Biologicals 41 (2013) 224–230

Contents lists available at SciVerse ScienceDirect

Biologicals

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

Immune response variations to *Salmonella enterica* serovar Typhi recombinant porin proteins in mice

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ARTICLE INFO

Article history:
Received 13 December 2012
Received in revised form
14 May 2013
Accepted 20 May 2013

Keywords:
OmpC
OmpF
OmpA
Salmonella enterica ssp. Typhi
Immunity

ABSTRACT

Objectives: Typhoid fever is caused by *Salmonella enterica* serovar Typhi. OmpC, OmpF and OmpA, the three major outer membrane proteins (OMPs), could serve as vaccine candidates.
Methods: The porins antigenicity was predicted *in silico*. The OMP genes were amplified, cloned and expressed. Sero-reactivities of the recombinant proteins purified by denaturing method were assayed by ELISA. BALB/c mice were immunized with the recombinant porins followed by bacterial challenge.
Results: Bacterial challenge of the animal model brought about antibody triggering efficacy of the antigen in OmpF > OmpC > OmpA order. Experimental findings validated the *in silico* results. None of the antigens had synergic or antagonistic effects on each other from immune system induction points of view. Despite their high immunogenicity, none of the antigens was protective. However, administration of two or three antigens simultaneously resulted in retardation of lethal effect. Porins, in addition to their specific functions, share common functions. Hence, they can compensate for each other's functions.
Conclusions: The produced antibodies could not eliminate the pathogenicity by blockade of one or some of the antigens. Porin antigens are not suitable vaccine candidates alone or in denatured forms. Native forms of the antigens maybe studied for protective immunogenicity.
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1. Introduction

Salmonella enterica serovar Typhi is a Gram-negative bacterium, the causative agent of human typhoid fever. Typhoid fever spreads by fecal-oral route via contaminated food and water [1,2]. A recent study estimated approximately 22 million cases of typhoid each year with at least 200,000 deaths [3,4]. Gram-negative bacteria have an outer membrane layer in their cell wall structures. Several proteins including porins present in outer membrane form water filled channels on it that allow small hydrophilic solutes to pass through the pore [5,6]. Furthermore these proteins involve in a variety of the pathogen functions [7,8]. *S. enterica* serovar Typhi synthesizes three major outer membrane proteins (OMPs): OmpC and OmpF with homotrimers structures, and OmpA with monomeric structure [9]. Expression of OmpC and OmpF genes in *S. enterica* serovar Typhi is controlled by *ompR–envZ*, members of a two-component signal transduction system. Shifting of osmolarity conditions in *S. enterica* serovar Typhi only affects the OmpF expression. OmpF is expressed primarily in low osmolarity conditions, and OmpC levels remain constant and expressed under low and high osmolarity condition [10]. Several researches have shown that OmpA, like porins and LPS, is also a target of the host immune response but its role in immuno-protection is not clearly demonstrated [11].

The OmpA folding is in majority and minority conformers. The majority conformers fold in to a structure with two large domains, the N-terminal domain and the C-terminal domain but the minority conformer forms channels allowing the diffusion of solutes up to several hundred daltons in size [12]. It was also shown that *Salmonella* porins play a role in pathogenesis and are important antigens against which the host immune response is directed to induce both humoral and cell mediated immunity [13]. The OMPs

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http://dx.doi.org/10.1016/j.biologicals.2013.05.005

of *S. enterica* serovar Typhi and many other Gram-negative bacteria are widely recognized as important immunogens that contribute to the pathogenesis of the disease and to protection against challenge [14]. Antibodies to porin antigens of *S. enterica* serovar Typhi induced during typhoid infection in human maybe of diagnostic value in typhoid infections [2]. Protection generated by ant-Vi Abs is induced by immunization of plain Vi polysaccharide or a Vi PS conjugate vaccine. This is currently the only licensed and efficacious subunit vaccine for typhoid [3]. Immunization with OMPs from other gram-negative bacteria also induces a protective status in experimental animals [15]. Since the purification of native porin proteins by routine methods is a lengthy procedure associated with contaminations particularly with lipopolysaccharide (LPS) [2]. Here, we report cloning, expression and purification of OmpA, OmpC and OmpF from *S. enterica* serovar Typhi PTCC 1609. Their immunogenicity and potential protective efficacy were studied in BALB/c mice. The current study was undertaken to define the role of OmpA, OmpC and OmpF of *S. enterica* serovar Typhi in the induction of protective immunity against a challenge with live bacteria.

2. Materials and methods

2.1. In silico analyses

2.1.1. Similarity studies

Protein sequences of OmpC (Acc. NP_804453.1), OmpF (Acc. NP_805701.1) and OmpA (Acc. NP_805619.1) served as queries for BLAST against all protein sequences of *S. enterica* ssp I serovars at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The first sequences of the OMP hits with E-value of 0.0 and the highest total score were retrieved in FASTA format to more precise similarity analyses. Therefore, alignment of these sequences was performed by PRALINE at http://www.ibi.vu.nl/programs/pralinewww/ for each type of the tree OMPs.

2.2. Prediction of antigen probability

The OMP sequences were submitted to VaxiJen v.2.0 at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html in order to determine their antigenic probability.

VaxiJen is the first server developed to allow antigen classification based on the physicochemical properties of proteins without recourse to sequence alignment. Accuracy of its predictions is 70%–89% [16].

2.3. Chemicals and enzymes

T4DNA ligase and restriction endonucleases were purchased from Fermentas (Vilnius, Lithuania). The gel purification and plasmid extraction kits were from Bioneer (Daejeon, Korea). Nickel–nitrilotriacetic acid (Ni–NTA) agarose was from Qiagen (Valencia, USA). Other chemical reagents were procured from Merck (Darmstadt, Germany). The primers were synthesized by Bioneer.

2.4. Bacterial strains, plasmids, culture media and human sera

S. enterica serovar Typhi PTCC (Persian Type Culture Collection) 1609 and *Escherichia coli* BL21 (DE3) were from our research laboratory. The plasmid pET28a (+) was a Novagen product (USA), Luria–Bertani (LB) broth or LB agar and Salmonella–Shigella (SS) agar were of Hi-media products. Serum samples were collected from 10 individuals with acute typhoid fever and positive Widal test (15–40 years old) referred to Razi laboratory of Andimeshk city of Iran. Sera from 10 healthy volunteers served as a control.

2.5. PCR amplification and cloning of OmpA, OmpC and OmpF genes

Heterologous expression in *E. coli* BL21 (DE3) along with its signal peptide is toxic to *E. coli*. While the removal of signal peptide leads to the formation of cytoplasmic inclusion bodies (IBs) [6], so the signal peptides from 5′-terminal were omitted in primer designing. The genes coding for OMPs were amplified from genomic DNA. The amplification of the OmpA, OmpF and OmpC genes was carried out using the following primers:

Forward (OmpC): 5′-GCAGCGAATCATATGGAAATTTATAATAAA-GAC-3′
Reverse (OmpC) [6]: 5′-AACATCTTTGGATCCTTAGAACTGGTAA AC-3′
Forward (OmpA): 5′-CGCTGAATTCATGCCGAAAGATAACACCTG-3′
Reverse (OmpA): 5′-CAAAAAGCTTTTAAGCCTGCGGCTGAGTTAC-3′
Forward (OmpF): 5′-CGCAGAATTCATGGCAGAAATTTATAATAA AGA-3′
Reverse (OmpF): 5′-AGTCAAGCTTTCAGAACTGGTAAGTAATACCG AC-3′

PCR reactions of 25 μl each for OmpA, OmpC and OmpF contained 2 μl of each primer (20 pM), 250 μM each dNTP. 1 μl DNA (50 ng/ml), 2 μl $MgSO_4$ (50 mM) and 0.5 μl of pfu DNA polymerase in a final volume of 25 μl was put in a thermal cycler (Techne Gradient). 30 PCR cycles were run as follows: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 1 min, primer annealing at 57 °C (OmpC), 60 °C (OmpA) and 66 °C (OmpF) for 1 min, and extension at 72 °C for 1 min, finally extended for 5 min at 72 °C. The amplified DNA products were electrophoresed in a 0.8% (*w*/*v*) agarose gel. PCR products were purified using a PCR purification kit as per the manufacturer's instructions. The PCR product (OmpC) was digested with BamHI and NdeI and OmpA, OmpF were digested with HindIII and EcoRI. They were cloned into pET28a (+) vector digested with the relevant endonucleases. The new constructs were named pET28a–OmpA, pET28a–OmpC and pET28a–OmpF. The ligated products were transformed into *E. coli* BL21 (DE3) as the expression host. The recombinant clones were selected on LB plates containing ampicillin (50 μg/ml). After mini-scale isolation of the plasmid DNA using the plasmid extraction kit, the presence of the open reading frame (ORF) was confirmed by restriction analysis and by sequencing.

2.6. Expression and purification of recombinant porins (OmpA, OmpC and OmpF)

The transformed *E. coli* BL21 cells were grown in LB medium (500 ml) and were then induced with 0.5 mM iospropyl b-D-thiogalactoside (IPTG). The expressed protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* BL21 cells harboring the OmpA–pET28a, OmpC–pET28a and OmpF–pET28a constructs were grown overnight at 37 °C in 10 ml of LB medium containing 50 μg/ml ampicillin under constant shaking (200 rpm). This culture was then used for inoculating 500 ml of LB medium. 0.5 mM isopropyl b-D-thiogalactoside (IPTG) was added at the optical density of 0.6 at 600 nm to induce expression. The expressed proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cells were further incubated at 37 °C for 6 h and were then harvested by centrifugation at 14,000× *g* for 10 min at 4 °C. The cell pellet was re-suspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea) with addition of lysozyme at 1 mg/ml, and was sonicated 5 times for 1 min at intervals of 1 min. 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 12,000× *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 purified protein was determined by Lowry et al. method [17].

2.7. Western blot with anti-His-tag antibody

In order to detect recombinant OmpA, OmpC or OmpF in *E. coli*, the induced and uninduced lysates were resolved on 12% SDS-PAGE. Protein samples were electrophoresed on a 12% SDS-PAGE gel and were then electroblotted onto a nitrocellulose membrane at a constant current of 300 mA at 48 °C for 1.5 h. The membrane was blocked using 5% skimmed milk in PBS–0.05% Tween under constant shaking at room temperature for 1 h. After washing the membrane thrice with PBS–T (PBS + 0.05% Tween-20, pH 7.4), the membrane was incubated with horseradish peroxidase (HRP)-coupled anti-polyhistidine antibodies diluted (1:1000) in PBS–0.05% Tween for 1 h at room temperature, then washed thrice with PBS–0.05% Tween. Diaminobenzidine (DAB) substrate was used to visualize the membrane.

2.8. Seroreactivity of the recombinant porin proteins

The IgG antibody levels of three porins were measured by ELISA. Sera from five healthy (typhoid negative) persons served as a control. 0.1 μg/well from each recombinant purified antigen was coated in the microtiter plate. BSA, serving as a negative control, was coated at the same concentration in the microtiter plate. Sera samples were tested at 1:100–1:128,000 dilution range.

2.9. Immunization of mice

Groups of 4–6 weeks old female BALB/c mice ($n = 10$/group), weighing 16–22 g were procured from the Pasteur 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 [18]. The animal care protocol was approved by Shahed University.

The animals were injected intraperitoneally (IP) with 10 μg from each of the purified recombinant proteins. The initial injections were emulsified with 10 μg of complete Freund's adjuvant (Sigma), and the subsequent injections were with 10 μg incomplete Freund's adjuvant (Sigma). Each mouse received three injections each at 15 days intervals. The blood samples were collected 10 days after the last injection through infra-orbital route. An additional 10 BALB/c mice served as the control group. An immunization schedule of the combination of the recombinant proteins was also carried out. 10 μg from each of the proteins were administered to mice in the following combinations: OmpA + OmpC, OmpA + OmpF and OmpC + OmpF. In another set of animal groups a combination of all the three recombinant proteins each at 10 μg level was administered intraperitoneally to the animals.

2.10. Analysis of antibody response in mice injected with recombinant proteins

The sera were assayed for the IgG antibody titer against the recombinant proteins by ELISA. Briefly, 96-well microtiter plates were coated with 2 μg purified recombinant OmpA, OmpC and OmpF proteins and incubated for 12–18 h at 4 °C. The wells were then washed three times with PBS plus 0.05% Tween 20 (PBST), and then incubated with 100 ml of PBST plus 5% skimmed milk for 1 h at 37 °C. Serial dilutions of each serum ranging from 1:400 to 1:51,200 were added to the wells in triplicates and were incubated at 37 °C for 1 h. The plates were washed 3 times again as described above. Antibodies binding to the antigen were detected using anti-mouse IgG conjugated with HRP at 1:2000 dilutions. The immune reaction was detected by adding 100 μl TMB (3,3′,5,5″-tetramethylbenzidine) as a substrate for 15 min. The reaction was stopped by adding 100 μl H_2SO_4 (2 M).

2.11. Cross-reactivity of the recombinant porins

The sera were assayed for cross-reactivity of the IgG antibody titer against recombinant proteins by ELISA as mentioned under "Analysis of antibody response in mice injected with recombinant proteins".

2.12. Western blotting with antisera

In order to precisely analyze the cross-reactivity, the purified recombinant OmpA, OmpC, OmpF, the IPTG induced *E. coli* lysates and Bovine Serum Albumin (BSA) were resolved and electrophoresed on a 12% SDS-PAGE gel. They were then electroblotted onto a nitrocellulose membrane at a constant current of 300 mA at 48 °C for 1.5 h. The membrane was blocked under constant shaking for 1 h with using a 5% skimmed milk in PBS–0.05% Tween at room temperature. After washing membrane thrice with PBS–T (PBS + 0.05% Tween-20, pH 7.4), they were incubated separately with diluted (1:100) mouse anti-OmpC, anti-OmpF and anti-OmpA serum for 1 h. They were again washed with PBS–T and were then incubated with mouse IgG conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. The membrane was then washed three times in PBS–T. The membrane was visualized with diaminobenzidine (DAB) substrate until brownish bands were observed. Color development was then terminated by washing in PBS.

2.13. Determination of lethal dose (LD_{50})

As mouse is not a natural host of *S. enterica* ssp Typhi, thus intraperitoneal injection must be performed for developing infection in mice [19]. We inoculated the mice intraperitoneally (i.p.) with *S. enterica* ssp Typhi. 200 μl of the bacterial suspensions at 3×10^4 to 3×10^9 CFU/ml were administered intraperitoneally to six groups of five BALB/c mice per group. Mortality rate was recorded for three consecutive post-challenge days. LD_{50} was defined as the volume (CFU/ml) of bacterial load that brought about death in half of the population size.

2.14. Animal challenge

Seven days after the last booster dose, the mice were challenged intraperitoneally with 10^8 of *S. enterica* ssp Typhi (PTCC 1609) suspensions in 100 μl PBS at microbial log phase. Control group was exposed to the same bacterial load.

2.15. Statistical analysis

Initially, the raw data were tested for normality using the SPSS software No.20 and the main data were then analyzed for the traits using analysis of variance and Duncan's multiple range test ($P < 0.01$). Independent samples *t*-test was used for comparison of the IgG titer in different sera and different porin proteins and BSA (Control). The Graph pad prism version No 5 software was used for drawing the graphs.

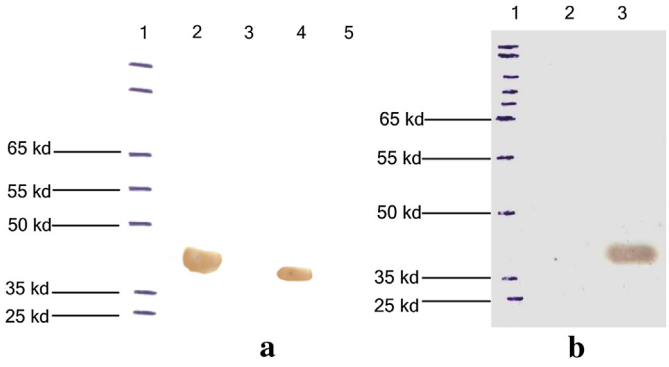

Fig. 1. Western blot analysis of recombinant OmpF and OmpA (a) and OmpC (b).

3. Results

3.1. In silico analyses

3.1.1. Similarity studies

No significant differences were found among same OMPs in various *S. enterica* ssp I serovars (Data not shown).

3.1.2. Antigen probability

Antigenicity of the three OMPs was predicted as: OmpF (0.83) > OmpC (0.78) > OmpA (0.69).

3.2. Amplification, expression and purification of OmpA, OmpF and OmpC

The OmpA, OmpC and OmpF genes of *S. enterica* ssp Typhi PTCC 1609 were successfully amplified by PCR and the fragments of appropriate size and digestion patterns of amplified porins with restriction enzymes were observed on 1% agarose gel. The fragments were cloned into pET28a (+) vector. This construct was confirmed by DNA sequencing and the PCR products were ligated into pET28a and transformed into BL21 (DE3) *E. coli* cells for expression of the recombinant porins (OmpA, OmpC and OmpF). The recombinant proteins were over-produced after IPTG induction. Following His-tagged affinity chromatography, the purified recombinant proteins analysis with SDS-PAGE revealed single bands of approximately 37.62 kDa (OmpA), 40.7 kDa (OmpC), 39.05 kDa (OmpF). A minor increase in molecular weights is because of 12 histidine tags in C and N terminals.

3.3. Western blot analysis

The specificity of the recombinant proteins was determined by Western blot analysis. The bacterial lysates of induced and uninduced cultures of OmpA, OmpC and OmpF were separated on 12% SDS-PAGE with a prestained protein molecular mass marker and were transferred onto the nitrocellulose membrane. Western blotting with anti-His-tag antibody detected 37.62 kDa, 40.7 kDa, 39.05 kDa proteins. The proteins were seen in the induced bacterial lysates, representing the recombinant OmpA, OmpC and OmpF, respectively, but the same bands were absent in the uninduced bacterial lysates (Fig. 1).

3.4. Seroreactivity with typhoid patients' sera

A significant difference between the OD values in sera of typhoid patients and healthy control in serial dilutions 1:100 could be observed for OmpA ($P < 0.05$), OmpC ($P < 0.01$) and OmpF ($P < 0.001$), as shown in Fig. 2.

The *t*-test results showed significant differences between IgG titer in typhoid patients' sera and normal sera, and between IgG titer in typhoid patients' sera and BSA in different porin proteins, while there was no significant difference between IgG titer in normal sera and BSA.

3.5. Immunogenic property of the recombinant proteins

Mice immunized with single (Fig. 3), two (Fig. 4) and three (Fig. 5) purified proteins showed significant rise of IgG antibodies. The antibody titer increased after the second booster, whereas animals received adjuvant and PBS, as a control had no porins-specific antibodies in serum. The combined protein administration had no significant difference with those of the single protein injections.

3.6. Cross-reactivity analyses

Anti-OmpC had cross-reactivity with other two porins. OmpF showed higher reactivity than OmpA. Similar results were seen for anti-OmpF, i.e. OmpC showed higher cross-reactivity than OmpA. Anti-OmpA showed low cross-reactivity with the other two porins (Fig. 6). Cross-reactivity was noted with IPTG induced *E. coli* lysate. No reactivity was observed with BSA (Fig. 7).

3.7. Determination of LD_{50}

LD_{50} was determined as 3×10^7 CFU/ml/mouse via intraperitoneal injection route. Mortality in the mice was recorded for two consecutive post-challenge days.

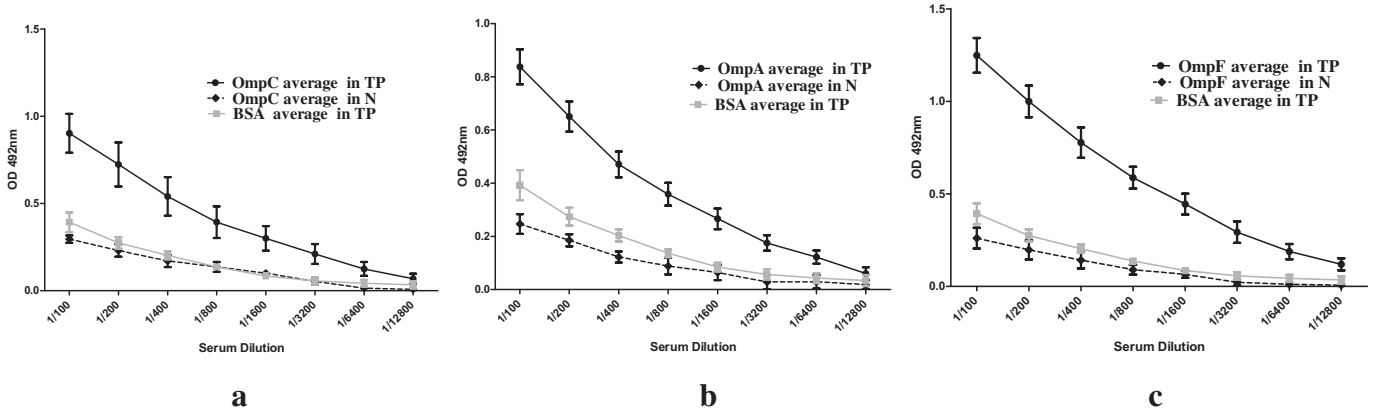

Fig. 2. Comparison of IgG titer against OmpA, OmpC and OmpF in healthy and typhoid patients' sera.

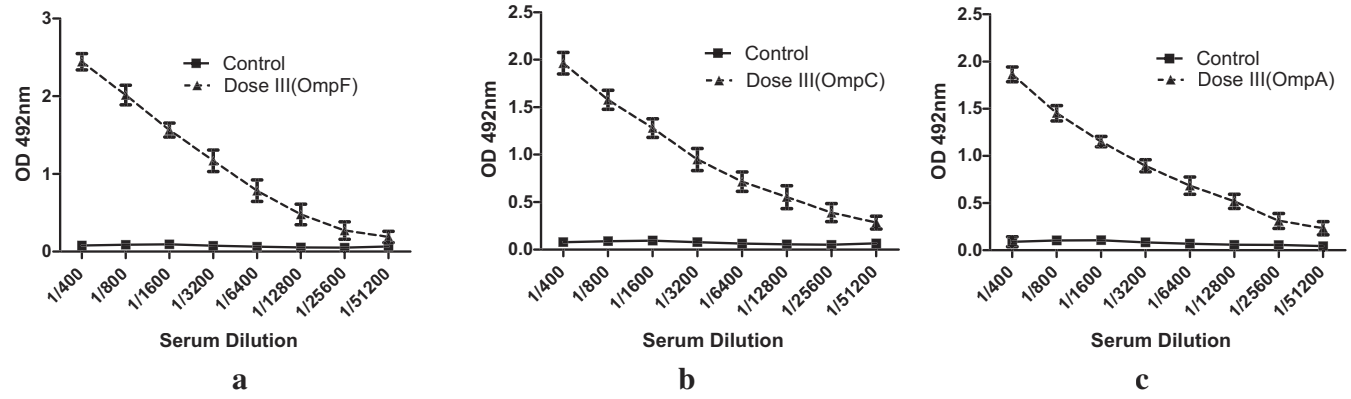

Fig. 3. Comparison of IgG titer in mice sera after third dose of immunization with single protein.

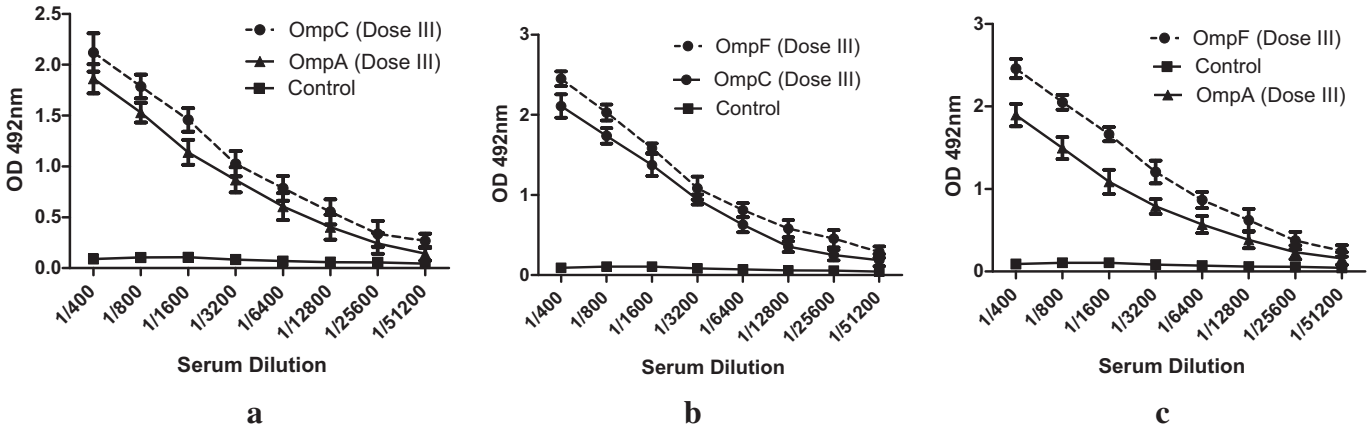

Fig. 4. Comparison of IgG titer in mice sera after third dose of immunization with combination of two proteins.

3.8. Mice challenge

Percent immunized mice survived is summarized in (Fig. 8). Despite induction of a strong immune response, porin proteins conferred no significant protection against *S. enterica* ssp Typhi in BALB/c mice. Control and all mice immunized with a single recombinant porin died within 24 h of challenge. Mice immunized with two OMPs could survive longer than mice immunized with a single recombinant porin. Mice immunized with three recombinant porins survived significantly ($P < 0.01$).

The analysis of variance showed significant differences among the treatments ($P \leq 0.01$). Comparison of mean using Duncan's multiple comparison test indicated that there were no significant differences among main effects (OmpA, OmpC and OmpF) and interaction (OmpA + OmpC, OmpA + OmpF, OmpF + OmpC) but there were high significant differences among control and main effects and interactions ($P \leq 0.01$) (Fig. 8).

4. Discussion

Typhoid fever, a major public health problem in developing countries, is established by a Gram-negative pathogen, *S. enterica* ssp Typhi [3,20]. Porins are part of outer membrane proteins in Gram-negative bacteria stimulating immune system [2,5,9]. Researchers showed that denatured forms of recombinant OmpC and OmpF of *S. enterica* ssp Typhi are immunogenic proteins in murine models [2]. They suggest those two OMPs as suitable vaccine candidates. However, no in vivo challenge study was performed on various types of porins [2]. In spite of high immunogenicity of recombinant OMPs, no evidence is available about protective immunogenicity. Kumar et al. [21] introduced two variable regions in OmpC and OmpF porin proteins useful for a typhoid fever diagnostic test based on antigen–antibody interaction. Our homology analyses revealed no significant differences in protein sequences of the OMPs among various *S. enterica* ssp I serovars. The issue implies that antibodies produced against the recombinant porins could interact with the proteins in other *S. enterica* ssp I

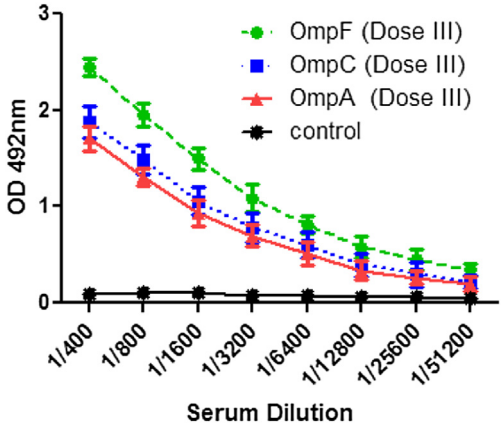

Fig. 5. Comparison of IgG titer in mice sera after third dose of immunization with combination of three proteins.

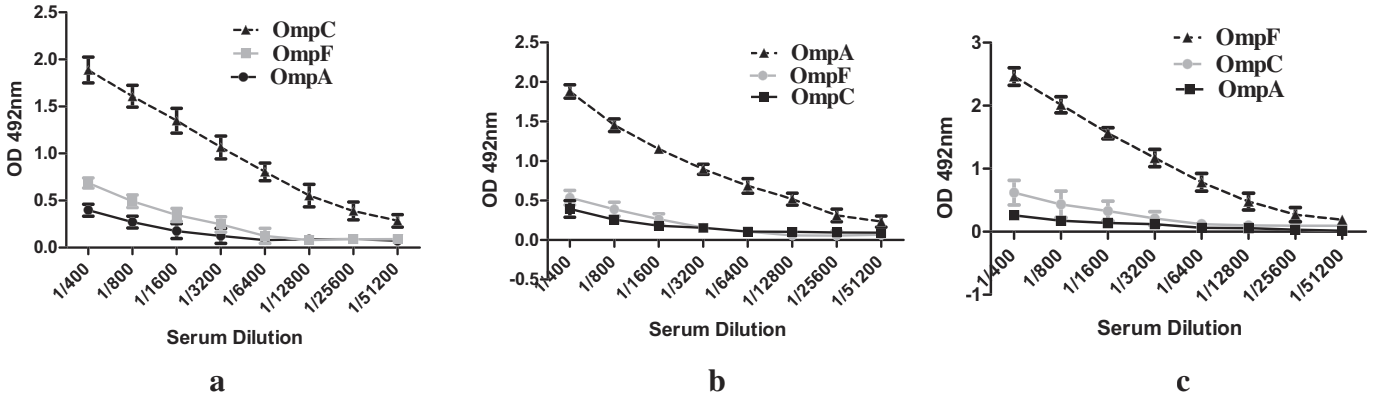

Fig. 6. Degree of cross-reactivity of antibody raised against each Omp.

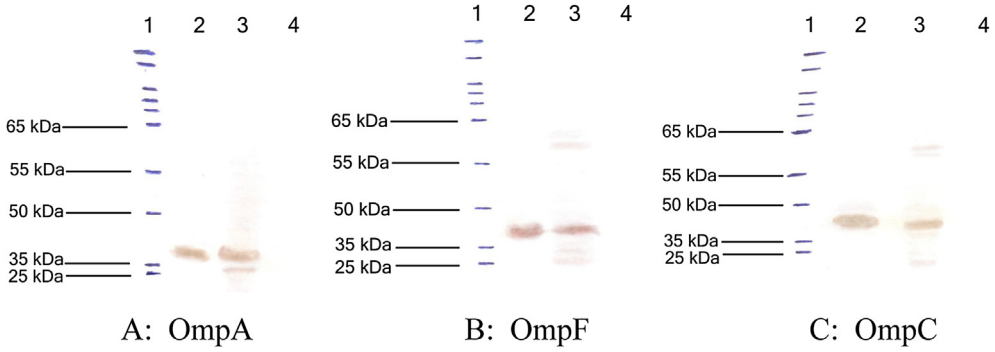

Fig. 7. Western blot analysis of recombinant OmpA, OmpC and OmpF with mouse antiserum.

serovars. Presence of signal peptide in the protein sequence of recombinant OmpC, resulted in toxic effects in *E. coli* expressing the protein [6]. Thus we deleted signal peptides of three investigated porins in the present study. Native antigens possess both linear and conformational B cell epitopes [22]. Because immune system responds to native forms of pathogen antigens during infection, antibodies raised against antigen proteins in typhoid patients may detect continuous as well as discontinuous B cell epitopes. In the current research, purification of proteins was done by a denaturing approach [23,24]. Therefore, the patient serum antibodies raised against linear B cell epitopes could only react with our recombinant proteins. ELISA test revealing antibodies raised against the antigen (P-value < 0.05) confirmed immunity developed against the porins.

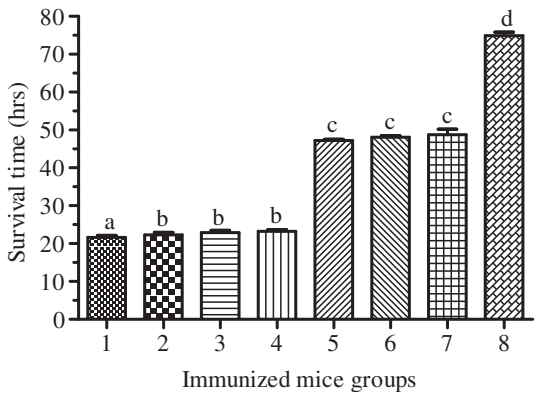

Fig. 8. Survival of mice immunized with *S. typhi* OMPs.

Interestingly, titer of antibodies showed that none of the antigens immunodominant, and none of them has synergic or antagonistic effects on each other in immune system induction. However, protective property must be validated by challenge results. Cross-reactivity seen among the recombinant OMPs at lower levels suggests possibility of presence of specific regions within the sequences which could be appropriate targets for diagnostic purposes. On the other hand, consensus regions of the sequences could be useful in designing vaccines against broad range of Gram-negative pathogens. Combined injection of the three recombinant porins showed no significant rise of antibody titer. On the other hand, the immune system could recognize all of them and raise specific antibodies against each. ELISA test results of the three porins revealed IgG titer against the OMPs in the following order: anti-OmpF > anti-OmpC > anti-OmpA. Several researchers employed bioinformatic tools to design and analyze recombinant antigen proteins [25,26]. Antigenicity of proteins could be theoretically estimated [18,25,27]. Estimated antigenicities of the OMPs were in accordance with experimental observations: OmpF (0.83) > OmpC (0.78) > OmpA (0.69). Results from ELISA test of patients' sera (Fig. 2) confirm the *in silico* analyses of VaxiJen. Since no adjuvant was used for immune system induction in the patients, we could therefore deduce from our findings as well as from the bioinformatic results that unlike a previous report [2] impact of different adjuvants in the discrepancy could not strongly be rationalized. None of the porins alone could elicit protective immunity in mice for a long duration at least in denatured forms of the proteins, however, mice survival period increased upon combined immunization. It has been shown that administration of porin proteins could elicit protection against the pathogen [28]. These evidences suggest that only blockade of all the porins could develop

protection. Type of elicited antibodies plays a pivotal role in protection development [29,30]. Since porins in addition to their specific functions, share common functions, they can compensate for each other's functions. Thus, the produced antibodies could not protect the host from the consequences of infection. It is noteworthy that as mice are not natural hosts for the pathogen, the results could presumably be different in the main hosts i.e. human and chimpanzees.

In conclusion, despite high immunogenicity of the porin antigens, they are not suitable vaccine candidates alone or at least in denatured forms. Combination of the three recombinant proteins could confer significant protection. However, similar investigations could be conducted on native forms of the antigens to study their suitability to serve as a vaccine.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported by Shahed University, Tehran, Iran.

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