J Appl Biotechnol Rep. 2021 Mar;8(1):41-45

doi 10.30491/JABR.2020.234981.1238

Journal of
Applied Biotechnology
Reports

Original Article

Recombinant Flagellin Protein Can Efficiently Protect Mice Against *Salmonella* Typhi

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Received June 12, 2020; **Accepted** August 9, 2020; **Online Published** February 4, 2021

Abstract

Introduction: Globally, *Salmonella enterica* serotype Typhi is responsible for more than 10 million enteric fever cases, annually. Because of the emergence of multidrug resistance strains of many bacteria, including *Salmonella* Typhi, vaccination may be a preferred strategy to combat infectious diseases. In the present study, the efficiency of flagellin protein as a recombinant vaccine candidate was evaluated in BALB/c mice.

Materials and Methods: For this aim, flagellin protein was expressed in *E. coli* BL21 (DE3). Mice were grouped into two groups: test and control. Test group were immunized by the intraperitoneal administration of recombinant protein in combination with Freund's adjuvant. Following the completion of the immunization period, the mice were challenged by IP injection of 10 LD_{50} of live *Salmonella Typhi* and subsequent culture of their spleens and livers.

Results: Flagellin protein expression was confirmed by SDS-PAGE and Western blotting. ELISA showed the proper stimulation of the humoral immunity of the immunized mice. The bacterial count decreased significantly in the spleens and livers of the immunized animals in comparison to the control ones.

Conclusions: Findings of this study show the efficiency of flagellin recombinant protein in protecting mice against *Salmonella Typhi*.

Keywords: *Salmonella* Typhi, Flagellin Recombinant Protein, Vaccine Candidate, Enteric Fever

Citation: Ghorbani N, Assmar M, Amirmozafari N, Issazadeh K. Recombinant flagellin protein can efficiently protect mice against *Salmonella* typhi. J Appl Biotechnol Rep. 2021;8(1):41-45. doi:10.30491/JABR.2020.234981.1238.

Introduction

Enteric fever is caused by *Salmonella enterica* serovars Typhi, which causes typhoid fever, and Paratyphi A, B and C, which cause paratyphoid fever. Globally, these serovars are responsible for 14.3 million cases of enteric fever, annually[1] of which the majority are caused by *Salmonella* Typhi (76.3%). Enteric fever is mostly associated with poor sanitation and hygiene, so that with quality improvement in public health, the morbidity and mortality due to the disease has reduced dramatically.[2] Different population-based studies show an age-specific incidence of the disease with higher incidence in children under 19.[3] The emergence of multidrug resistant strains of enteric pathogens, including *Salmonella*,[4,5] vaccination is considered as a promising way to prevent the infection by the bacteria.[6]

There are some approved commercial vaccines against *Salmonella* Typhi, including two WHO-approved vaccines: Ty21a and Vi capsular polysaccharide (Vi CPS).[7] Ty21a is the only licensed live attenuated oral vaccine against typhoid fever with about 50% efficiency in preventing salmonellosis. Another WHO-licensed anti-typhoid vaccine is Vi CPS that developed in the 1980s with the efficiency of 54%.[8,9] Ty21a and Vi CPS are administered to people above 5 and 2, respectively and none of them are suitable for use in infants. Because of medium protectivity as well as not applicable in infants, there are many attempts in developing new vaccines against the bacteria.

Recombinant subunit vaccines, due to many advantages, mainly safety and easy production, are considered as proper candidate vaccines against different pathogens. In the case of *Salmonella*, the potential of cross-protection is another advantageous. Many surface antigens have been known and introduced which can be applied for the protection against *S.* Typhi.[10-12] Flagellin protein, encoded by *fliC* gene, is an acidic protein with the molecular weight of 47 kDa. The protein is a secreted protein which polymerizes to form the filaments of bacterial flagella. The protein is a mediator of epithelial activation as well as systemic inflammation induced by *Salmonella*. This protein induces nitric oxide synthase and some proinflammatory mediators. It also causes cardiovascular dysfunction.[13] It is present among many bacterial species, including bacteria from Enterobacteriaceae

family. It has been shown that flagellin from *Salmonella* Typhimurium can protect mice against this pathogen; however, the immunogenicity of its homolog, i.e. flagellin from *Salmonella* Typhi has not been evaluated yet. So, in the present study, we investigated the immunogenicity and protectivity of *Salmonella* Typhi's flagellin protein against this pathogen in mice.

Materials and Methods

Ethic Statement

Animal experiments were carried out according to institutional guideline of Animal Care and Use Committee at Islamic Azad University of Lahijan, Iran. The ethical code was 1242 dated back July-20-2018.

Chemicals, Enzymes and Media

Acetic acid and ethanol were prepared from Mojallali Co. (Iran). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and prestained protein ladder were prepared from SinaClon (Iran). PCR reagents and DNA ladders were purchased from GoldBio (China). HRP-conjugated antibodies and anti-his tag antibodies and antibiotics (ampicillin and kanamycin) were prepared from Sigma (Germany). Media cultures, Luria-Bertani broth and agar were purchased from Difco Laboratories (USA).

Bacterial Strains

Expression vector *E. coli* BL21 (DE3) was prepared from Royan Institute (Iran). For challenge, *Bacillus subtilis* PY79 was prepared from Iranian Research Organization for Science and Technology (Iran).

Flagellin Protein Expression and Purification

fliC gene sequence was adopted from GeneBank with the accession number of AY353376. Codon optimization and other bioinformatics analyses, including codon adaptation index (CAI), codon frequency distribution (CFD) and GC% of the sequence have been described elsewhere (detailed data are publishing).

The codon-optimized gene was synthesized and cloned in pET28a (+) between BamHI and XhoI restriction sites by Bioneer Company (South Korea). The recombinant plasmid was transferred into competent *E. coli* BL21 (DE3) cells using the heat shock method.[14] To confirm the transformation of *E. coli* cells, polymerase chain reaction (PCR) was performed using universal T7 promoter primers to amplify *fliC* gene.

To express flagellin protein, the transformed bacteria were grown in LB broth medium. When, the OD of the media culture reached 0.6, isopropyl β- d-1-thiogalactopyranoside (IPTG) with the final concentration of 1mM was added to the media and expression induction was continued for 4 h. The cells were collected and washed with phosphate-buffered saline. Then, 300 µL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris base, 8M urea, pH 8) was added to each 1 mL of initial culture media and the pellet was dissolved by pipetting. Following 1 hour incubation at 37°C, the samples were centrifuged at 13 000 g for 15 minutes. 20 µL of the supernatant was mixed with 5 µL of sample buffer (10 % SDS, 50 % glycerol, 1 % bromophenol blue, 0.5 % 2-mercaptoethanol, 1 mM Tris, pH 6.8) and electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. To visualize the proteins, including recombinant flagellin, the gel was stained in Coomassie brilliant blue staining buffer for 15 min and destained in destaining buffer (10 % acetic acid, 30 % methanol) for 30 min.[15] Western blotting was done to confirm flagellin protein.[16] As the secondary antibody, mouse HRP-conjugated anti-His tag antibody with the final concentration of 1:5000 (Abcam, USA) was used. Recombinant flagellin was purified by using a nickel-nitrilotriacetic acid (NiNTA) column in non-denaturing condition[17] and the purification was evaluated by SDS-PAGE. The concentrations of proteins were determined by Bradford assay and the purified proteins were stored at -20°C until use.

Immunization of Animal Models

Female BALB/c mice (about 8-week-old) were used for immunization schedule. The mice were grouped into 2 groups, each with 10 mice, one test and one control. Mice were immunized subcutaneously (SC) on days 0, 14, 28 and 42 with 15 µg of flagellin admixed with Freund's adjuvant (Freund's complete adjuvant for the first administration and Freund's incomplete adjuvant for the second, third and fourth administrations. Mice in the control group received only adjuvant.

Evaluation of Humoral Immune Response by IgG Assay

Anti-flagellin antibodies were measured in sera of immunized mice as previously described.[15] Briefly, 5 µg of recombinant flagellin was coated in each well of a microtiter plate (Nunc). Wells were washed by washing buffer (PBST) and blocked with 5% skimmed milk. Following the addition of 100 µL/well of mouse sera and incubation at 37°C for 1 hour, HRP-conjugated anti-mouse IgG was added to the wells. Then, substrate [0.1 M citrate/phosphate buffer, pH 5, containing 0.02% H_2O_2 and 0.2 mg/mL σ-phenylenediamine (OPD)] was added to the wells and after 10 minutes, the reaction was stopped by addition of 100 µL of 1 M H_2SO_4 and the and the optical density was determined at 492 nm by an ELISA reader.

Challenge Experiment

The immunogenicity of the formulation was evaluated by IP injection of 10 LD_{50} of live *Salmonella* Typhi to mice 2 and 4 weeks after the last immunization.[18] The mice were killed using cervical dislocation. Under sterile conditions, the spleens and livers of the mice were collected and completely homogenized in 1500 µL Triton X-100. The samples (10 µL of each) were cultured on Trypticase Soy Agar (TSA) media. Following 24 hours incubation at 37°C, the colonies were counted.[19]

Statistical Analysis

For statistical analyses as well as the graphics generation, GraphPad Prism 5 was used. To investigate the statistical significance of the data, *t* test was used. Data are presented as the mean ± standard errors SEM for at least 3 samples per group of mice. In all determinations, a P value < 0.05 was

considered significant.

Results

Production of Recombinant Flagellin Protein

Bioinformatics analyses details have been described elsewhere (Data are publishing). Briefly, all required parameters were in a good situation; CAI was 0.98, CFD was 0 and the sequence's GC content was 50.95%.

Transfer of the recombinant plasmid into the competent *E. coli* BL21 (DE3) was confirmed by colony-PCR (Figure 1). Amplification of the synthetic gene was conducted the universal primers (T7 promoter and terminator). Considering the addition of a 264 bp fragment to the *fliC* sequence, the amplified fragment has a size of 1830 bp.

Flagellin protein expression was evaluated by SDS-PAGE (Figure 2). Two different colonies were examined for the expression of the recombinant protein and as it is seen in the figure both colonies were able to overexpress the flagellin protein. Western blot analysis further verified the recombinant flagellin expression (Figure 3). Following the confirmation of the recombinant protein, it was purified by Ni-NTA column (owing to its His-tag) (Figure 4).[20] Gradual lowering the pH

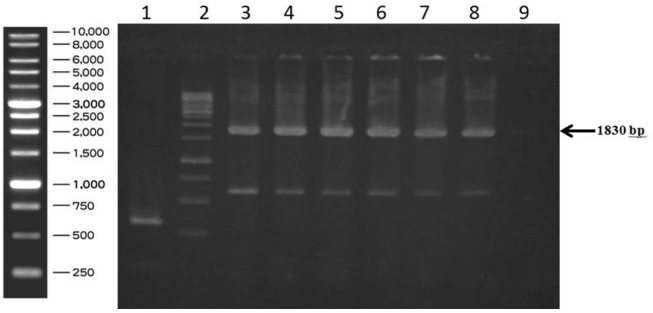

Figure 1. Colony PCR for the Confirmation of the Transformation Process Using T7 Universal Primers; Lane 1) pET28a intact plasmid; Lane 2) 1 kb DNA Ladder; Lane 3-8) six different transformed colonies; Lane 9) Negative control (no DNA as the template).

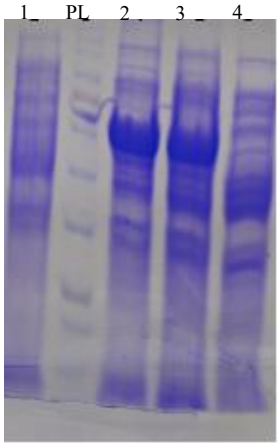

Figure 2. Evaluation of the Expression of the Recombinant Flagellin Protein. Two transformed colonies were induced by 100 mM IPTG for 4 h and the result was analyzed by SDS PAGE. Lane 1) un-induced clone 1; Lane 2) induced clone 1; Lane 3) induced clone 2; Lane 4) un-induced clone 2. PL: Protein ladder.

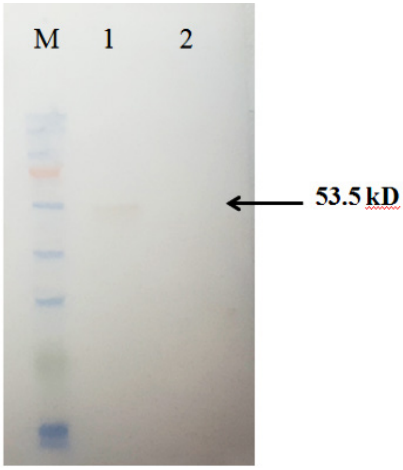

Figure 3. Confirmation of the Recombinant Flagellin by Western Blot Analysis Using HRP-Conjugated Anti-His Tag Antibody. M, protein size marker; Lane 1 and 2) IPTG-induced and un-induced samples.

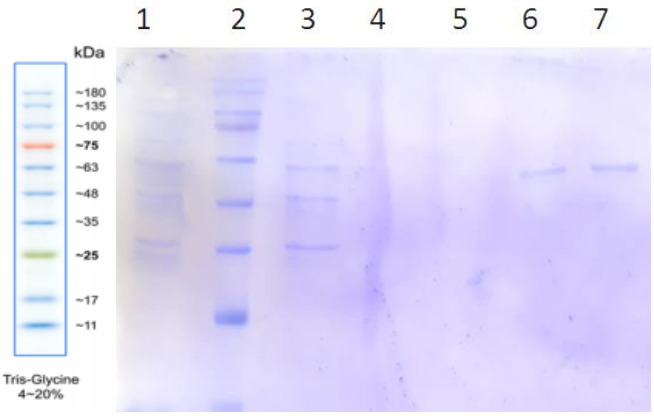

Figure 4. Flagellin Purification Under Non-denaturing Condition. Lane 1) cell lysate; Lane 2) prestained protein ladder; Lane 3) flow-through following the introduction of lysate to the column; Lane 4-7) washing the column with buffer B (pH 8), buffer C (pH 6.3), buffer D (pH 5.8) and buffer E (pH 4.5).

value of the elution buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea) from 8.0 to 4.5 dissociated the protein from the column. At last, protein concentration was determined using the Bradford method.[18]

IgG Antibody Response

The humoral immune response of the vaccinated mice was investigated by measuring serum IgG level of the animals using indirect ELISA method which the result can be seen in Figure 5. As it is shown in the figure, the recombinant flagellin protein elicited the humoral immune response efficiently (P <0.001).

Animal Challenge

Mice were treated with live *Salmonella* Typhi. Two days after the treatment, the mice were anesthetized and their spleens and livers were extracted for further analysis. Following the homogenization, these organs were cultured on trypticase soya agar (TSA) media. The grown bacteria were counted

after 16 h incubation at 38°C. Figure 6 shows the number of bacterial cells: 4.64×10^8 and 2.06×10^8 for control and test groups, respectively. The number of the grown bacteria in two groups were significantly different ($P < 0.0001$).

Discussion

Annually there are more than 12·5–20·6 million cases and 200 000 death due to typhoid fever.[19,21] 3% of the infected patients become chronic carriers and children are more susceptible to this infection.[22] Adequate sanitation and Improvement of water quality has a great impact on the infection control.[23] However, because of the emergence of multidrug resistant strains of the causative agents of infectious diarrheal diseases, including *Salmonella* Typhi, there is a great need for developing efficient vaccines and therapeutics against the disease.[4,24,25] In the present study the efficiency of *Salmonella's* flagellin protein as a vaccine candidate was investigated.

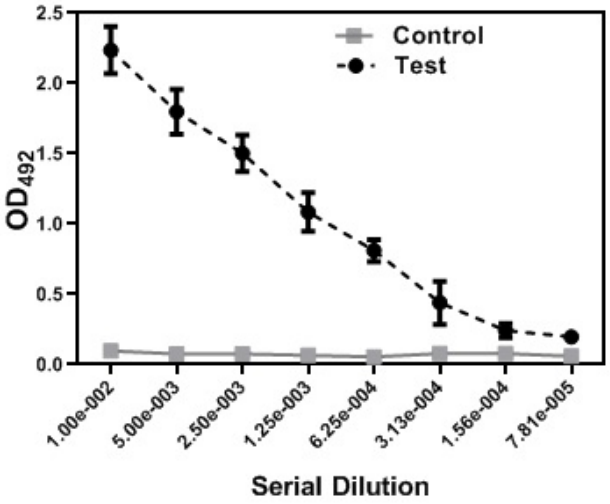

Figure 5. Serum IgG Response Following the Immunization Period. Indirect ELISA was performed to obtain the IgG antibodies titer in which FliC protein was coated in the plate and rabbit anti-mouse IgG conjugated with HRP were used as the secondary antibody for the detection.

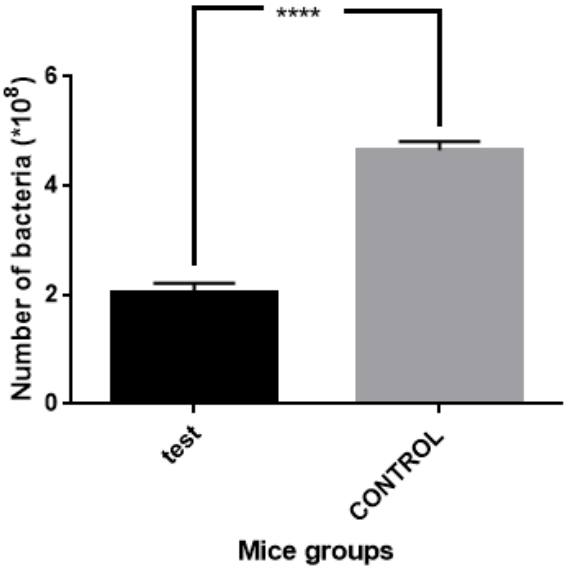

Figure 6. Culturing the Spleens and Livers of Test and Control Mice on TSA Medium. Mice in the test group were immunized with FliC and those in the control group just received PBS.

The flagellin protein was produced recombinantly in *E. coli* expression system. For the efficient expression, it was codon-optimized according to *E. coli* codon preference and some parameters such as CAI, GC% and CFD was set to optimized amount.[26,27] The *flagellin* gene was cloned into pET28a (+), an efficient expression vector. In pET vectors the multiple cloning site is located downstream of the T7 promoter so the cloned gene can be efficiently transcribed in *E. coli* (DE3) cells, which have *T7 RNA polymerase* gene in their genomes.[28] The results of the expression analysis confirmed the appropriate optimization of the sequence.

The results of the challenge showed that the flagellin protein is a potent immunogen which can elicit a good protection against *S* Typhi. The immunogenicity of flagellin from other bacterial species has been already shown. For example, Lee et al showed that a truncated form of campylobacter jejuni's flagellin protein is able to elicit a protective immune response against this bacteria.[29] Roy et al found that flagellin protein from enterotoxigenic *E. coli* (ETEC) is a proper immunogen which can be used in ETEC vaccines.[30] In a study conducted by Lee at al, they showed that a flagellin protein from *Vibrio vulnificus* can induce protective immunity.[31] In *Salmonella* genus, the immunogenicity of flagellin protein has been proven. Strindelius et al investigated the elicited immune response following the oral or nasal immunization of mice with flagellin from *Salmonella* Enteritidis and found that the immunized mice had significantly lower degree of infection by the bacteria in comparison to the control group.[32] Bobat et al showed that the recombinant flagellin from *Salmonella* Typhimurium can induce Th2 response in animal models.[33] Here we showed that this protein from *S*. Typhi is able to confer protective immunity against this bacterium.

Conclusions

Since the efficiency of the available vaccines against *S*. Typhi is relatively low,[34] our finding is of a great importance in developing a potent candidate vaccine against this bacterium. Surely, fusion of this protein with other immunogenic antigens from this bacterium can further promote its immunogenicity.

Authors' Contributions

All authors contributed equally to current study.

Conflict of Interest Disclosures

There is no potential conflict of interests relevant to this paper.

Acknowledgements

This work is a part of N. Ghorbani's PhD thesis. We appreciate all students and staff of the Department of Microbiology, Islamic Azad University of Lahijan for their warm and kind help.

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