Analysis of Immunological Activity of Recombinant Outer Membrane Protein of *Fusobacterium Necrophorum*

Hang Jia¹, Shengxin Wang¹, Xiaoli Zhang¹, Yang Guo¹ and Jing Xu²,*

¹Medical Laboratory Technology, College of Medical Technology, Qiqihar Medical University, Qiqihar 161006, China
²Department of Biochemistry and Molecular Biology, Qiqihar Medical University, Qiqihar 161006, China

*Corresponding author

Abstract. To investigate the prokaryotic expression of 93 ku outer membrane protein (OMP) obtained from *Fusobacterium necrophorum* and the response to immunization and cytotoxicity in mice. The recombinant plasmid pET-28a-OMP was transformed into *E. coli* BL21 (DE3) competent form, and a recombinant outer membrane protein containing 2 His tags (label size 3.8 ku) was expressed. The mice were immunized as immunization antigens by Freund's incomplete adjuvant. SDS-PAGE and Western blot analysis showed that the recombinant protein had a molecular weight of about 93 ku. After the immunization, the serum antibody titer of the experimental group was higher, at the same time, in the challenge experiment, the liver has certain toxicity, and the protection rate is 83.3%. The recombinant 93 ku outer membrane protein of *Fusobacterium necrophorum* has a good immune response level and provides basic data for the study of the subunit vaccine of *Fusobacterium necrophorum*.

Keywords: *Fusobacterium necrophorum*; Outer membrane proteins; Immunoprotective.

1. Introduction

*Fusobacterium necrophorum* (FN) is a zoonotic pathogen, which is generally chronic. Infected animals often experience weight loss, miscarriage and death. The bacteria are also main pathogens of inflammation liver hepatic abscess, rot disease, and human acute and chronic throat etc [1-4]. FN is a Gram-negative anaerobic bacterium, no spores, no capsules, no flagella, often found in the intestines, mouth of animals and humans. Usually FN is divided into 2 sub-species, namely Fnn and Fnf. Fnn mainly infects livestock and has low virulence to humans, while Fnf mainly infects humans, including A, B and AB genotypes. The bacterium is the main pathogen of ruminants, such as cattle, sheep, deer, etc. Infected animals can cause cross-contagion if not treated in time, seriously affecting the development of animal husbandry, and even cherish the breeding of animals, etc. If the infection continues to be frequent, it will cause great economic losses. This infection of zoonotic bacteria is also worthy of the attention of many researchers. At present, vaccination is the most direct and effective method to prevent FN infection. Outer membrane proteins (OMP) are unique structures in the cell wall of Gram-negative bacteria, which maintain the structure of bacteria, transport of substances and transport agents and antibacterial agents. Regulation and other plays a vital role, and it is also one of the main virulence factors of bacteria, and has good immunological activity. It is an important potential target for the development of vaccines [5-9]. Based on the prokaryotic expression of *Fusobacterium necrophorum* and the primary extraction of OMP, this study determined that the recombinant protein 93ku OMP was expressed in 44.5 ku OMP...
The immunogenicity study of recombinant 93 ku OMP provides basic data for the screening of new FN subunit vaccines.

2. Materials and Methods

2.1. Primary Reagent
Restriction enzymes Xho I, EcoR I (BioLabs); ProteinMolecular Weight Standard-high (TaKaRa); Coomassie Brilliant Blue R-250 (Sigma); Protein Pre-stained Marker (Fermentas); HEPEs (Beijing Huamei Bioengineering Co., Ltd); HRP-labeled goat anti-mouse IgG, DAB chromogenic kit (Beijing Zhongshan Jinqiao Biotechnology Company)

2.2. Strains and Vectors
Fusobacterium necrophorum (FN) AB strain, a virulence strain isolated, identified and preserved by the Institute of Special Products of the Chinese Academy of Agricultural Sciences; E. coli TOP10 competent strain, deposited by the Institute of Special Products of the Chinese Academy of Agricultural Sciences; E.coli BL21 (DE3), pET-28a was purchased from Novagen; the recombinant plasmid pET28a-OMP was constructed by our laboratory.

2.3. Experimental Animal
Kunming BALB/c mice, clean grade 8 weeks mice (25g) were purchased from the Experimental Animal Center of Jilin University and passed the test of Jilin Provincial Laboratory Animal Quality Testing Center.

2.4. Recombinant Outer Membrane Protein Expression
Construction of recombinant plasmid pET28a-OMP, expression of the outer membrane protein of Fusobacterium necrophorum 93ku[11].

2.5. MTS Test
The mouse macrophage (AnA-1) was adjusted to a cell concentration of 10^4-10^5 cells/mL with a culture medium, and inoculated into a 96-well cell culture plate, 100 μL of the above-mentioned concentration cells were added to each well, and a control empty (medium, MTS, PBS, cells), zero-well (medium, MTS), about 20 h, discard the culture medium in the well, and wash the surface of the cells once with PBS. Adjust the expressed OMP concentration to 100 μg/mL, filter with 0.45 μm, preset 6 gradients (2-fold gradient dilution), 3 replicate wells per gradient, and add it to the well-packed 96-well plate. After 16 h of incubation, 120 μL of MTS dilution (100 μL + 20 μL MTS) per well was added to a 96-well plate in the dark, and cultured in a cell culture incubator for about 3.5 h. The light absorption value of each well was measured by a microplate reader under OD490 absorption light.

2.6. Preparation of Immune Serum
Sixty Kunming mice were randomly divided into 4 groups, 15 in each group. Group 1 blank control, multiple HEPEs (20 mM), 0.2 mL/n in the back and abdominal cavity; 2nd group negative control, 0.2 mL/F incomplete Freund's adjuvant (IFA); Group 3 experiment Group, back and abdominal multi-point injection of recombinant OMP 100 μg/protein (protein antigen 0.1 mL, IFA 0.1 mL) emulsified with Freund's complete adjuvant, group 4 positive control, multi-point inoculation of super-breaking bacteria in the back and abdominal cavity Body pellet (20 mL culture, 2 mL 20 mM HEPEs resuspended), 0.2 mL/only. Each group was immunized once every other week for a total of three immunizations. In the case of booster immunization, the antigen was emulsified with Freund's incomplete adjuvant. After 10 days of the last immunization, fasting for 10 hours or so, blood collection of 3/group, 4,000 r/min centrifugation. Serum was prepared at 10 min and stored at -80 °C until use.
2.7. Western-blotting Test
SDS-PAGE electrophoresis, antigen is recombinant OMP, primary antibody is mouse anti-OMP (FN strain) serum (1:200), secondary antibody is HRP-labeled goat anti-mouse IgG (1:3000), DAB chromogenic kit color.

2.8. Protection Rate Detection
The remaining mice after the above three exemptions were tested for protection rate, and the back and abdominal cavity were inoculated with the super-degraded Bacillus nebula bacterial cell pellet (100 mL culture solution, 10 mL 20 mM HEPEs resuspended), 0.2 mL only, note Observe the mice's eating, metabolism, coat color, limbs, weight, activity, death and so on.

3. Results and Analysis

3.1. SDS-PAGE Verification
SDS-PAGE identified that the recombinant OMP of Fusarium oxysporum had a molecular weight of 93 ku and was stained with Coomassie Brilliant Blue R-250. A clearly visible band appeared at approximately 93 ku, as shown in Figure 1.

3.2. Western-blotting Identification
Western blotting showed that a band appeared at 93 ku, indicating that the expressed protein has good specificity, as shown in Figure 2.

3.3. MTS Test
Recombinant OMP 100 μg/mL, preset 6 gradients (2-fold gradient dilution: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64) to the small paved MTS dilutions were added to the mouse macrophage (AnA-1) plate in the
dark. The OD490 light absorption value of each well was measured by a microplate reader, and the results showed that the absorbance values were lower at 1/2 and 1/4 of the dilution factor, as shown in Figure 3.

![Figure 3. MTS reduction test of the AnA-1 cell](image)

3.4. Immune Serum Antibody Titer Test
The serum antibody titer was detected. The serum of the mice immunized with recombinant OMP (3 in each group) was determined by ELISA. The results showed that the serum titer of the experimental group was 1:320000. The control group and the blank group were not detected. Antibody production indicates that the method yields high serum.

3.5. Mouse Protection Rate Test
After the mice were exempted, the remaining 12 groups in the first group of blank group, 3 had blood collection test; the remaining 2 groups in the second group of negative control group, 3 had blood test; the remaining 10 groups in the third group, 3 had blood test 2 had died; the remaining 4 in the 4th positive control group, 3 had blood test and 5 had died. The super-degraded Bacillus necrotic bacteria (100 mL culture medium, 10 mL 20 mM HEPEs were resuspended), 0.2 mL/only, after multiple inoculation in the back and abdominal cavity, after two weeks, the results showed the first and second. All the mice in the group died, and the remaining mice in the third and fourth groups were 7 (3 deaths) and 5 (2 deaths), and the protection rates were 70% and 71.4%, respectively (protection rate = number of surviving mice / The total number of test mice × 100%), as shown in Table 1.

| Group                  | Group 1 blank group | Group 2 negative control | Group 3 experimental group | Group 4 positive control |
|------------------------|---------------------|--------------------------|---------------------------|-------------------------|
| Total                  | 15                  | 15                       | 15                        | 15                      |
| Remaining              | 12                  | 12                       | 10                        | 7                       |
| Protection             | 12                  | 12                       | 3                         | 2                       |
| Remaining              | 0                   | 0                        | 7                         | 5                       |
| Protection rate        | 0%                  | 0%                       | 70%                       | 71.4%                   |

4. Discussion
In recent years, necrobacillus is a zoonotic disease that is widely concerned by scholars at home and abroad. It is widely distributed, causing many types of diseases, the treatment effect is not obvious, and it is easy to relapse. Therefore, prevention and control are very difficult. The immunological activity and immunoprotection of outer membrane proteins have been studied in many bacteria and been the target of subunit vaccine research. The outer membrane protein is the main structure of the bacterial cell wall, which directly contacts the external environment and plays an important role in the
invasion, adhesion, and prevention of host serum defense and killing [16-17]. The study found that many human diseases are related to the outer membrane proteins of bacteria, so the outer membrane protein is concerned by many scholars, such as Oprl (a major OMP of Pseudomonas aeruginosa), which can induce small mice after immunization with recombinant protein against Pseudomonas aeruginosa [18]. The pathogenic role of pathogenic bacteria is achieved by immune evasion to adapt to the host's defense mechanism. When the pathogen is adsorbed to the susceptible part of the organism, it is easy to cause disease; the pathogen that adsorbs to the epithelial cells of the respiratory tract plays a key role, avoiding the mechanical removal of the host, thus achieving the first step of the invasion [19-22].

In this study, the virulence factor outer membrane protein of Fusobacterium necrophorum was selected as the research object. The prokaryotic expression of Fusobacterium necrophorum recombinant 93 ku OMP was emulsified by incomplete Freund's adjuvant, and then it was used as an antigen to immunize Kunming mice to detect serum antibody effect. The price is higher, and it has better protection in the experiment. The Western blotting shows that it has good reaction specificity, and it is the same protein as the crude protein of 44.5 ku. OMP lays the foundation for the development of FN subunit vaccine.

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