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Cloning and expression of *Mycobacterium bovis* secreted protein MPB51 in *Escherichia coli*

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Abstract The purpose of this study is to clone, identify, and express the mature secreted protein MPB51 from *Mycobacterium bovis* and to lay a good foundation for the diagnosis of *M. bovis*, for applying *M. bovis* vaccine into clinical practice, and for detection of immunity effectiveness. The gene encoding MPB51 was amplified from *M. bovis* Vallee111 chromosomal DNA by using PCR technique, PCR product was approximately 800 bp DNA segment. Clone vector pGEM-T-51 was successfully constructed by the PCR product that was cloned into pGEM-T vector by using T-A clone technique. pGEM-T-51 and pET28a(+) were digested by *Bam*H I and *Eco*R I double enzymes. The prokaryotic expression vector pET28a-51 was constructed by using the purified MPB51 gene that was subcloned into the expression vector pET28a(+). Plasmid containing pET28a-51 was transformed into competence *E. coli* BL21 (DE3). The bacterium was induced by IPTG and its lysates were loaded directly onto SDS-PAGE. An approximately 30 kDa exogenous protein was observed on the SDS-PAGE. The protein was analyzed by using Western-blotting and it had the antigenic activity of *M. bovis*. These results could serve as a basis for further studies on the usefulness of the gene and its expression product in the development of subunit vaccine and DNA vaccine against bovine tuberculosis.

Keywords *Mycobacterium bovis*, MPB51 gene, cloning, prokaryotic expression

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1 Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis in a range of animal species and human beings. More than 10% of human tuberculosis is caused by *M. bovis*. World Health Organization (WHO) indicates that if a country is contaminated by bovine tuberculosis, the residents there will always be menaced by it. If effective actions are not taken to eliminate it, the control of human tuberculosis will be unsuccessful. Up to now, many developed countries and regions, such as USA, Australia, North European countries, etc., have eliminated bovine tuberculosis to some extent, but the prevalence of tuberculosis in human beings and wildlife makes these countries detect it all the time. In many developing countries, bovine tuberculosis still prevails severely. The positive ratio of bovine tuberculosis is 10% only in China. The prevention of bovine tuberculosis mainly adopts allergic detection by using purified protein derivative (PPD). The positive cattle will be isolated or killed. However, by using this method, this disease is not successfully controlled, and on the contrary, the incidence is still increasing (Tollefsen et al., 2003). Nowadays, the prevention of human tuberculosis often adopts inoculation of Bacille Calmette-Guerin (BCG). But among different people in different regions, the inoculation of BCG shows remarkable differences (Colditz et al., 1994), especially among adults. As for cattle and other animals, after inoculation of BCG, the allergic detection will always show the positive results, so artificial immunization and natural infection are difficult to differentiate. Furthermore, it will have a great influence on the bovine quarantine and international trade, so a kind of new vaccine must be developed to prevent bovine tuberculosis. Some studies have indicated that culture fluids of *M. bovis* contain actively secreted proteins and these proteins can be identified by CD₄⁺ and CD₈⁺ T cells, which can protect the experimental animals (Andersen et al., 1992; Andersen et al., 1994). MPB51 is one of the secreted proteins found in culture fluids (Rinke et al., 1993; Ohara et al., 1995). MPB51 is the main protective antigen of *Mycobacterium tuberculosis*. In this study, the mature protein gene MPB51 was cloned and its prokaryotic expression plasmid was constructed and was expressed in *E. coli*, so

a solid foundation can be laid for the newly developed vaccines.

2 Materials and methods

2.1 Materials

2.1.1 Strains

M. bovis Vallee111 was purchased from China Institute of Veterinary Drug Control (IVDC); *E. coli* JM109 was conserved in our laboratory; *E. coli* BL21 (DE3) was presented by Yang Lianyu.

2.1.2 Vectors

pGEM-T vector system was purchased from Promega Co. (U.S.A.); pET28a(+) expression vector was presented by Zhang Yanyu.

2.1.3 Chemicals

Proteinase K was purchased from Merck Co. (Germany); EX *Taq* DNA Polymerase, *Bam*H I, *Eco*R I, and Nucleic acid weight marker were purchased from TaKaRa Biotechnology Co. (Japan); Agarose was purchased from Spanish Co. (Spain); Lysase, dNTPs and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) were supplied by Bebcos Co. (U.S.A.); Ampicillin (Amp), Kanamycin (Kan), IPTG and RNase A, DTT were purchased from Sigma Co. (U.S.A.); T4 DNA ligase was supplied by Promega Co.; DNA Gel Extraction Kit was purchased from V-gene Biotechnology Limited in Shanghai; Yeast Extract and Tryptone were purchased from Oxoid Co.; Protein marker of low weight was supplied by Biochemistry Institute in Shanghai; Polyvinylidene fluoride (PVDF) transfer membrane was purchased from Glemann Co.; Polyclonal antibody of bovine tuberculosis and horse radish peroxidase (HRP) conjugated rabbit anti-bovis IgG were developed and conserved in our laboratory.

2.2 PCR amplification and cloning of MPB51

Based on the MPB51 gene sequence of *M. bovis* BCG Tokyo strain in Gen Bank (D26486), a pair of PCR primers were designed for amplifying the mature protein gene, whose gene sequences were Primer-1: 5'-CTAGGATCCACCATGGC CCCATACGAGAA-3' and Primer-2: 5'-ATG GAATTC-GCATCGGCACCTGGCTTAG-3' (The lineats are *Bam*H I and *Eco*R I enzyme digestion sites, respectively). Chromosomal DNA of *M. bovis* Vallee111 was extracted as described by Cai et al. (1999). The mature protein gene MPB51 was amplified by using the template of *M. bovis* chromosomal DNA and specific primers (Primer-1 and Primer-2) and the reaction condition of PCR was as follows: The total volume

is 50 μ L [10 \times Ex *Taq* Buffer 5 μ L; P₁, P₂ (20 pmol \cdot μ L⁻¹) 1 μ L respectively; dNTPs (each 2.5 mmol \cdot μ L⁻¹) 4 μ L; template DNA 2 μ L; deionized water 36.5 μ L and Ex *Taq* DNA Polymerase 0.5 μ L (5 U \cdot μ L⁻¹)]. The reaction was at 98°C for 8 min; 95°C for 1 min; 55°C for 1 min; 72°C for 1.5 min, in total 32 circles, and finally it was extended for 10 min at 72°C. After purification, the PCR products were linked with pGEM-T vector system by T4 DNA ligase. The linked products were transformed into competence *E. coli* JM109. Through α -complementation, the weight of plasmid, enzyme digestion and PCR amplification, the recombinant plasmid was identified, so the positive recombinant plasmid pGEM-T-51 was successfully constructed and its sequence was analyzed by Dalian TaKaRa Co.

2.3 Construction of prokaryotic expression recombinant plasmid and expression of MPB51 gene

The recombinant plasmid pGEM-T-51 and vector pET28a(+) were digested by *Bam*H I and *Eco*R I double enzymes. After being purified and ligated by T4 DNA ligase, the products were transformed into competence *E. coli* BL21(DE3), and then the positive recombinant plasmid pET28a-51 was acquired after filtration. The prokaryotic expression recombinant was inoculated in 5 mL LB liquid medium (containing 50 μ g \cdot μ L⁻¹ Kan sulfate) and was cultured overnight at 37°C in 200 r/min, then a 2 mL culture was inoculated into 100 mL LB liquid medium and cultured up to OD_{600} of 0.6–0.8 at 37°C in 250 r/min. The bacteria were induced by 1 mmol/L IPTG and taken out every 1 h up to 10 h. Similarly, the *E. coli* BL21 (DE3) containing pET28a(+) was induced up to 10 h as a control.

2.4 SDS-PAGE analysis

The OD_{600} values of bacteria cultures acquired every hour were adjusted to the same value 0.68. 1.5 mL bacteria cultures were centrifuged at 4°C then the bacteria were harvested and lysed by 100 μ L deionized water and 100 μ L 2 \times SDS loading buffer, and then they were mixed and boiled for 5 min in boiling water, centrifuged at 15 000 r/min for 10 min. The 15 μ L lysates were loaded directly onto 120 g/L SDS-PAGE according to the procedure described in the preface (Sambrook et al., 1992).

2.5 Western-blot analysis

After SDS-PAGE of expression products, they were transferred onto PVDF membrane by BIO-RAD system following the procedure described in the preface (Sambrook et al., 1992). The membrane was blocked by bovine serum albumin (BSA), then dipped into bovine tuberculosis polyclonal antibody, HRP conjugated rabbit anti-bovine immunoglobulin, at last diaminobenzidine in 10 mmol/L Tris-HCl buffer (pH 7.6) as substrate.

3 Results and analysis

3.1 Cloning and sequential analysis of MPB51 gene

The gene encoding mature protein MPB51 from *M. bovis* Vallee111 chromosomal DNA was amplified by using PCR technique. PCR products were detected by 10 g/L agarose electrophoresis, afterwards, a DNA segment about 800 bp was seen obviously, which was the same as expected.

The purified PCR product of MPB51 was linked to pGEM-T vector, then the recombinant plasmid pGEM-T-51 was successfully constructed, through α -complementation, enzyme digestion analysis, PCR amplification and sequential analysis, and the positive recombinant was selected. The recombinant pGEM-T-51 was digested by *Bam*H I and *Eco*R I double enzymes. Through digestion analysis, two segments were acquired, which were pGEM-T linear fragment about 3 000 bp and the inserted fragment about 800 bp. The sequential analysis indicated that the inserted DNA fragment was 801 bp, the homogeneity with *M. bovis* BCG Tokyo strain MPB51 mature protein gene reached 99.8%, but to the nucleotide sequence, the 151st base and 591st base were mutated from A and C in BCG Tokyo strain to G and T in Vallee111. As to the amino acid sequence, the difference only lay in 51st amino acid, and in BCG Tokyo strain, it was M but in Vallee111, it was mutated to V.

3.2 The construction of prokaryotic expression plasmid and the identification of recombinant

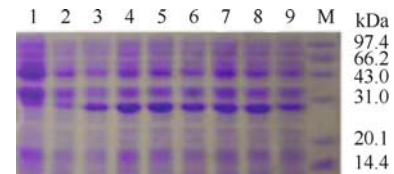
The pGEM-T-51 and pET28a(+) were digested with *Bam*H I and *Eco*R I double enzymes and purified. Through the linking of T4 DNA ligase, the recombinant plasmid pET28a-51 was constructed successfully. Through the weight of plasmid, restriction endonuclease analysis and PCR amplification, the positive recombinant was filtered. After pET28a-51 was digested with *Bam*H I and *Eco*R I double enzymes, two fragments were obtained. They were pET28a linear fragment about 5 300 bp and the inserted fragment about 800 bp, then the Prokaryotic expression plasmid of MPB51 mature protein was constructed successfully.

3.3 SDS-PAGE analysis

The pET28a-51 induced by IPTG was expressed in *E. coli* BL21 (DE3). Its expression condition is shown in Fig. 1. MPB51 gene was successfully expressed. With the increase of induced time, its expression quantity increased, and when it was induced up to 6 h, the expression quantity peaked. The molecular weight of this protein was about 30 kDa, which was the same as MPB51 mature protein, but the control of *E. coli* BL21 (DE3) containing pET28a(+) did not have an interrelated expression band.

3.4 Western-blot analysis

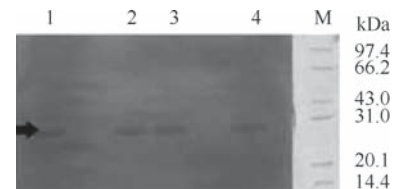
After the SDS-PAGE of expression products, the expression bands were transferred onto PVDF membrane, and antigen



1: pET28a expression result in *E. coli* BL21 with IPTG induced 10 h, as control; 2: pET28a-51 expression result in *E. coli* BL21 with non IPTG induced; 3–9: pET28a-51 expression results in *E. coli* BL21 with IPTG induced 2 h, 6 h, 7 h, 3 h, 4 h, 5 h, 1 h, respectively; M: low molecular weight protein Marker.

Fig. 1 pET28a-51 expression in *E. coli* BL21

bands were detected by using polyclonal antibody of bovine tuberculosis and HRP conjugated rabbit anti-bovine immunoglobulin, with diaminobenzidine in 10 mmol/L Tris-HCl buffer (pH 7.6) as substrate. The result is shown in Fig. 2. A distinct MPB51 band, the major protein about 30 kDa, could be seen on it. And hence, based on the preceding analysis, it could be confirmed that the expression product had the antigenicity of *M. bovis*.



1–4: expression product of the recombinant plasmid pET28a-51 in *E. coli* BL21; M: low molecular weight protein Marker.

Fig. 2 Western-blot analysis of the expression product of the recombinant plasmid pET28a-51

4 Discussion

In recent years, there have been many measures to control bovine tuberculosis, such as quarantine, isolation and killing, etc., but up to now, it has not been eliminated thoroughly. In fact, the positive ratio has increased in recent years. Practical and effective vaccines are not currently available. Thus, developing novel, safe and effective vaccines has become the key research subject for the elimination of bovine tuberculosis.

MPB51 is the mainly secreted protein of *M. bovis*, and the main target antigen of novel vaccine. In this study, the gene encoding MPB51 was amplified from *M. bovis* Vallee111 chromosomal DNA by using PCR technique and cloned into pGEM-T vector system, through enzyme digestion and PCR identification. The results supported that the cloned gene was as big as the target gene. Moreover, through sequential determination and DNA star analysis, between the cloned MPB51 gene and *M. bovis* BCG Tokyo strain MPB51 gene, the sequence homogeneity reached 99.8% and the amino acid sequence homogeneity reached 99.6%. The preceding analysis indicated that MPB51 gene was very conservative in *M. bovis*. MPB51 gene that was cloned into pGEM-T system was subcloned into pET28a(+) expression system, then the

prokaryotic expression plasmid was constructed successfully. The expression plasmid was transformed into *E. coli* BL21 (DE3) and induced by IPTG. When it was induced up to 6 h, its expression quantity reached the peak. The N-terminal of the protein fused six his tags in favor of further purification of this protein. The expression protein analyzed by using Western-blot proved that it had antigenic activity of *M. bovis*. On the cloning of MPB51 gene and its expression in *E. coli*, there were no interrelated reports, so these results could serve as a basis for further studies in the development of diagnostic reagent, subunit vaccine and DNA vaccine of MPB51 in *M. bovis* against bovine tuberculosis.

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