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Production of recombinant flagellin to develop ELISA-based detection of *Salmonella* Enteritidis

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ABSTRACT

Food-borne diseases, caused by the pathogenic bacteria, are highly prevalent in the world. *Salmonella* is one of the most important bacterial genera responsible for this. *Salmonella* Enteritidis (SE) is one of the non-typhoid *Salmonellae* that can be transmitted to human from poultry products, water, and contaminated food. In recent years, new and rapid detection methods such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have been developed. In this study, recombinant FliC (rFliC) was produced to be used as an antigen. The immunization was conducted in mice with the purified recombinant FliC (rFliC). The mice were subcutaneously immunized with rFliC and elicited significant rFliC specific serum IgG antibodies. An indirect ELISA system was established for the detection of *Salmonella* Enteritidis. Our results confirmed that the recombinant flagellin can be one of the excellent indicators for the detection of *Salmonella* Enteritidis.

Introduction

Salmonella represents a group of important gram-negative bacterial pathogens that cause intestinal and systemic diseases in human and animal hosts after the ingestion of contaminated water and food such as poultry meat and eggs.[1] Approximately one million cases of *Salmonella* infections are reported every year in the United States.[2] In previous descriptive studies from different places and samples in Iran, the prevalence of *Salmonella* was found to be as 9.2% in 272 stool samples and 8% in 369 stool. In a study, 610 samples were obtained from children under 12 years of age with 37.5% prevalence of gastroenteritis, which is also caused by an important enteric pathogen bacterium.[3–6] More than 2500 serovars have been identified for *Salmonella* Enteritidis, based on antigenic differences in O, H1, and H2 antigens.[7] Among the 30 *Salmonella* serovars that are responsible for 73% cases of salmonellosis in the United States, *Salmonella enterica* subsp. *enterica* serovar Enteritidis or *Salmonella* Enteritidis is an important and dominant bacterial pathogen. It was a prevalent cause of human salmonellosis and causative agents of foodborne illnesses worldwide during the early 1980s to the late 1990s.[8–10] Different methods (e.g., conventional, immunological, and molecular-based methods) have been developed for the detection of *Salmonella*. Although the current

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culture-based methods for the detection of *Salmonella* are sensitive and inexpensive but at the same time they are very time and material-consuming and need initial enrichment. For example, the conventional method for the detection of *Salmonellae*, including *Salmonella* Enteritidis, from eggs takes 5–7 days, is labor-intensive and involves the isolation of the organism using pre-enrichment as well as selective enrichment procedures and serological confirmation tests. This method is useful for the detection of small numbers of *Salmonella* Enteritidis.

Molecular methods (PCR) are good but they also have few limitations. For PCR-based methods, the pathogen needs to be grown and a high concentration of nucleic acid is required to be extracted.[11,12] Bacterial flagellin is one of the outer membrane proteins that serve many functions like mobility, pathogenicity, and adjuvanticity and shows toll-like receptor (TLR)-ligand activity. It is effective at very low doses[13,14] and binds to toll-like-receptor 5 (TLR 5), which is present on the immune-system cells (epithelial cells, dendritic cells, and macrophage). One of the outmost flagellin proteins is FliC which has a molecular weight of 50–60 kDa.[15,16] The binding of FliC with TLR5 leads to a cascade of reactions that results in the production of pro-inflammatory cytokines like TNF-α, IL-6, and IL-12.[17]

In this study, we produced recombinant flagellin (r-FliC) for the detection of *Salmonella* Enteritidis (SE) using enzyme-linked immunosorbent assay (ELISA) and proposing its usefulness in ELISA for the detection of *Salmonella*.

Materials and methods

Kits, enzymes, and reagents

The plasmid extraction and gel purification kits were procured from iNtRON (Seongnam, Korea). Nickel-nitrilotriacetic acid (Ni-NTA) agarose was obtained from Qiagen (Maryland, State, USA). Primers were synthesized by Sinaclon (Tehran, Iran). Restriction endonucleases were obtained from Sinaclon (Tehran, Iran). T4 DNA ligase was supplied by Fermentas (Vilnius, Lithuania). All other reagents were of at least analytical grade and obtained from Sigma–Aldrich or Merck, Germany, including kanamycin (40 μg/mL, Sigma), nitrocellulose membrane (PROTRAN), anti-polyhistidine antibodies and anti-mouse IgG conjugated with horseradish peroxidase (HRP) (RAY Biotech), and an ELISA reader (Bio-Rad, Berkeley, CA, USA).

Bacterial strains and culture conditions

The standard strain of *Salmonella enterica* serovar Enteritidis (SE) (ATCC – 13076, Institute Pasteur of Iran) was used as the source of *flic* gene. It was grown in Luria-Bertani (LB) broth or LB agar at 37 °C. Bacterial genome was extracted by the CTAB-NaCl method, and the DNA concentration was measured by a spectrophotometer (Cecil, UK, OD 260 and 280 nm). The quality of the isolated DNA was assessed by electrophoresis on 1% agarose gel.

Amplification of flic gene

A colony of *Salmonella* Enteritidis was grown in Luria-Bertani broth (LB broth) overnight at 37 °C under constant agitation at 150 rpm. The genomic DNA was extracted from the *Salmonella* strain and *flic* gene was amplified by polymerase chain reaction (PCR) using the following two specific primers (*flic* F: 5-tatagaattcatggcacaagtcattaatac-3 containing an *Eco*RI-engineered restriction site and *flic* R: 5-tatataagcttttaacgcagtaaagagagg-3 containing a *Hind*III-engineered restriction site). The primers were designed to over the complete sequence of *flic* gene (1518 bp) located on the chromosomal DNA of *Salmonella enteritidis* as mentioned in the database available at the National Center for Biotechnology Information (NCBI). For the amplification of the *flic* gene, the polymerase chain reaction (PCR) was standardized using 10 pM of each gene specific primers, 2 μL of 25 mM $MgCl_2$, 10 mM of each dNTPs, 2.5 μL of 10× enzyme buffer and 0.5 U of Taq DNA polymerase (Fermentas) in a-25 μL final reaction volume. The amplification was carried out with the initial denaturation of DNA at 95 °C for 5 min followed by 30 cycles at 95 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, and then a final extension at 72 °C for 5 min. The amplified product was analyzed by the electrophoresis using 1% agarose gel and ethidium bromide as a tracking dye. For the cloning of *flic* gene, the amplification was carried out with pfu DNA polymerase (Fermentas, Lithuania) in a reaction mixture (25 μL) containing DNA in the presence of 4 mM magnesium sulfate and 10 pM of each primer. The cycling conditions were initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 55 °C for 45 s, 72 °C for 60 s and the final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Cloning and expression of flagellin

The amplified PCR product was double-digested with *Eco*RI and *Hind*III, and cloned into the prokaryotic expression vector pET-28a (+) at *Eco*RI/*Hind*III site to form the expression plasmid pET-*fliC* with kanamycin resistance as a selectable marker. *Escherichia coli* DH5α transformants grown overnight on LB plates containing kanamycin (20 μg/mL) were screened. For ligation, the *flic* gene PCR product and vector plasmid were used in the ratio of 2:1. The ligated product was initially propagated in *E. coli* DH5α competent cells.[15] The transformed colonies were screened by colony PCR, restriction enzyme analysis, and sequencing. The recombinant pET-*fliC* plasmid extracted from *E. coli* DH5α cells was purified and transformed into *E. coli* BL21 (DE3) strain for the expression of flagellin. The expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to growing culture of the transformed *E. coli* BL21 (DE3) when OD600 reached 0.6, at 37 °C, under constant shaking at 150 rpm. A zero-time aliquot (uninduced culture) was used as the control. The samples were collected after 24 h and analyzed for the protein expression in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant protein was further confirmed by Western blot analysis.

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