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Immunogenicity of *Salmonella enterica* serovar Enteritidis virulence protein, InvH, and cross-reactivity of its antisera with *Salmonella* strains

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ABSTRACT

Acellular vaccines containing bacterial immunodominant components such as surface proteins may be potent alternatives to live attenuated vaccines in order to reduce salmonellosis risk to human health. *invH* gene, an important part of needle complex in type three secretion system (TTSS) plays important role in efficient bacterial adherence and entry into epithelial cells. In this work we hypothesize that use of a 15 kDa recombinant InvH as *Salmonella enterica* serovar Enteritidis surface protein could provoke antibody production in mouse and would help us study feasibility of its potential for diagnosis and/or a recombinant vaccine. The purified InvH provoked significant rise of IgG in mice. Active protection induced by immunization with InvH against variable doses of *S. enterica* serovar Enteritidis, indicated that the immunized mice were completely protected against challenge with 10^4 LD_{50}. The immunoreaction of sera from immunized mice with other *Salmonella* strains or cross reaction with sera of *Salmonella* strains inoculated mice is indicative of possessing by *Salmonella* strains of the surface protein, InvH, that can be employed in both prophylactic and diagnostic measures against *S. enterica*. Bacteria free spleen and ileum of the immunized mice in this study indicate that the *invH* gene affects bacterial invasion. Efficacy of the virulence protein, InvH, in shuttling into host cells in injectisome of *S. enterica* serovar Enteritidis and inhibition of this phenomenon by active immunization was shown in this study. In conclusion immunization with InvH protein can develop protection against *S. enterica* serovar Enteritidis infections. InvH in *Salmonella* strains can be exploited in protective measures as well as a diagnostic tool in *Salmonella* infections.

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1. Introduction

Salmonella enterica serovar Enteritidis is one of the main causes of food-borne illness. It grows under natural conditions (Braden 2006). Epidemic of human infections mediated by *S. enterica* serovar Enteritidis was observed during the last two decades of the 20th century (Abasht et al. 2008; Scallan et al. 2011; Matheson et al. 2010). Poultry and poultry products such as fowl (Braden 2006), shell eggs (Guard-Petter 2001) are the most prevalent sources of infection of *S. enterica* in humans (Braden 2006). Attempts were made to control poultry infection through both passive and active immunizations (Wisner et al. 2011; Chakravortty et al. 2005; De Buck et al. 2005; Nandre et al. 2011; Lamont et al. 2002; Rychlik et al. 2009). Serological and bacteriological monitoring of poultry and products, improved biosecurity and hygiene measures, restocking from Salmonella-free grandparent flocks, slaughter of affected animals, use of competitive exclusion products, antibiotics and vaccines are of the measures taken to control infection (McSorley and Jenkins 2000; Mead and Barrow 1990). Bacterins, attenuated, and subunit vaccines are three major classes of the currently available vaccines against salmonellosis. Protection induced by bacterins in poultry is generally modest (Barbour et al. 1993; Gast et al. 1993). An extensive review on the use of live vaccines against *Salmonella* in food-animals is available (Simon et al. 2011). Varying degrees of protection in chickens are offered by live attenuated *Salmonella* vaccines (Cooper et al. 1996). Acellular vaccines containing immunodominant components of the bacteria may be potent alternatives. These components include outer-membrane proteins, lipopolysaccharide, porins, fimbriae and flagellae epitopes (Liu et al. 2001; McSorley and Jenkins 2000). Use of adjuvants is necessary to enhance the duration of the immune response, and stimulate humoral, cellular, and mucosal immune responses (Arbos et al. 2002). Present vaccines have limited efficacy and hence there is a need to develop stable, efficacious, and safe vaccines to prevent

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human and poultry salmonellosis (Arbos et al. 2002; Liu et al. 2001; Toyota-Hanatani et al. 2008). To further reduce the risk to human health of salmonellosis, one aim remains as the development of a multivalent *Salmonella* vaccine (Woodward et al. 2002). Bacteria deliver protein effectors across eukaryotic cellular membranes by a complex nanomachine, the type three secretion injectisome. *invH* gene is an important part of needle complex in type three secretion system (TTSS) (Galan and Wolf-Watz 2006). For efficient bacterial adherence and entry into epithelial cells, the role of *invH* gene is essential (Altmeyer et al. 1993). Except for *S. enterica* subspecies *arizonae*, *invH* is present in all *Salmonella* strains. No homologous sequences were detected in *Yersinia*, *Shigelia*, *Proteus*, and several strains of enteroinvasive and enteropathogenic *Escherichia coli* (Altmeyer et al. 1993). The previous explanations express limitations in efficacy of vaccines against infections mediated by *S. enterica*. In this work we hypothesize that use of InvH as a bacterial surface protein could provoke antibody production in mouse and would help us study feasibility of its potential for diagnosis and/or a recombinant vaccine against *S. enterica* serovar Enteritidis.

2. Materials and methods

2.1. Kits, enzymes and reagents

The plasmid extraction and gel purification kits were procured from Bioneer (Daejeon, Korea). Nickel-nitrilotriacetic acid (Ni-NTA) agarose was from Qiagen (Valencia, USA). Primers were synthesized by Bioneer. Restriction endonucleases were obtained from Cinnagen (Tehran, Iran). T4 DNA ligase was from Fermentas (Vilnius, Lithuania). All other chemical reagents were from Sigma (Munich, Germany) or Merck (Darmstadt, Germany).

Nitrocellulose membrane (PROTRAN), anti-polyhistidine antibodies and anti-mouse IgG conjugated with HRP (RAY Biotech), ELISA reader (TECAN), microtiter plates (Caspian), Salmonella Shigella agar plates (MERCK).

2.2. Bacterial strains and culture conditions

S. enterica serovar Typhimurium, *S. enterica* serovar Typhi (PTCC 1609), *S. enterica* serovar Paratyphi A (PTCC 1230), *S. enterica* serovar Paratyphi B (PTCC 1231), *S. enterica* serovar Paratyphi C (Shahed university isolate), *E. coli* (ATCC 43889) and *S. enterica* serovar Enteritidis (ATCC-13076) was grown in Luria–Bertani (LB) broth or LB agar at 37 °C.

2.3. DNA extraction and construction of plasmid

The *invH* gene (GenBank accession No. AM933172) was amplified from its genomic DNA by PCR using the forward primer 5′-AAGAATTCAAACCGGTACAGCAACCTG-3′ and the reverse primer 5′-AAAAGCTTTTATAAGGATTGCAGTCTTTCATG-3′. Primers were used to amplify the *invH* gene with the stop codon truncated in order to be fused with a downstream His-tag sequence on the vector. Forward primer was designed to contain an *EcoRI* site and reverse primers carried a *HindIII* site. The *invH* gene was amplified in 35 PCR-cycles as follows: initial denaturation at 94 °C for 5 min and 35 cycles of 30 s at 94 °C/1 min at 62 °C/1 min at 72 °C, followed by an additional 5 min at 72 °C. PCR products were purified with the Wizard PCR preps kit and was digested with *EcoRI* and *HindIII* and cloned into pET28a(+) vector which adds a six-histidine tag at the C-terminus of the recombinant protein to allow its purification by affinity chromatography. The recombinant DNA plasmid, pET28a–*invH*, was transformed into *E. coli* strain BL21(DE3) as an expression host and were grown in LB agar containing 70 μg/mL kanamycin at 37 °C for 12 h. DNA plasmid was purified using a plasmid extraction kit, and was then sequenced in order to verify the cloned insert integrity.

2.4. InvH expression and purification

E. coli BL21 cells harboring the *invH*–pET28a construct were grown overnight at 37 °C in 10 mL of LB medium containing 70 mg/mL kanamycin, under constant shaking at 200 rpm. This culture was then used for inoculating 200 mL of LB medium. At the optical density of 0.6 at 600 nm, 1 mM isopropyl b-D-thiogalactoside (IPTG) was added to induce expression. The cells were further incubated at 37 °C for 6 h and were then harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell pellet was resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea), addition of lysozyme at 1 mg/mL, and was sonicated 5 times for 1 min at 200 W with 1 min intervals. Protein was purified from the supernatant by affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, CA). The supernatant was separated from cellular debris by centrifugation at 8000 × *g* for 30 min at 4 °C. Protein was eluted from the Ni-NTA matrix using elution buffer (10 mM Tris, 10 mM, pH 4.5). The fractions were individually collected and analyzed by SDS-PAGE. Concentration of the purified protein was determined by the Bradford method (Bradford 1976).

2.5. Western blotting

Protein samples were separated by SDS-PAGE in 12% gel and electrotransferred to nitrocellulose membrane at a constant current of 300 mA at 4 °C for 1.5 h. The membrane was blocked under constant shaking for 1 h with 5% skimmed milk in PBS–0.05% Tween-20 (PBS-T), pH 7.4 at room temperature. After washing the membrane thrice with PBS-T, the membrane was incubated with horseradish peroxidase (HRP)-coupled with anti-polyhistidine antibodies diluted (1:1000) in PBS-T for 1 h at room temperature and was then washed thrice with PBS-T. Diaminobenzidine (DAB) substrate was used to visualize the membrane.

2.6. Animals husbandry

BALB/c mice, 4–6 weeks old (16–22 g), were procured from the Razi Institute, Tehran, Iran. Mice were housed in clean standard and well-aerated conditions in the animal care facility at Shahed University. Research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed (Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011). The animal care protocol was approved by Shahed University.

2.7. Immunization of mice

The immunization trials were carried out in five to six-week-old female BALB/c mice procured from Razi Institute, Tehran, Iran. Two groups of five mice each were injected subcutaneously on days 0, 15, 30 and 45 with 10 μg of the recombinant protein per mouse using Freund's complete adjuvant at the first dose and incomplete adjuvant in the subsequent doses. A mice group injected with 100 μL of PBS each time simultaneously with the test group served as negative control.

2.8. Anti InvH antibody titers by ELISA

For detection of InvH-specific antibody by ELISA, 5 μg per well of the recombinant protein was used to coat the surface of a

96-well microtiter plates by incubation at 4 °C overnight. Serial dilutions of sera ranging from 1:400 to 1:51,200 were added to the wells. 100 μL of (anti-mouse IgG conjugated with HRP) secondary antibody diluted to 1:3000 was added to each well. The immunoreaction was detected by adding orthophenylenediamine (OPD) for 30 min and the reaction was stopped with H_2SO_4 (2 M). The microtiter plates were analyzed by ELISA reader at OD_{492}. The first blood samples collected prior to vaccination with InvH or before inoculation of the animals with *S. enterica* serovar Enteritidis, were used as control. The post vaccination or bacterial inoculation samples collected after 2 weeks, 1–5 months at one month increments were used in order to measure stability of antibody titer.

2.9. Determination of bacterial lethal dose (LD_{50})

The 50% lethal dose (LD_{50}) was determined in the following manner: *S. enterica* serovar Enteritidis at doses ranging from $3 \times l0^4$ to $3 \times l0^9$ CFU/mL were orally administered to 6 groups of 6 mice per group. The animals were monitored for 14 days and lethal effect was noted. LD_{50} was estimated from the number of survivals on day 14 (Cawthraw et al. 2011; Strindelius et al. 2002).

2.10. Animal challenge with S. enteritidis

The microbial population was precipitated $3000 \times g$ and was then suspended in 0.2 mL PBS. The immunized and control mice were orally challenged with various doses of *S. enterica serovar Enteritidis* ranging from 2×10^8 to 3×10^{13}.

2.11. Whole-bacterial cell ELISA

S. enterica serovar Enteritidis, *S. typhi*, *S. typhimurium* and *S. paratyphi* A, B, C and *E. coli* to be used in the bactELISA were plated for confluent growth in LB until an optical density equivalent to 5×10^8 bacteria per mL was reached. The cells were 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 bacterial coatings were dried using hair drier. In order to collect sera from inoculated mice groups, *S. enteritidis* and all serovars of *S. paratyphi* were given via oral route at doses below LD_{50}. *S. typhi* was injected intraperitoneally. *S. typhimurium* was lethal to the mice even at lower concentrations and hence no sera could be collected. Serial dilutions of sera ranging from 1:400 to 1:6400 were added to the wells. 100 μL of (anti-mouse IgG conjugated with HRP) 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).

2.12. Cross-reactions between sera from mice immunized with InvH against different serovars of Salmonella

ELISA experiments were carried out with mice antisera against *S. enterica* serovar Enteritidis, *S. typhi*, and *S. paratyphi* serovars A, B and C. Five micrograms per well of the recombinant protein was used to coat the surface of a 96-well microtiter plates by incubation at 4 °C overnight. Serial dilutions (1:200–1:3200) of mice sera against *S. enterica* serovar Enteritidis, *S. typhi*, *S. paratyphi* serovars A, B and C were added to the wells. 100 μL of (anti-mouse IgG conjugated with HRP) secondary antibody diluted to 1:3000 was added to each well. The immunoreaction was detected as in whole-bacterial cell ELISA.

2.13. Assessing the effect on bacterial shedding of immunization with InvH

To assess the effect of immunization on bacterial shedding, 15 mice were gavaged with 1×10^7 infective live *S. enterica* serovar Enteritidis suspended in 200 μL of PBS buffer one week following the last immunization. The number of bacteria shed in feces of the immunized and control animals were determined. Briefly, individual mouse was placed in a separate collection cage and the fecal pellets excreted over a 1 h period were collected. 100 mg of feces of each mouse were suspended and incubated in 1 mL selenite cysteine broth. 1, 10 and 100 μL of the sample was cultured in Salmonella Shigella agar (SS agar) plates and number of bacteria present in the fecal pellets was counted.

2.14. Recovery of S. enterica serovar Enteritidis from tissue organs

Ten immunized and non-immunized mice were orally inoculated with 10^7 *S. enterica* serovar Enteritidis. The animals were sacrificed after 72 h and spleen, liver and ileum were removed under aseptic conditions. The surfaces of the samples were washed thoroughly with sterile phosphate buffered saline (PBS) to remove non adherent bacteria. Tissues were homogenized, and incubated in 1 mL selenite cysteine broth and were subsequently plated on SS agar plates.

2.15. Histopathology

Morphological changes brought about by *S. enterica* serovar Enteritidis infection were assessed histopathologically. Spleen, liver and ileum removed from mice were fixed in 10% formalin, and embedded in paraffin blocks. Transverse sections (5 μm) cut with microtome, were stained with eosin and hematoxylin.

2.16. 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.

3. Results

3.1. Gene amplification, cloning, expression and purification

The *invH* gene was successfully amplified. The size of PCR amplicon was 350 bp. 1 mM IPTG induced the expression of the recombinant protein in *E. coli* BL21 (DE3). A single band of approximately 15 kDa was obtained. The recombinant protein was purified by Ni-NTA affinity chromatography. All the fractions were analyzed by SDS-PAGE.

3.2. Humoral response

The sera collected after second and third immunizations were assessed for specific antibodies of IgG class. Mice immunized with purified InvH showed significant rise of IgG. The antibody titer increased significantly ($P < 0.001$) after the second booster, whereas animals received adjuvant and PBS, as control had no InvH-specific antibodies in serum. The samples collected after 2 weeks, 1–5 months at one month increments, from vaccinated or bacterially inoculated mice showed stability of antibody titer. At the end of fifth months the antibody titers in immunized and infected mice were significantly higher than the control group. *P* values were

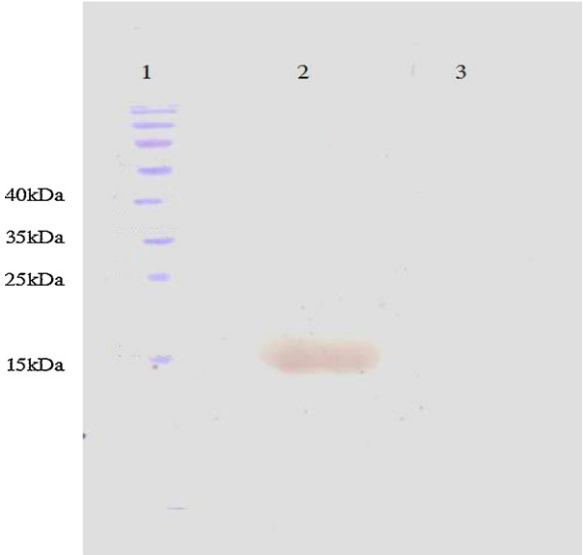

Fig. 1. Western blot analysis of recombinant InvH. Lane 1: protein weight markers; lane 2: purified recombinant InvH; lane 3: non-induced bacterial cell lysate.

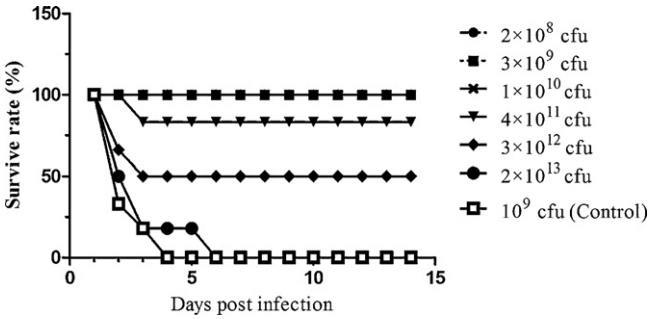

Fig. 2. Control and immunized mice response to *Salmonella enterica* serovar Enteritidis challenge. The first three lower loads of bacterial inoculation overlap each other as a result of immunized mice resistance to bacterial load.

$P < 0.001$ and $P < 0.01$ for mice immunized with InvH protein and mice inoculated with *S. enterica serovar* Enteritidis respectively.

3.3. Western blot analysis

The recombinant InvH was determined by western blotting test. The HRP-coupled anti-polyhistidine antibodies were successfully fused to His-tag in recombinant protein C-terminal and reacted with DAB (Fig. 1).

3.4. LD_{50} determination

LD_{50} was determined for unprotected and protected mice as 3×10^8 and 2×10^{13} respectively by oral route (Fig. 2).

3.5. Whole-bacterial cell and cross-reaction ELISA tests

The immunoreactions of sera from mice immunized with InvH (Table 1) or cross reaction between sera from mice infected with different *Salmonella* strains or immunized with InvH compared with sera from control mice (Table 2) showed immune response and antibody production. Antibody response against whole-bacterial cell ELISA with sera from immunized mice or inoculated with other *Salmonella* strains compared with *invH* deficient bacteria such as *E. coli* were significantly higher than the control

Table 1
Immunoreactions of sera from mice immunized with InvH or infected with *Salmonella* strains.

Serum sample	Bacterial coat	(OD_{492}) at dilution 1/400	(OD_{492}) at dilution 1/800	(OD_{492}) at dilution 1/1600	(OD_{492}) at dilution 1/3200	(OD_{492}) at dilution 1/6400	*P* value
S. enteritidis	*S. enteritidis*	1.6 ± 0.089	1.56 ± 0.056	1.32 ± 0.032	1.11 ± 0.06	0.9 ± 0.002	<0.001
InvH immunized	*S. enteritidis*	1.38 ± 0.14	1.17 ± 0.04	0.90 ± 0.016	0.63 ± 0.01	0.436 ± 0.03	<0.01
Negative control (unimmunized healthy mice)	*S. enteritidis*	0.02 ± 0.001	0.03 ± 0.001	0.021 ± 0.001	0.02 ± 0.001	0.01 ± 0.001	
S. typhi	*S. typhi*	1.36 ± 0.08	1.2 ± 0.05	1.01 ± 0.02	0.95 ± 0.01	0.89 ± 0.009	<0.001
InvH immunized	*S. typhi*	1.10 ± 0.14	1.09 ± 0.03	0.86 ± 0.01	0.75 ± 0.09	0.62 ± 0.04	<0.01
Negative control (Unimmunized healthy mice)	*S. typhi*	0.01 ± 0.001	0.012 ± 0.001	0.01 ± 0.001	0.013 ± 0.001	0.013 ± 0.001	
S. typhimurium	*S. typhimurium*	Nd	Nd	Nd	Nd	Nd	<0.001
InvH immunized	*S. typhimurium*	1.31 ± 0.1	1.11 ± 0.02	0.563 ± 0.01	0.311 ± 0.01	0.155 ± 0.002	<0.01
Negative control (unimmunized healthy mice)	*S. typhimurium*	0.01 ± 0.001	0.02 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	
S. paratyphi A	*S. paratyphi* A	1.36 ± 0.09	1.12 ± 0.08	1.07 ± 0.07	0.75 ± 0.05	0.718 ± 0.023	<0.001
InvH immunized	*S. paratyphi* A	0.98 ± 0.05	0.76 ± 0.02	0.62 ± 0.02	0.48 ± 0.02	0.32 ± 0.014	<0.01
Negative control (unimmunized healthy mice)	*S. paratyphi* A	0.03 + ± 0.001	0.01 ± 0.001	0.005 ± 0.001	0.016 ± 0.001	0.012 ± 0.001	
S. paratyphi B	*S. paratyphi* B	1.06 ± 0.09	0.88 ± 0.06	0.65 ± 0.02	0.55 ± 0.02	0.38 ± 0.001	<0.001
InvH immunized	*S. paratyphi* B	0.88 ± 0.02	0.75 ± 0.02	0.56 ± 0.02	0.42 ± 0.001	0.32 ± 0.005	<0.01
Negative control (Unimmunized healthy mice)	*S. paratyphi* B	0.03 ± 0.001	0.01 ± 0.001	0.04 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	
S. paratyphi C	*S. paratyphi* C	1.23 ± 0.1	1.12 ± 0.09	0.89 ± 0.075	0.78 ± 0.07	0.41 ± 0.023	<0.001
InvH immunized	*S. paratyphi* C	0.75 ± 0.001	0.65 ± 0.01	0.54 ± 0.02	0.32 ± 0.001	0.12 ± 0.01	<0.05
Negative control (unimmunized healthy mice)	*S. paratyphi* C	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	
E. coli	*E. coli*	1.44 ± 0.09	1.43 ± 0.082	1.32 ± 0.08	1.18 ± 0.06	0.42 ± 0.03	<0.001
InvH immunized	*E. coli*	0.09 ± 0.001	0.08 ± 0.008	0.07 ± 0.005	0.052 ± 0.002	0.042 ± 0.002	<0.001
Negative control (unimmunized healthy mice)	*E. coli*	0.05 ± 0.001	0.04 ± 0.001	0.03 ± 0.001	0.03 ± 0.001	0.02 ± 0.001	

Nd, not done. The animals did not survive even after minimal inoculation.

Table 2
Cross-reactions (OD_{450}) against InvH of immunized mice sera or sera from mice infected with different strains of *Salmonella*.

Serum dilutions	Unimmunized mice (negative control 1)	*E. coli* (negative control 2)	Immunized mice (positive control)	*S. enteritidis*	*S. typhi*	*S. paratyphi* A	*S. paratyphi* B	*S. paratyphi* C
1/200	0.021 ± 0.001	0.065 ± 0.003	2.8 ± 0.12	0.855 ± 0.22	0.75 ± 0.05	0.54 ± 0.05	0.735 ± 0.095	0.69 ± 0.1
1/400	0.018 ± 0.001	0.08 ± 0.003	2.42 ± 0.12	0.71 ± 0.27	0.554 ± 0.04	0.49 ± 0.04	0.630 ± 0.11	0.59 ± 0.1
1/800	0.015 ± 0.001	0.06 ± 0.002	2.1 ± 0.11	0.60 ± 0.28	0.47 ± 0.04	0.37 ± 0.06	0.37 ± 0.07	0.35 ± 0.02
1/1600	0.014 ± 0.009	0.055 ± 0.0001	1.85 ± 0.11	0.55 ± 0.09	0.281 ± 0.03	0.02 ± 0.001	0.082 ± 0.05	0.15 ± 0.006
1/3200	0.011 ± 0.008	0.052 ± 0.0001	1.65 ± 0.11	0.42 ± 0.08	0.087 ± 0.0025	0.01 ± 0.005	0.04 ± 0.04	0.02 ± 0.003
P value				<0.01	<0.05	<0.05	<0.05	<0.05

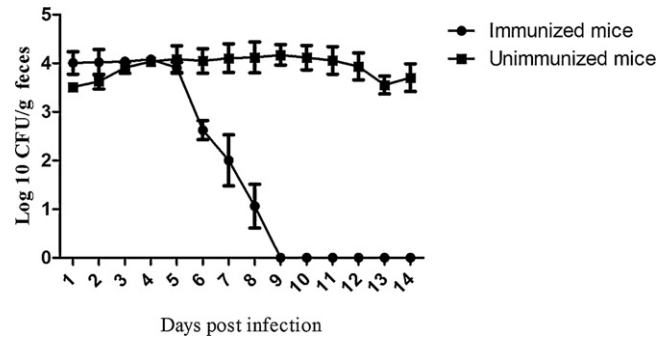

Fig. 3. Trend of decreasing *Salmonella enterica* serovar Enteritidis shedding in immunized mice as compared to the unimmunized group. Mice were gavaged with infective live *S. enterica* serovar Enteritidis at Sub LD_{50} level of 1×10^7 suspended in 200 μL of PBS buffer one week following the last immunization.

group. Specificity of the reactions were high with *S. enterica* serovar Enteritidis (Tables 1 and 2).

3.6. Effect of immunization on bacterial shedding or bacterial uptake in liver, spleen and ileum

Immunized mice shed high levels of bacteria in outset and reduced gradually to stop completely after nine days. Non immunized mice exhibited high levels of bacterial shedding (9×10^4) over the two-week sampling period (Fig. 3).

Bacterial loads of $(1.4 \pm 0.33) \times 10^2$ and $(6.1 \pm 0.41) \times 10^3$ were detected per gram of spleen and ileum of the positive group respectively while no bacterial load was detected in the same organs of the immunized mice or control groups.

3.7. Histopathology of S. enterica serovar Enteritidis-infected ileum

Histopathology results showed considerable effect of immunization to inhibit establishment of *S. enterica serovar* Enteritidis infection in ileum. Protection against tissue damage compared to unimmunized mice was evident. Immunization with InvH reduced ileal invasion of *Salmonella*. Liver and spleen from infected mice did not show tissue damage. Ileum from infected mice exhibited considerable villous height reduction, with associated extrusion of enterocytes and influx of inflammatory cells into the epithelium and submucosal region (Fig. 4a). Immunized animals showed no damage (Fig. 4b).

4. Discussion

The type three secretion systems (TTSS) encoded by *Salmonella* pathogenicity islands (SPI) 1 and 2 are major virulence factors of *Salmonella* (Gallois et al. 2001). Large clusters of genes are typically accommodated in pathogenicity islands contributing to a particular virulence phenotype (Marcus et al. 2000). *invH* gene, first identified in 1993 in *S. enterica* serovar Choleraesuis and *S. enterica* serovar Typhimurium (Altmeyer et al. 1993) is located at one border showing a different distribution among *Salmonella* serotypes than the genes encoding the SPI1 TTSS apparatus. InvH was present in the peptidoglycan (Pucciarelli and Garcia-del Portillo 2003) and hence there is a need for it in order to assemble parts of outer ring in needle complex system in TTSS. In the present study we successfully produced a recombinant InvH protein of approximately 15 kDa immunization with which resulted in a significant immune response. The purified InvH provoked significant rise of IgG in mice increasing the antibody titer significantly ($P < 0.001$) after the

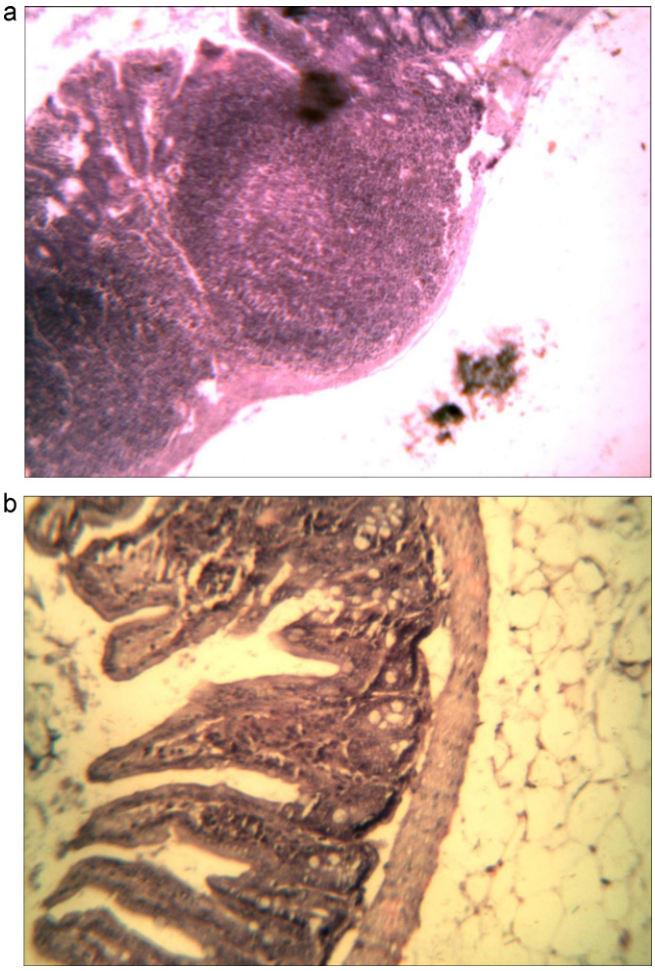

Fig. 4. (a) Micrograph (×400) showing localized mucosal lymphatic infiltration with lymphoid follicle formation. (b) Micrograph (×400) showing healthy and intact ileum of immunized mice challenged with *Salmonella enterica* serovar Enteritidis.

second booster. The *invH* mutation has been reported to significantly reduce the secretory and inflammatory responses that it was shown the important role of InvH in immunity response (Marcus et al. 2000). In our study the antibody titer was stable for at least 5 months in both bacterially inoculated and InvH immunized animals. Researches on mutation in *invH* gene confirm that mutants contain a reduced number of needle complexes suggesting that *invH* may modulate organelle assembly (Sukhan et al. 2001). Active protection induced by immunization with InvH against variable doses of *S. enterica* serovar Enteritidis, indicated that the immunized mice were completely protected against challenge with 10^4 LD_{50}. The immunoreaction (Table 1) of sera from immunized mice with other *Salmonella* strains or cross reaction (Table 2) with sera of *Salmonella* strains inoculated mice indicates that InvH is a surface protein (Daefler and Russel 1998) and antibody produced in immunized mice has the ability of attaching to this protein on bacterial surface. Immune response against InvH protein was shown to cross react with sera from mice infected with different *Salmonella* strains. The findings suggest applicability of InvH in both prophylactic and diagnostic measures against *S. enterica*. Recovery of $(1.4 \pm 0.33) \times 10^2$ and $(6.1 \pm 0.41) \times 10^3$ bacterial loads per gram from spleen and ileum of the positive group respectively, indicate that the *invH* gene affects bacterial invasion (Altmeyer et al. 1993; Watson et al. 1995; Watson et al. 1998). On the other hand bacteria free spleen and ileum of the immunized mice in this study supports the report that mutations in *invH* reduce invasion by *Salmonella* strains into eukaryotic cells (Watson et al. 1998) and reduce the recovery of *S. enterica* serovar Enteritidis from ileum and spleen and *S. typhimurium* from bovine mucosa following oral inoculation (Watson et al. 1998). This is further supported by *S. enterica* serovar Enteritidis shed by control and immunized mice. The pathogen has failed to attach the immunized host cells and hence decreased colonization or shedding was noted in this group. The observations are in agreement with InvH involvement in the ability of *Salmonella* to attach to cultured epithelial cells (Altmeyer et al. 1993; Sua'rez and Rassmann 2010; Watson et al. 1998). The ability to enter cells of the intestinal epithelium is an essential step in the pathogenic life cycle of the enteric pathogen, *Salmonella*. This organism passes through the intestinal epithelium in membrane-bound vesicles, reaching the lamina propria and cells of the reticuloendothelial system (Altmeyer et al. 1993; Kim 2003). In the present work liver and spleen from infected mice did not show tissue damage. Depending upon the infecting serotype or the infected host, *Salmonella* may penetrate to deeper tissues such as liver and spleen or may remain localized in the lamina propria of the intestinal epithelium (Mittrucker et al. 2000). In our study considerable villous height reduction of ileum in infected mice was noted with associated enterocytes extrusion and inflammatory cell influx into the epithelium and submucosal region. Previous researches indicated that IgG can be transported bidirectionally across epithelial barriers by the human neonatal Fc receptor FcRn. Most IgG of the human gastrointestinal, respiratory, and genital tracts mucosal surfaces is derived from the serum (Meckelein et al. 2003; Spiekermann et al. 2002; Yoshida et al. 2004). In this study it is possible that IgG antibodies were responsible for protection against *S. enterica* serovar Enteritidis in intestinal epithelium. The tissue organs maintained intact in immunized mice show efficacy of the virulence protein, InvH, in shuttling into host cells in injectisome of *S. enterica* serovar Enteritidis. This phenomenon has been inhibited by active immunization in this study.

5. Conclusions

Recombinant InvH protein can induce production of antibody in mice. Immunization with InvH protein can develop protection against infection with *S. enterica* serovar Enteritidis. With respect to the presence of *invH* in all *Salmonella* strains, the gene expression product, i.e. InvH, can be used for protective measures as well as a diagnostic tool in *Salmonella* infections.

Conflict of interest statement

The authors declare no conflict of interests.

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