

Cloning and Expression of *Mycobacterium bovis* Secreted Protein MPB83 in *Escherichia coli*

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The gene encoding MPB83 from *Mycobacterium bovis* Vallee111 chromosomal DNA was amplified by using polymerase chain reaction (PCR) technique, and the PCR product was approximately 600bp DNA segment. Using T-A cloning technique, the PCR product was cloned into pGEM-T vector and the cloning plasmid pGEM-T-83 was constructed successfully. pGEM-T-83 and pET28a(+) were digested by *Bam*HI and *Eco*RI double enzymes. The purified MPB83 gene was subcloned into the expression vector pET28a(+), and the prokaryotic expression vector pET28a-83 was constructed. Plasmid containing pET28a-83 was transformed into competence *Escherichia coli* BL21 (DE3). The bacterium was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) and its lysates were loaded directly onto sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), approximately 26 kDa exogenous protein was observed on the SDS-PAGE. The protein was analyzed using Western-blotting. The results indicated that the protein was of antigenic activity of *M. bovis*. The results were expected to lay foundation for further studies on the subunit vaccine and DNA vaccine of MPB83 gene in their prevention against bovine tuberculosis.

Keywords: Cloning, MPB83 gene, *Mycobacterium bovis*, Prokaryotic expression

Mycobacterium bovis is the causative agent of bovine tuberculosis in a range of animal species and human beings. More than 10 percents of human tuberculosis is caused by *M. bovis*. WHO indicated that if a country is contaminated by bovine tuberculosis, the human beings will always be menaced by it. If they do not take actions to eliminate it, the

control of human tuberculosis will be unsuccessful. Up to now, many developed countries and regions, such as USA, Australia and north Europe etc, have eliminated bovine tuberculosis to some extents, but the prevalence of tuberculosis of human beings and wildlife made these countries detect it all the time. In many developing countries, bovine tuberculosis still prevails severely. The prevention of bovine tuberculosis mainly adopts allergic detection by using purified protein derivative (PPD). The positive cattle will be isolated or killed. But by this way, this disease is not under control, on the contrary, the incidence is increasing (Tollefsen *et al.*, 2003).

Now, the prevention of human tuberculosis often adopts incubation of Bacille Calmette-Guerin (BCG). But among different people and different regions, the protection of BCG has difference in large extents (Colditz *et al.*, 1994), especially for the adult. For the cattle and other animals, after incubation of BCG, the results of allergic detection will always be the positive, so artificial immunization and natural infection are difficult to differentiate. Furthermore, it will affect the bovine quarantine and international trade, so we must develop a kind of new vaccine to prevent the bovine tuberculosis.

Some studies have indicated that culture fluids of *M. bovis* contained actively secreted proteins and these proteins can be identified by CD₄⁺ and CD₈⁺ T cells. And they can protect the experimental animals (Andersen *et al.*, 1992; Andersen, 1994). MPB83 is one of the proteins found in culture fluids and a exported lipid protein presenting on the surface of *M. bovis* (Harboe *et al.*, 1995; Wiker *et al.*, 1996; Harboe and Wiker, 1997; Harboe *et al.*, 1998; Wiker *et al.*, 1998). MPB83 is the mainly protective antigen of *Mycobacterium tuberculosis*. In this study, the mature protein gene MPB83 was cloned and its prokaryotic expression plasmid was constructed and was expressed in *E. coli*, so we can lay a solid foundation for the newly developed vaccines.

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Materials and Methods

Strains, vectors, enzymes, and chemicals. *M. bovis* Vallee111 was purchased from China Institute of Veterinary Drug Control (IVDC); *E. coli* JM109 and *E. coli* BL21(DE3) were conserved in our laboratory; pGEM-T vector system was purchased from Promega Co.; pET28a(+) expression vector was purchased from Novagen Co.; Proteinase K was purchased from Merk Co.; EX *Taq* DNA Polymerase, *Bam*HI, *Eco*RI, Nucleic acid weight marker were purchased from TaKara Biotechnology Co.; Agarose was purchased from Spanish Co.; dNTPs, Lysase, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) were supplied by Bebc Co.; Ampicillin, IPTG, RNaseA were purchased from Sigma Co..

Designation and composition of primers. Based on the MPB83 gene sequence of *Mycobacterium bovis* BCG Tokyo strain in Gen Bank (D64165), a pair of PCR primers were designed for amplifying the mature protein gene: Primer-1 (29-mer), 5'-CAG GGATCCACCATGTTCTTAGC-GGGTTG-3'; Primer-2 (25-mer), 5'-TGGCGAATTC-TTACTGTGCCGGGG-3'. The lineats are *Bam*HI and *Eco*RI enzyme digestion sites. The primers were composited by DaLian Takara company.

Culture of *M. bovis* and abstraction of chromosomal DNA. *M. bovis* was incubated in Löwenstein-Jensen medium. After seven weeks, chromosomal DNA was extracted as described by Cai (Cai *et al.*, 1999).

PCR amplification and cloning of MPB83 gene. The mature protein gene MPB83 was amplified by using the template of *M. bovis* chromosomal DNA and specific primers Primer-1 and Primer-2, the reaction condition as follows: 98°C 5 min; 95°C 1 min; 56°C 1 min; 72°C 1 min, total 32 circles, at last 72°C extent 10 min. After purification, the PCR products were linked with pGEM-T vector system by using T4 DNA ligase. The linking products were transformed into competence *E. coli* JM109 (Sambrook *et al.*, 1992). Through α -complementation, the weight of plasmid, enzyme digestion and PCR amplification, the recombinant plasmid was identified, so the positive recombinant plasmid pGEM-T-83 was successfully constructed and its sequence was analyzed by DaLian Takara company.

The construction of prokaryotic expression recombinant plasmid and expression of MPB83 gene. The recombinant plasmid pGEM-T-83 and vector pET28a(+) were digested by *Bam*HI and *Eco*RI double enzymes. The prokaryotic expression vector pET28a-83 was constructed by using the purified MPB83 gene that was subcloned into the expression vector pET28a(+). Plasmid containing pET28a-83 was transformed into competence *E. coli* BL21(DE3). Then the positive recombinant plasmid pET28a-83 was acquired, which contained the right insertion. The prokaryotic expression recombinant was innoculated in 5 ml LB liquid medium (containing 50 μ g/ μ l Kanamycin sulfate) and was cultured overnight at 37°C, then 2 ml cultures were innoculated into 100 ml LB liquid medium and cultured up to OD₆₀₀ of 0.6-0.8. The bacteria were induced by 1 mM IPTG and taken out every one hour up to ten hours. Similarly, the *E. coli* BL21(DE3) containing pET28a(+) was induced up to ten hours as a control.

SDS-PAGE analysis. The OD₆₀₀ values of bacteria cultures acquired every hour were adjusted to the same value 0.68. 1.5 ml bacteria cultures were centrifuged at 13,000 \times g for 10 min at 4°C then the bacteria was harvested. The bacteria were lysed by 100 μ l deioned water and 100 μ l 2 \times SDS loading buffer, then we mixed them and boiled them 5 min in boiling water, centrifuged at 20,000 \times g for 10 min. The 15 μ l lysates were loaded directly onto 12% SDS-PAGE following the procedure described by Sambrook *et al.* (1992).

Western-blotting analysis. After SDS-PAGE of expression products, they were transferred onto Polyvinylidene fluoride (PVDF) membrane by BIO-RAD system following the procedure described by Sambrook *et al.* (1992). The membrane was blocked by bovine serum albumin (BSA), then dipped into bovine tuberculosis polyclonal antibody (The polyclonal antibody was positive bovine serum of natural infection which was produced by our experiment. In clinical practice, we often make some clinical examinations, regarding the doubtful bovis, we will diagnose by clinical and experimental methods. If the result of allergic detection was positive, PPD-ELISA analysis was strong positive, through clinical anatomy, there was obviously tuberculosis focus.). The antigen bands were detected by using horse radish peroxidase (HRP) conjugated rabbit anti-bovine immunoglobulin with diaminobenzidine in 0.01 M Tris-HCl buffer (pH7.6) as substrate.

Results

***M. bovis* chromosomal DNA.** The extracted chromosomal DNA was detected through 0.5% agarose electrophoresis, there was a strong band near 23 kb (Fig. 1).

The PCR amplification of MPB83 gene. The gene encoding mature protein MPB83 from *Mycobacterium bovis* Vallee111 chromosomal DNA was amplified using PCR technique. PCR products were detected by 1% agarose electrophoresis, then a DNA segment about 600 bp was seen obviously, which was the same with our expectation (Fig. 2).

The cloning of target gene and selection of recombinant. The purified PCR product of MPB83 was linked to pGEM-T vector, then the recombinant plasmid pGEM-T-83 was successfully constructed, through α -complementation, enzyme digestion analysis, PCR amplification and sequential analysis,

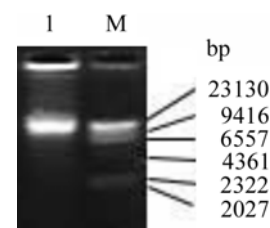


Fig. 1. Electrophoresis of chromosomal genome DNA of MB. Lane M: λ DNA digested with *Hind*III (TaKaRa); Lane 1: chromosomal genome DNA of MB.

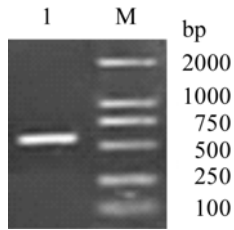


Fig. 2. Electrophoresis of MPB83 amplified with PCR. Lane 1: PCR product of MPB83; Lane M: DNA Marker/DL2000 (TaKaRa).

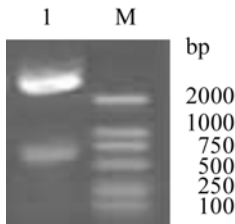


Fig. 3. Restriction analysis map of pGEM-T-83. Lane 1: product of pGEM-T-83 digested with enzyme; Lane M: DNA Marker/DL2000 (TaKaRa).

the positive recombinant was selected. The recombinant pGEM-T-83 was digested by *Bam*HI and *Eco*RI double enzymes. Through digestion analysis, then two segments were acquired, which were pGEM-T linear fragment about 3000 bp and the inserted fragment about 600 bp (Fig. 3). The sequential analysis indicated that the inserted DNA fragment was 603 bp, the homogeneity with *M. bovis* BCG Tokyo strain MPB83 mature protein gene reached 99.7%, but to the nucleid sequence, the 310th base and 384th base were mutated from G and A to A and G in Vallee111. To the amino acid sequence, the difference only lied in 104th amino acid, in BCG Tokyo strain, it was V but in Vallee111, it has mutated to I, the homogeneity between them was 99.5%.

The construction of prokaryotic expression plasmid and the identification of recombinant. The pGEM-T-83 and pET28a(+) were digested with *Bam*HI and *Eco*RI double enzymes and purified. Through the linking of T4 DNA ligase, the recombinant plasmid pET28a-83 was constructed successfully. Through the weight of plasmid, restriction endonuclease analysis and PCR amplification, the positive recombinant was filtered. pET28a-83 was digested with *Bam*HI and *Eco*RI double enzymes, two fragments were obtained being pET28a linear fragment about 5,300 bp and the inserted fragment about 600 bp (Fig. 4), then the Prokaryotic expression plasmid of MPB83 mature protein was constructed successfully.

SDS-PAGE analysis. The pET28a-83 was transformed into *E. coli* BL21(DE3) and induced with IPTG. Its expression condition was shown in Fig 5. MPB83 gene was successfully expressed. With the increase of induced time, its expression quantity increased, when it was induced to 4 hours, the

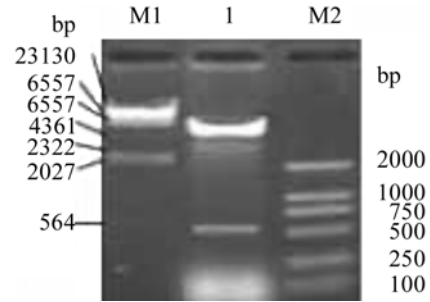


Fig. 4. Restriction analysis map of pET28a-83 plasmid. Lane M1: λ DNA digested with *Hind*III (TaKaRa); Lane 1: product of pET28a-83 digested with enzyme; Lane M2: DNA Marker/DL2000 (TaKaRa).

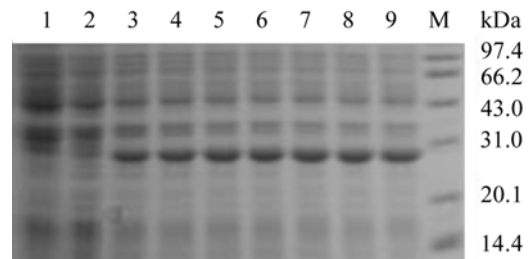


Fig. 5. pET28a-83 expression in *E. coli* BL21. Lane1: pET28a expression result in *E. coli* BL21 with IPTG induced 10h, as control; Lane 2: pET28a-83 expression result in *E. coli* BL21 with non IPTG induced; Lanes3-9: pET28a-83 expression results in *E. coli* BL21 with IPTG induced 1, 2, 3, 4, 5, 6, 7 h, respectively; Lane M: low molecular weight protein Marker.

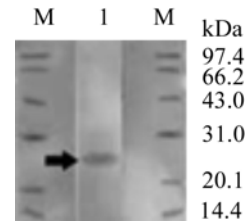


Fig. 6. Western blotting analysis of the expression product of the recombinant plasmid pET28a-83. Lane1: expression product of the recombinant plasmid pET28a-83 in *E. coli* BL21; Lane M: low molecular weight protein Marker

expression quantity peaked. The Mr of this protein was about 26 kDa, which was the same as MPB83 mature protein, but the control of *E. coli* BL21 containing pET28a did not have interrelated expression band (Fig. 5).

Western-blotting analysis. After the SDS-PAGE of expression products, the expression bands were transferred onto PVDF membrane, antigen bands were detected by using horse radish peroxidase (HRP) conjugated rabbit anti-bovine immunoglobulin with diaminobenzidine in 0.01 M Tris-HCl buffer (pH 7.6) as substrate. The result was shown in Fig 6. A distinct MPB83 band, the major protein about 26 kDa, could be seen on it. So

based on the preceding analysis, it can be confirmed that the expression product had the antigenicity of *M. bovis*.

Discussion

In recent years, awareness of the importance of tuberculosis in domestic animals and wildlife has increased. With emphasis on the detection, diagnosis and management of tuberculosis. But bovine tuberculosis is not only as a potential reservoir of infection for domestic animals, but also as a threat to valuable wildlife species even human beings. Up to now, it have not been eliminated thoroughly. Few management options are currently available let alone practical and effective vaccine. So vaccination is the hot subject of much research for the elimination of bovine tuberculosis. But further developments need to be done before it can be used to control tuberculosis in any animals.

MPB83, a effective lipoprotein locating on the surface of *M. bovis*, maybe the main target protein that can be identified by antibody as soon as the infection begin (Wiker *et al.*, 1998). The plasmid DNA encoding MPB83 can induce strong T-cell immunoreaction, mixing antibody of IgG1 and IgG2 and protection of anti-*M. bovis* if mouse, guinea pig and cattle were incubated with the plasmid (Chambers *et al.*, 2000; Chambers *et al.*, 2002). So MPB83 is the ideal antigen of novel vaccine. In this study, the gene encoding MPB83 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 MPB83 gene and *M. bovis* BCG Tokyo strain MPB83 gene, the sequence homogeneity reached 99.7%, amino acid sequence homogeneity reached 99.5%. The preceding analysis indicated that MPB83 gene was very conservative in *M. bovis*. MPB83 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 to 4 hours, its expression quantity reached the peak. The N-terminal of the protein fuse six His tags, which were in favor of the purification of this protein. The expression protein analyzed by using western-blotting proved that it had antigenic activity of *M. bovis*. About the cloning of MPB83 gene and its expression in *E. coli*, there were no interrelated reports in china, so 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.

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