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Research Article

Preliminary Study of High Efficiency Vaccine of *Rhipicephalus (Boophilus) microplus* in South Xinjiang, China

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Abstract

The direct damage and spread of pathogens of *Rhipicephalus (Boophilus) microplus* to cattle -based livestock is extremely serious, the traditional chemical acaricides control have many disadvantages, and vaccine prevention and control is a potential alternative. The commercially available vaccine, based on *Rhipicephalus (Boophilus) microplus* Bm86, has been favored by farmers and veterinarians in some areas, but it also has drawbacks such as reduced effectiveness due to genetic variation. Based on the fact that Bm91 and Bm86 sharing can enhance the immune effect of Bm86, as well as the characteristics of DNA vaccines, the combination of DNA vaccine and protein vaccine enhanced the immune effect. In this study, adopts the local *Rhipicephalus (Boophilus) microplus* strains, using prokaryotic expression system preparation Bm86 and Bm91 proteins, and using the eukaryotic expression vector pVAXI containing the CpG sequences constructed Bm86 and Bm91 double gene eukaryotic expression system. This research provides materials for the next step of Co-immunizing animals with Bm86 and Bm91 mixed proteins with Bm86 and Bm91 dual-gene carriers, it also provides a way for co-immunization with multi-antigen protein and multi-gene carriers to control ticks.

Keywords: *Rhipicephalus (Boophilus) microplus, Bm86, Bm91, Co-expression*

Çin'in Güney Xinjiang Bölgesinde *Rhipicephalus (Boophilus) microplus*'a Karşı Yüksek Verimli Aşı İçin Ön Çalışma

Öz

Sığır besiciliğinde *Rhipicephalus (Boophilus) microplus*'un doğrudan oluşturduğu hasar ve yayılımı oldukça ciddi bir problem olup, geleneksel kimyasal akarisitlerin kontrolde pek çok dezavantajları vardır ve aşı ile koruma ve kontrol alternatif olabilir. *Rhipicephalus (Boophilus) microplus* Bm86 temelli hazırlanan ve ticari olarak mevcut olan aşı bazı bölgelerde yetiştiriciler ve veteriner hekimler tarafından tercih edilmektedir, ancak genetik varyasyondan dolayı azalmış etkisi gibi negatif tarafları bulunmaktadır. Bm91 ve Bm86'nin birlikte bulunması Bm86'nın bağışıklık oluşturma etkisini artırabileceğinden aynı zamanda DNA aşıların özelliklerinden dolayı DNA aşısının protein aşı ile kombinasyonu bağışıklık etkisini artırabilir. Bu çalışmada yerel *Rhipicephalus (Boophilus) microplus* türü ile Bm86 ve Bm91 proteinlerinin prokaryotik ekspresyon sistemi ve CpG sekansı içeren pVAXI prokaryotik ekspresyon vektörü aracılı Bm86 ve Bm91 çift gen ökaryotik ekspresyon sistemi kullanıldı. Bu çalışma, Bm86 ve Bm91 proteinleri karışımı ile Bm86 ve Bm91 çift gen taşıyan aşı materyali oluşturmuş, böylece kene kontrolünde çoklu antijen protein ve çoklu gen taşıyan aşı ile koimmunizasyon için bir araç oluşturmuştur.

Anahtar sözcükler: *Rhipicephalus (Boophilus) microplus, Bm86, Bm91, Koekspresyon*

INTRODUCTION

The cattle tick *Rhipicephalus (Boophilus) microplus* is parasitic on cattle hematophagous ectoparasite, which spread of various pathogens [1-8]. This parasite was found in West Africa, East Africa, South Africa, the Middle East, Latin America and Asia [6,8-12]. In China, *R. microplus* tick is the most widespread tick species and widely distributed in 23 provinces [12]. The traditional method to control *R. microplus* tick is to use chemical insecticides, but there are many disadvantages,

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such as food and environmental pollution, chemical residues and increase of antidrug ticks [13-17]. The vaccine that protects cattle against ticks is a promising alternative way to control ticks, which has additional advantages compared with chemical control agents [14,17-21]. At present, the only commercial anti-tick vaccine in the world is from Cuba, named GavacTM, which is based on the recombinant Bm86 antigen of the midgut membrane protein of *R. microplus* tick [16,17,22-24]. The main protective mechanism of this vaccine is the production of antibodies against Bm86 protein. This humoral immune response directly affects the intestinal tract of ticks, reduces the amount of blood sucking and reduces the weight and fecundity of female ticks [23,25-28]. This antigen does not grant enough protection to several *R. microplus* tick populations [27,29,30]. There is evidence that improving the efficacy of this vaccine against *R. microplus* tick has been achieved satisfactorily by adding more than one antigen into the vaccine [31]. Bm 91 is an antigen isolated from the salivary glands and midgut of *R. microplus* tick. When inoculated to cattle, this antigen will significantly reduce the ability of sucking and laying eggs of ticks infesting the cattle [32]. The anti-tick effect of Bm91 on the immune response against ticks is not as obvious as Bm86 [33]. But, Bm91 induced long-term immune response and showed an increased efficacy of Bm86 vaccine for *R. microplus* tick when co-administered [33,34]. In addition, the sequence variation of antigen sites between *R. microplus* ticks isolated from different geographical areas have been proved to affect vaccine efficacy [35]. Therefore, it is necessary to select the predominant strains in a local region for preparation of effective vaccines [15]. The DNA vaccine has a potential advantage over other types of vaccines, they can induce strong cellular immune responses in addition to the humoral immune response, and they have no risks associated with the use of traditional attenuated vaccines, and the purification of plasmid DNA is easier and cheaper than recombinant protein. Furthermore, the DNA vaccine is stable at room temperature [28,36,37]. At the same time, some studies have shown that the co-immunisation of DNA and protein vaccines boosts the immune effect [38,39].

From the above, this study investigated the preparation of Bm86 and Bm91 recombinant mixed proteins and dual gene plasmid DNA adopts the local *Rhipicephalus (Boophilus) microplus* strain, and laid the foundation for the joint immunological study to enhance the immunological effect of anti-tick vaccine.

MATERIAL and METHODS

Materials

Research Areas and R. microplus Collection

In 2013, *R. microplus* were collected from cows in Makit of Kashgar Prefecture, Xinjiang (1179 m above sea level; 38°54'N, E77°39'E). These tick specimens were placed in sampling vials with sufficient air and transported immediately to the laboratory for cryopreservation.

Methods

RNA Extraction, RT-PCR and Sequence Analysis

The cryopreserved *R. microplus* ticks specimens were washed twice with 0.1% DEPC, then frozen in liquid nitrogen and ground using a mortar. Total RNA was prepared from *R. microplus* ticks using TRIzol® reagent (BBI, Shanghai, China, Code No. B610409) in accordance with the manufacturers' protocols. Total RNA was used for the synthesis of cDNA using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara, Dalian, China, Code No. RR019A) in accordance with the manufacturer's manual. The cDNA was used for *R. microplus* Bm86 (1953 bp) gene and Bm91 (1833 bp) gene amplification with gene-specific primers. The primers sequence used for Bm86 and Bm91 gene amplification were 5'-ATGCGTGGCATCGCTTTGTTCG-3', 5'-TTACAACGATGCTGCGGTGACTG-3 and 5'-ATG GGCGT TGCCTTTATAGAAGGCT-3', 5'-TCATAACGAGATGTTTTTCC AGC-3', the annealing temperature was 57ºC and 58ºC, respectively. All PCR amplicons were bi-directionally sequenced using ABI PRISMTM 3730 XL DNA Analyzer. The sequencing results were analyzed in online BLAST *(https://blast.ncbi.nlm.nih.gov/)*. The obtained sequences in this study were submitted to GenBank under the accession numbers "GenBank accession NO.: MH165269 and MH165270".

Vector Constructs for Expression Recombinant Protein

The DNA fragments of Bm86 and Bm91 were amplified using the cryopreserved Bm86 and Bm91 gene amplification products using the Premix Taq™ Version 2.0 Kit (Takara, Dalian, China, Code No. R004A). The primers sequence used for Bm86 gene amplification were P1: 5'-CG*G GATCC* TGCGTGGCATCGCTTTGTTC G-3' with the restriction enzyme sites *Bam*H I) and P2: 5'-TCC*CCG GAATCC*TTACAA CGATGCTGCGGTGACTG-3' with the restriction enzyme sites *Eco*R I); The primers sequence used for Bm91 gene amplification were P3: 5'-TCC*CCGGAATCC*ATGGGCGTT GCCT TTATAGAAGGCT-3' with the restriction enzyme sites *Eco*RI) and P4: 5'-TT *GCGGCCGC* TCATAACGAGATGTTTTTCCAGC-3' with the restriction enzyme sites *Not* I). Using Bm86 and Bm91 gene amplification products as the template, the dual DNA fragments Bm were amplified with the P1 and P4 primers. Collect and purify the target fragments of Bm86, Bm91 and Bm using the OMEGA™ Gel Extraction Kit (OMEGA, China, Code No. D2500) in accordance with the manufacturer's manual. The DNA fragments of Bm86, Bm91 and Bm were ligated into pEASY-Blunt vector using the pEASY-Blunt Simple Cloning Vector (TransGen, China) in accordance with the manufacturer's manual. The plasmids were introduced into *E. coli* DH5α, the positive clone strains were detected by the technique of colonies PCR, using the following primers: P1 and P2 for Bm86, P3

and P4 for Bm91, P1 and P4 for Bm. The positive colony were cultured and extracted plasmids using the OMEGA™ Plasmid Mini Kit (OMEGA, China, Code No. D6943) in accordance with the manufacturer's manual. The extracted plasmids were double-enzyme cut to identify, using the following restriction enzyme sites: *Bam*H I and *Eco*R I for Bm86; *Eco*R I and *Not* I for Bm91; *Bam*H I and *Not* I for Bm. Moreover, the vectors were verified by DNA sequencing. The DNA fragments of Bm86 and Bm91 were ligated into pET28a via the restriction enzyme sites *Bam*H I/*Eco*R I and *Eco*R I/*Not* I using T4 DNA ligase. The plasmids were introduced into *E. coli* DH5α, the positive clone strains were detected by the technique of colony PCR, using the following primers: P1 and P2 for Bm86, P3 and P4 for Bm91. The positive colony were cultured and extracted plasmids using the OMEGA™ Plasmid Mini Kit (OMEGA, China, Code No. D6943) in accordance with the manufacturer's manual. The extracted plasmids were double-enzyme cut to identify, using the following restriction enzyme sites: *Bam*H I and *Eco*R I for Bm86; *Eco*R I and *Not* I for Bm91. The correct strain and plasmid DNA were kept for use.

Expression and Purification of the Recombinant Protein

The recombinant plasmid was confirmed by sequencing and introduced into *E. coli* expression strain BL21, the positive clone strains were detected by the technique of colony PCR, using the following primers: P1 and P2 for Bm86, P3 and P4 for Bm91. The positive colony were cultured and purified Bm86 and Bm91 protein by IPTG induction. *E. coli* cells harboring recombinant plasmids, pET28a-Bm86 and Bm91, were respectively grown under continuous shaking at 37°C in LB broth containing kanamycin. The cells were induced at OD600=0.5 with 0.6 mM IPTG, and grown for an additional 20 h at 18°C, and then harvested by centrifugation (5000 g, 15 min) and the pellets were frozen at −80°C until used. All purification steps were carried out at 4°C. Bacterial pellets were thawed in 100 mL lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 10% glycerine, 0.5% TritonX-100, 2 mM EDTA, pH 8.0), supplemented with the appropriate protease inhibitor cocktail (Roche, Switzerland). Bacterial cells were lysed using an ultrasonic processor to generate the crude bacterial extract and centrifuged for 30 min at 12.000 × g to collect the supernatant. The supernatants were passed over a Ni column (GE, USA) pre-equilibrated with binding buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 10 mM, pH 8.0) and washed with 20 column volumes of binding buffer. Then the column was washed with 10 column volumes of washing buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 20 mM, pH 8.0) and eluted with elution buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 200 mM, pH 8.0). The proteins were quantified using BCA Protein Assay Kits in accordance with the manufacturer's manual (GenStar, USA). Protein extracts (20 µg per lane) were resolved on 12% sodium dodecyl sulphate-polyacrylamide gels and electro blotted onto Bio-Rad Immun-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). After transfer, PVDF membranes were blocked in Tris-buffered saline-Tween 20 (TBST; containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature and incubated with the primary antibody of mouse His-TAG (Sigma-Aldrich, dilution 1:1000) overnight at 4°C. Membranes were washed three times (10 min) in TBST and incubated with the secondary antibody of anti-mouse immunoglobulin G (Sigma-Aldrich, dilution 1:2000) for 30 min. Subsequently, the membranes were washed three times (10 min) in TBST. Horseradish peroxidase activity was examined by Chemiluminescent Substrate (Roche) in accordance with the manufacturer's protocol. The proteins were collected for the following experiment.

Constructs for Eukaryotic Expression

The full-length DNA fragment of Bm was inserted into pVAX1-CpG vector between *Bam*H I and *Not* I sites for cell transfection and subsequent injection into cattle -based livestock. Cell transfection was performed using HEK293 cells (CRL-1573, American Type Culture Collection). Briefly, HEK293 cells were cultured in petri dish with DMEM medium (Hyclone, Code No.SH30243.01) added 10% FBS (Gemini, Code No.100-106) and 1% Penicillin-Streptomycin (Gibco, Code No.15140), put it in the incubator with 5% CO_2, 37°C. On the day before transfection, the cells are digested by trypsin, enriched by centrifugation or diluted with the medium according to their density, then replacing the medium using OPTI-MEM medium (Gibco, Code No.31985) with 10% FBS. Extracted 200 µL OPTI-MEM medium put into two 1.5 mL EP tubes, one EP tube added 4 µg plasmid DNA to be transfected, and another EP tube added 8 µL PEI (DNA: PEI=1 µg: 2 µL) gently mixed. The culture medium with PEI was added to the medium with the plasmid DNA, incubate at room temperature for 20 min. Add the HEK293 cells slowly to the mixture and gently mixed. The six orifice plates were cultured in the cell incubator, and the old medium was discarded after 4 h, and the new OPTI-MEM medium containing 10% FBS was added, then continued cultivation for 24 h before test.

Total RNA was prepared from transfection cells using TRIzol® reagent (Life Technologies, USA) and purified using a PureLink® RNA Mini Kit (Invitrogen, USA) combined with a PureLink® DNase Kit (Invitrogen), in accordance with the manufacturers' protocols. RNA concentration and quality were measured using a NanoVue spectrophotometer (GE Healthcare, USA). Approximately 2 µg total RNA was used for the synthesis of cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis Kit in accordance with the manufacturer's manual (TransGen, China). The cDNA was used for PCR reaction with gene-specific primers. The Bm fragment was amplified with P1 and P2 primers, and the pVAX1-Bm-CpG is preserved to provide nucleic acid to immune animal.

RESULTS

To obtain the recombinant protein in *E. coli*, we separately developed the Bm86 and Bm91 prokaryotic expression vector pET28a *(Fig. 1)*.

The field collected *R. microplus* ticks specimens were used for total RNA prepared. Total RNA was used for the synthesis of cDNA, and the cDNA was used for *R. microplus* Bm86 gene and Bm91 gene amplification with gene-specific primers. The gene fragments that were consistent with the desired fragment size were obtained *(Fig. 2)*. The PCR amplicons were bi-directionally sequenced, the sequencing results confirmed the Bm 86 gene with 1953 bp full open reading frame, encoding 650 aa and the Bm 91 gene with 1833 bp full open reading frame, encoding 610 aa, were obtained. The obtained Bm86 gene sequences results were similar with *B. microplus* cell surface glycoprotein Bm86 (GenBank accession NO.: TCKBM86A), and the similarity was 99% (1926/1953). The obtained Bm91 gene sequences results were similar with *Boophilus microplus* angiotensin-converting enzyme-like protein (Bm91) (GenBank accession NO: BMU62809), and the similarity was 98% (1801/1833).

Primers with internal *Bam*H I and *Eco*R I restriction sites were designed to amplify the cDNA of Bm86, and Primers with internal *Bam*H I and *Not* I restriction sites were designed to amplify the cDNA of Bm91. The PCR product was subcloned into the BamHI-*Eco*R I sites of pET28a to produce a fusion protein with a His tag at the N terminus, which was named as pET28a-Bm86 *(Fig. 3)*. The PCR product was subcloned into the *Bam*H I-*Not* I sites of pET28a to produce a fusion protein with a His tag at the N terminus, which was named as pET28a-Bm91 *(Fig. 3)*.

E. coli cells harboring recombinant plasmids were used to express and purify The *R. microplus* Bm86 and Bm91 proteins. Bm86 contained a 650 amino acid, which was predicted as a 71.5 KDa polypeptide and Bm91 contained a 610 amino acid, which was predicted as a 67.1 KDa polypeptide were obtained *(Fig. 4)*.

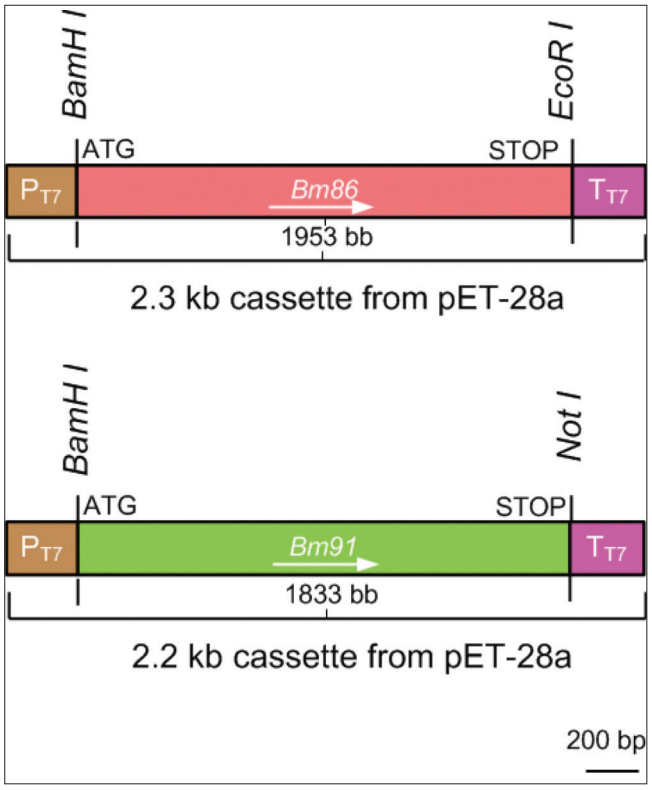

Fig 1. Construct maps of *pET28a-Bm86* and *pET28a-Bm91*. Construct maps of *pET28a-Bm86 (top)* and *pET28a-Bm91 (bottom)*. Arrows indicate the direction of transcription; P_{T7}, T7 promoter; T_{T7}, T7 terminator. The restriction enzymes used for cloning are indicated

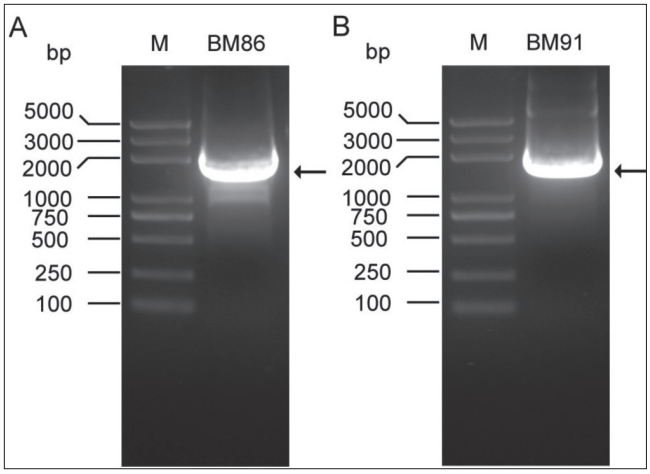

Fig 2. *R. microplus* Bm86 gene and Bm91 gene PCR amplification. **A-** The *R. microplus* Bm86 (1953 bp) gene PCR amplification with gene-specific primers, **B-** The *R. microplus* and Bm91 (1833 bp) gene PCR amplification with gene-specific primers

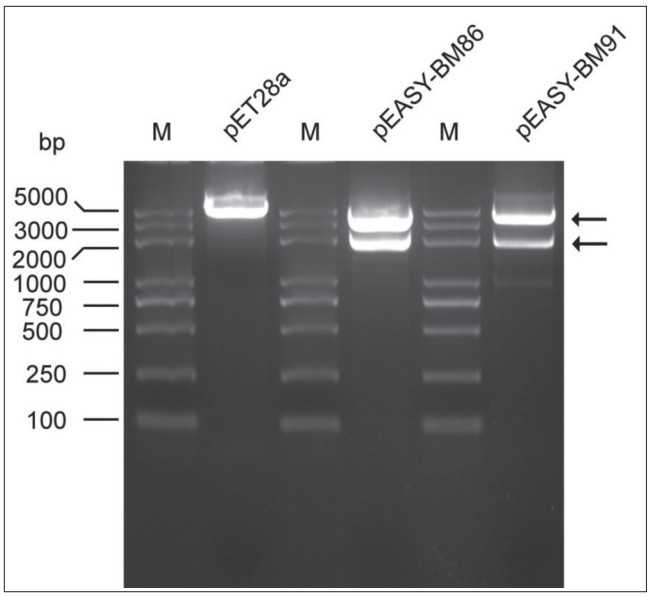

Fig 3. *pET28a,* Bm86 gene and Bm91 gene double enzyme cutting results. The *R. microplus* Bm86 (1953 bp) gene double enzyme cutting use *Bam*H I and *Eco*R I restriction sites and Bm91 (1833 bp) gene PCR amplification double enzyme cutting use *Bam*H I and *Not* I restriction sites

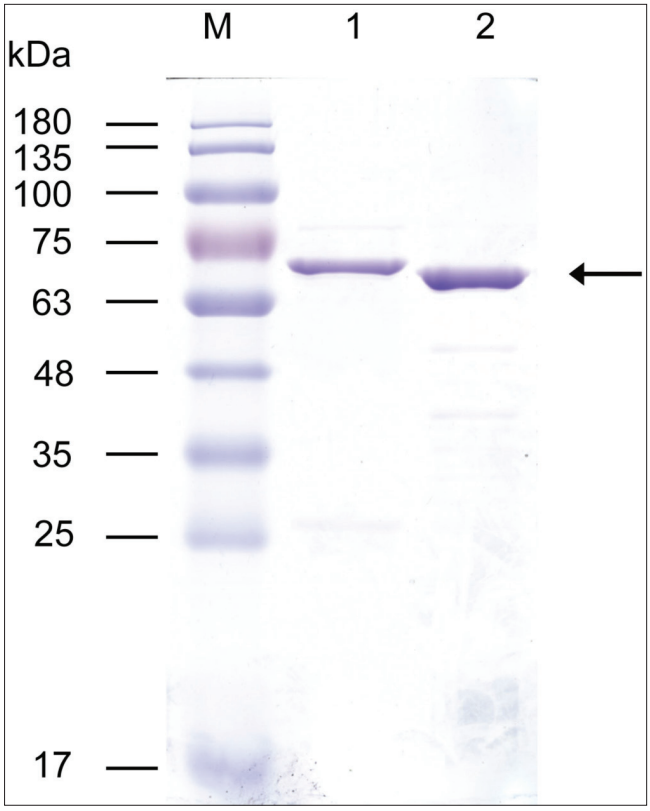

Fig 4. Bm86 and Bm91 recombinant protein SDS-PAGE electrophoresis detection. The *R. microplus* Bm86 protein (lane 1) and Bm91 protein (lane 2) SDS-PAGE electrophoresis detection. The arrow indicate the destination strip location. 5 μL protein was taken for electrophoresis, and the protein concentration of Bm86 and Bm91 were 0.98 mg/mL and 1.68 mg/mL, respectively

To further confirm the obtained proteins were the target proteins, the proteins were detected in the protein level with Western blot analyses *(Fig. 5)*.

Co-immunization DNA and protein vaccines boosts the immune effect, so we investigated the preparation of Bm86 and Bm91 recombinant mixed proteins and dual gene plasmid DNA Bm. To obtain the dual gene plasmid DNA Bm, we developed the double gene expression vector pVAX1-Bm-CpG *(Fig. 6)*.

Primers with internal *Bam*H I and *Not* I restriction sites were designed to amplify the cDNA of Bm. The PCR product was subcloned into the *Bam*HI-*Not* I sites of pVAX1-CpG to produce a double gene expression vector, which was named as *pVAX1-Bm-CpG (Fig. 7)*.

To further study whether the double gene Bm could express in mammalian cells. The double gene expression vector of Bm was performed cell transfection using HEK293 cells. Total RNA was prepared from transfection cells and used for the synthesis of cDNA. The cDNA was used for PCR reaction with gene-specific primers P1 and P2. As shown in the *Fig. 8*, the target band can be detected, and the pVAX1-Bm-CpG is preserved to provide nucleic acid to immune animal.

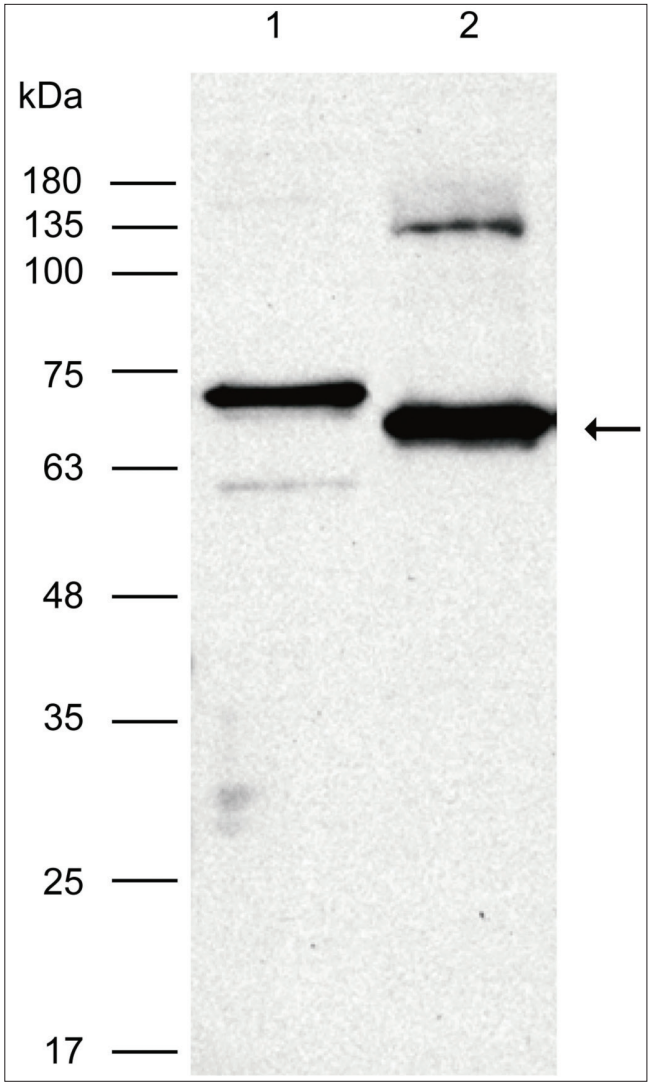

Fig 5. Bm86 and Bm91 recombinant protein SDS-PAGE Western blot detection. The *R. microplus* Bm86 protein (lane 1) and Bm91 protein (lane 2) SDS-PAGE electrophoresis detection. The arrow indicate the destination strip location. 5 μL protein was taken for Western blot, and the protein concentration of Bm86 and Bm91 were 0.98 mg/mL and 1.68 mg/mL, respectively

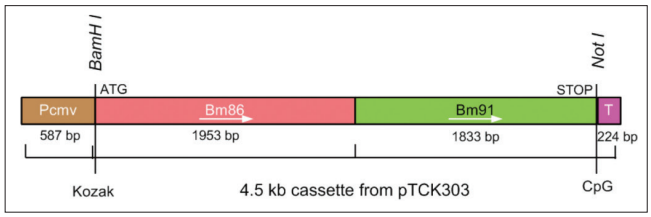

Fig 6. Construct maps of *pVAX1-Bm-CpG*. Construct maps of *pVAX1-Bm-CpG*. Arrows indicate the direction of transcription; P_{CMV}, CMV promoter; T_{BGH}, BGH terminator. The restriction enzymes used for cloning are indicated. CpG means CpG sequence and Kozak sequence GCCACC

DISCUSSION

Ticks rank second to mosquitoes, which are the pathogen carriers of human, livestock and wildlife diseases [2,40]. *Rhipicephalus (Boophilus) microplus* is the most influential

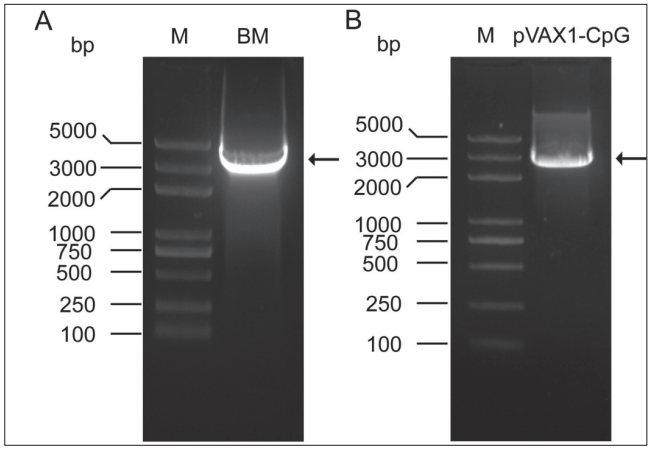

Fig 7. Bm gene and *pVAX1-CpG* vector double enzyme cutting results. The double gene Bm (3800 bp) gene double enzyme cutting use *Bam*H I and *Not* I restriction sites and *pVAX1-CpG* vector used the same double enzyme cutting to developed the double gene expression vector

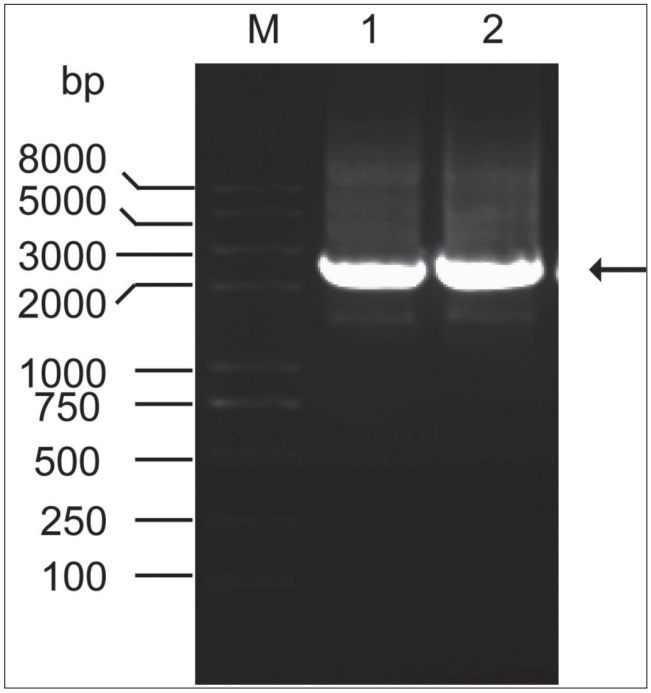

Fig 8. Expressions of *Bm* gene were identified in transfected mammalian cells. The expressions of *R. microplus Bm* gene were detected in the RNA level with reverse transcription polymerase chain reaction. lane 1 and 2 represent two repeats. The arrow indicate the destination strip location

tick species affecting cattle in the world [41,42]. It is known for its aggressiveness and rapid replacement of other species of the same subgenus [8]. *R. (B.) microplus* tick is a harmful species for animal husbandry resulting in huge economic losses to farmers from tropical to subtropical regions [43]. At present, acaricides are no longer effective enough to control ticks alone on farms. The vaccine may provide a complementary treatment in a comprehensive pest management program [17]. The glycoprotein Bm86 is located in *B. microplus* tick midgut cells, and Bm91 located in the salivary gland and B midgut of *B. microplus* [25,44].

At present, the vaccines based on bm86 are used to immunize cattle in order to induce immunoglobulin. When ticks swallow blood, these antibodies, together with other components of the immune system, such as complement, can cause the cleavage of intestinal epithelial cells, causing ticks to die or damage [22,45]. If Bm86 and Bm91 antigens used in combination, the effects of their antibodies will act on different parts of *R. (B.) microplus* ticks, and cause more serious damage, further reduce the fertility rate of ticks and the frequency of acaricide and achieve the result of control ticks and tick-borne diseases. Facts have been proved that the addition of the Bm91 antigen indeed improves the efficacy of Bm86 vaccine alone [33]. In addition, the efficacy of the vaccination with Bm86 and the amino acid sequence variations in the Bm86 protein in challenges with *R. microplus* was negatively correlated [17,46]. Therefore, using local *R. microplus* ticks to prepare multi antigen vaccines may have an ideal effect on prevention and control of local ticks.

Gene vaccine or DNA vaccine, which is encoded antigen DNA, has been evaluated as prophylactic vaccines and therapeutic treatments for the treatment of infectious diseases, allergies or cancer [28]. Studies have shown that pBMC2 DNA immunization potentially induces humoral and cellular immune responses against *B. microplus* [28]. But, using Bm86 antigen vaccines did not achieve an ideal effect on prevention and control of *B. microplus* in sheep, due to Bm86 antigen induces a protective immune response against *B. microplus,* and DNA vaccination did not result in sustained antibody production [47]. The effect of the *B. microplus* tick DNA vaccine and the double DNA vaccine of *B. microplus* tick immune bovine requires further verification. The combined use of different nucleic acid vaccines and recombinant protein vaccines immunization can enhance the humoral and cellular immune responses induced by DNA vaccine, such as raising antibody level, cell proliferation reaction, CTL activity and cytokine secretion, etc., so as to effectively improve the effect of the vaccine [48-50].

In addition, the expression vector of DNA vaccine is also important. The promoter strength of the expression vector is an important factor in determining the transcription efficiency, and the enhancer can promote the transcription ability of the promoter. PVAXl vector contains pCMV strong promoter and enhancer, which is an efficient new eukaryotic expression vector. Antigen gene expression unit and the CpG base motif adjuvant unit are two essential functional units of DNA vaccine. The CpG motif can induce the body to produce Thl immune response and increase the expression of costimulatory molecules [51]. In addition, in order to enhance the translation efficiency of eukaryotic genes, the kozak sequence (GCCACC) was increased at the front end of the antigen gene, which greatly improved the effect of DNA vaccine.

This study successfully expressed the *R. microplus* Bm86

and Bm91 protein using the prokaryotic expression system, combined with the advantages of pVAXl and CpG, successfully constructed the Bm86 and Bm91 dual gene carrier pVAX1-Bm-CpG, which provided the material for the next step of Co-immunizing animals with Bm86 and Bm91 mixed proteins and Bm86 and Bm91 dual-gene carriers, and laid the foundation for the development of the new vaccine. At present, the research progress is proceeding smoothly with the design of this study. It is expected that the double gene nucleic acid vaccine and the mixed protein vaccine have a good effect or provide the research ideas for the future development of the anti-tick vaccine.

Consent for Publication

Not applicable.

Availability of Data and Materials

All the sequences obtained in our laboratory have been uploaded to the GenBank database *(Bm86 and Bm91*: MH165269 and MH165270).

Competing Interests

The authors declare that they have no competing interests.

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Ethical Approval

Ethical treatment of animals was practiced in this study; however, the relevant document number is not available at Tarim University. Permission was obtained from the farm owners before collection of the specimens.

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