletion of *cpxR* gene is essential to elevate the ability of bacteria to adhere and invade the host cell. In the present research the reduction of bacterial recovery in immunized mice compared to unimmunized mice demonstrated that immunization with InvH restricted bacterial invasion.

Conclusion

Immunization with InvH protein can develop protection against *S. typhi* infections. InvH may be exploited in protective measures as well as a diagnostic tool in *Salmonella* infections.

Acknowledgment

Conflict of interests

The authors have no conflict of interests.

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