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Recombinant Mip-PilE-FlaA dominant epitopes vaccine candidate against *Legionella pneumophila*

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ABSTRACT

Legionella pneumophila is the main causative agent of Legionnaires' disease, which is a severe multi-system disease with pneumonia as the primary manifestation. We designed a recombinant Mip-PilE-FlaA dominant epitopes vaccine against *Legionella pneumophila* to prevent the disease and evaluated its immunogenicity and protective immunity. The protein structures of Mip, PilE and FlaA were analyzed using a computer, and the gene sequences of the dominant epitopes of the three proteins were selected to construct and optimize the vaccine. The optimized *mip*, *pilE*, *flaA* and recombinant *mip-pilE-flaA* gene sequences were cloned, expressed and purified. The purified proteins were used as dominant epitopes vaccines to immunize BALB/c mice and determine the protective immunity and immunogenicity of these purified proteins. The identification confirmed that the recombinant *mip-pilE-flaA* was successfully cloned and expressed. ELISA revealed that the Mip-PilE-FlaA group produced the highest IgG response, and this protein may considerably improve the production of some cytokines in BALB/c mice. Histopathology analyses of lungs from mice immunized with Mip-PilE-FlaA revealed a certain protective effect. Our work demonstrated that the recombinant dominant epitopes of Mip-PilE-FlaA exhibited strong immunogenicity and immune protection, and this protein may be an efficient epitopes vaccine candidate against *Legionella pneumophila*.

1. Introduction

Legionella pneumophila (*L. pneumophila*) is the etiological agent of Legionnaires' disease, and it widely exists in natural and man-made water environments and soil. The bacteria spread in the form of aerosols in the air and invade human alveolar macrophages and lung epithelial cells in the respiratory tract. In alveolar macrophages, the pathogen can effectively avoid endosomal and lysosomal bactericidal activities, and its replication eventually leads to the death of the host cell [1]. *L. pneumophila* causes approximately 90% of Legionnaires' disease, and *Legionella pneumophila* serogroup 1 (Lp1) is responsible for over 84% of clinical cases worldwide [2] and approximately 70% of European travel-associated cases [3]. The disease, characterized by pneumonia with systemic toxemia, is a sporadic and endemic disease worldwide. But there is no ideal preventive measure.

The known virulence factors of *L. pneumophila* include FlaA, IP, LPS, Mip, MompS and PilE, which play important roles in promoting the adhesion, invasion and colonization of *L. pneumophila* of the host cell, and advancing the pathogen's growth and reproduction in host cells [1,4]. Among the virulence factors, macrophage infectivity potentiator (Mip), type IV pilin (PilE) and flagellin (FlaA) exhibited the corresponding immunological function in many studies of *L. pneumophila* [5,6]. Therefore, we selected these three virulence factors of Lp1 as candidate proteins to construct a newly recombinant vaccine.

The Mip protein is expressed in the surface of *L. pneumophila,* and it belongs to the enzyme family of peptidyl-prolyl-cis/trans-isomerases (PPIases), which are FK506-binding proteins (FKBP) [7]. PPIases are involved in numerous biological processes, such as gene expression, signal transduction, protein secretion and tissue regeneration [8]. In *L. pneumophila*, Mip plays an important role in the resistance of intracellular killing from the host cell by affecting the aerobic oxidation and membrane depolarization of pulmonary

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macrophage cells [6], and it contributes to the bacterial dissemination within the lung tissue and the spread of Legionella to the spleen [7]. The *pilE* gene encodes the type IV pilin protein of *L. pneumophila.* This protein is not required for bacterial intracellular growth, but it may facilitate the attachment of the bacteria to host cells [4]. The expression of type IV pilus is also associated with competence for DNA transformation in *L. pneumophila* [9]. Another candidate protein, FlaA, is the major subunit of the monopolar flagellum of *L. pneumophila*. The flagellum positively affects infection via the promotion of the bacterial encounter with the host cell and enhancement of the invasion capacity of the bacteria [10]. Furthermore, FlaA exhibits strong immunogenicity, and it stimulates T-cell-mediated immune responses to induce significant protection against a lethal *L. pneumophila* challenge in A/J mice model [11].

Mip, PilE and FlaA are important attachment factors and protective immunogens of *L. pneumophila.* The protective immunity and immunogenicity of vaccines with multi-antigens are better than single antigen vaccines [12,13]. Therefore, our study designed the recombinant protein Mip-PilE-FlaA as a vaccine against Lp1 and selected dominant epitopes of the three proteins to remove unrelated peptide segments. The fusion expression vectors of pET-*mip*, pET-*pilE*, pET-*flaA* and pET-*mip-pilE-flaA* were constructed, and the proteins were obtained to immunize animals and examine the immunogenicity and protective immunity of these proteins after expression and purification. Our data demonstrated that the recombinant Mip-PilE-FlaA dominant epitopes vaccine exhibited good immunogenicity and protective effects in BALB/c mice. Therefore, this protein may be an efficient epitopes vaccine candidate against Lp1.

2. Materials and methods

2.1. Bacterial strains and media

Legionella pneumophila serogroup 1 (American Type Culture Collection, USA; no. 35133) were grown on BCYE-α medium in a candle urn at 37 °C for 4–5 days and harvested using PBS (pH 7.2). The bacteria were washed via centrifugation in PBS and resuspended to appropriate concentrations at 4 °C. The *E. coli* strains DH5α and BL21 (DE3) (Tiangen, Beijing, China) were used for gene cloning and protein expression, respectively. These strains were grown at 37 °C in Luria-Bertani (LB) medium containing kanamycin at a final concentration of 50 μg/ml, when required.

2.2. Dominant epitopes selection

We predicted the secondary structure and surface properties of Mip, PilE and FlaA (GenBank: AE017354.1) proteins of Lp1 using DNAStar software and analyzed the extracellular domains of the proteins using Expasy protein analysis system. The dominant epitopes sequences were selected from the secondary structure, hydrophilicity plot, flexible regions, antigenic index, surface probability plot and extracellular domains of the three proteins. The CTL epitopes in the three selected sequences were calculated by NetCTL 1.2 server, which included predictions of MHC/peptide binding for 12 MHC class I supertypes. Then, the sequences of the three optimized proteins were linked by two flexible peptide fragments to maintain a normal folding and structure of the newly recombinant protein Mip-PilE-FlaA. The secondary structure and surface properties of Mip-PilE-FlaA were predicted to assess the vaccine, and a 3D model and other information were estimated to ensure the correct function using PHYRE2 Protein Fold Recognition Server.

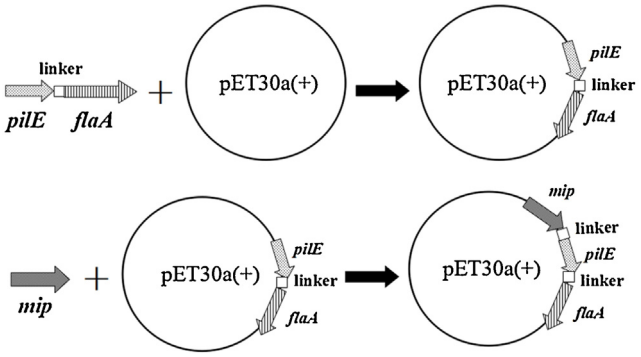

Fig. 1. The construction process of the recombinant plasmid pET-*mip-pilE-flaA*. The optimized *mip, pilE* and *flaA* genes were amplified using PCR. We linked the optimized *pilE* and *flaA* genes and inserted the ligation product into pET30a(+) to obtain the recombinant plasmid pET-*pilE-flaA*. Then, the optimized *mip* gene was cloned into pET-*pilE-flaA* to successfully construct the recombinant plasmid pET-*mip-pilE-flaA*.

2.3. Plasmid construction, expression, and purification of Mip-PilE-FlaA

The prokaryotic expression vector pET30a(+) (BioDee, Beijing, China) was used for the cloning of genes. The fusion expression plasmids pET-*mip*, pET-*pilE*, pET-*flaA* and pET-*mip-pilE-flaA* were constructed by PCR amplification and T4 DNA ligase. Fig. 1 shows the construction process of pET-*mip-pilE-flaA*, and Table 1 lists the primers of single and triple genes with two linkers designed into the triple gene primers. The TIANamp Bacteria DNA Kit and Universal DNA Purification Kit (Tiangen, Beijing, China) were used for genome DNA extraction and DNA fragment isolation, respectively. The E.Z.N.A. TM Plasmid Mini Kit (Omega) was used for plasmid purification. T4 Ligase and restriction enzymes used in this study were purchased from Thermo.

The plasmids were transformed into competent *E. coli* DH5α cells, and positive recombinant plasmids were obtained after kanamycin screening, white-blue plaque, colony and plasmid PCR, enzyme digestion, DNA sequencing and nucleotide BLAST. Positive recombinant plasmids were transformed into competent *E. coli* BL21 cells and induced by isopropyl-D-thiogalactopyranoside (IPTG) to express His-tagged fusion proteins. Fusion proteins were purified by HisTrap affinity columns (GE Healthcare) and dialysis to maintain specificity. Purified proteins were analyzed using SDS-PAGE and Western blot. A rabbit polyclonal anti-*L. pneumophila* antiserum (1:7500), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Sigma Chemical, USA) (1:4000), and ECL staining were used for protein identification and visualization. Finally, the concentrations of successfully purified proteins were measured by BCA method, and the endotoxin contaminants in the protein preparations were tested by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Genscript, China).

2.4. Immunization of mice and antibody detection

Specific-pathogen-free female BALB/c mice (n = 100), aged 6–8 weeks, were purchased from the Laboratory Animal Center of Sichuan University, Chengdu, China. All mice were randomly divided into 5 groups including non-vaccinated, Mip, PilE, FlaA and Mip-PilE-FlaA groups, with 20 mice in each group in a room with an automatically controlled temperature of 22 °C and 12 h of light. These groups were respectively immunized with purified Mip, PilE, FlaA and Mip-PilE-FlaA proteins (10 μg protein in 0.2 ml PBS/mouse) three times, each interval of a week, and PBS was used as the non-vaccinated control. Purified proteins were mixed with isochoric Freund's complete adjuvant and injected into