

Identification and Characterization of Immunogenic Genes from Genomic Expression Library of *Mycoplasma ovipneumoniae*

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Abstract

Mycoplasma ovipneumoniae is an important pathogen causing respiratory disease in sheep. At present, the immune-associated antigens of *M. ovipneumoniae* are still unknown, which significantly limits the development of new vaccines for *M. ovipneumoniae*. In order to identify and characterize the immune-associated antigen genes, genomic expression library of *M. ovipneumoniae* was constructed and identified, from which positive clones were recognized and screened by positive serum against *M. ovipneumoniae*. Sequence analysis showed that these 10 clones contained 5 different genes encoding P97-like protein, P102-like protein, Translation initiation factor (IF-1), Methionine aminopeptidase (MAP) and P56 membrane protein, respectively. Three proteins including IF-1, MAP and P97-like protein were expressed in *E. coli* and used to immunize lambs to verify their immunogenicity, respectively. Animal immunization test confirmed that the novel protein MAP displayed a strong immunogenicity, while the immunogenicity of P97-like protein and IF-1 were relatively weak. The identification of immunogenic protein MAP provided a potentially valuable antigen candidate for the development of serological diagnostic method and subunit vaccine against *M. ovipneumoniae* infection.

Keywords: *Mycoplasma ovipneumoniae*, Immune-associated antigen, Screening, Characterization

Mycoplasma ovipneumoniae'nin Genomik İfade Kütüphanesinden İmmünojenik Genlerin Belirlenmesi ve Karakterizasyonu

Öz

Mycoplasma ovipneumoniae, koyunlarda solunum sistemi hastalığına neden olan önemli bir patojendir. Günümüzde, *M. ovipneumoniae*'nin immün ilişkili antijenleri hala bilinmemektedir ve bu durum *M. ovipneumoniae*'ya karşı yeni aşuların geliştirilmesini önemli ölçüde sınırlamaktadır. Çalışmada immün ilişkili antijen genlerinin tanımlanması ve karakterizasyonu amacıyla, *M. ovipneumoniae*'ye karşı pozitif klonlar pozitif serum tarafından belirlenip taranarak *M. ovipneumoniae*'nin genomik ekspresyon kütüphanesi oluşturuldu. Sekans analizi, bu 10 klonun, sırasıyla P97-benzeri protein, P102-benzeri protein, translasyon başlatma faktörü (IF-1), Metiyonin aminopeptidaz (MAP) ve P56 membran proteinini kodlayan 5 farklı gen içerdiğini gösterdi. Sırasıyla IF-1, MAP ve P97-benzeri proteini içeren üç protein, *E. coli*'de eksprese edildi ve immünojenisitelelerini doğrulamak için kuzuların immünizasyonunda kullanıldı. Hayvan immünizasyon testi, yeni protein MAP'ın güçlü bir immünojenisite sergilediğini, P97 benzeri proteininin ve IF-1'in immünojenisitesinin nispeten zayıf olduğunu doğruladı. İmmünojenik protein MAP'ın tanımlanması, *M. ovipneumoniae* enfeksiyonuna karşı serolojik tanı yöntemi ve alt ünite aşı geliştirilmesi için değerli bir potansiyel antijen adayı elde edilmesini sağladı.

Anahtar sözcükler: *Mycoplasma ovipneumoniae*, İmmün ilişkili antijen, Tarama, Karakterizasyon



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INTRODUCTION

Mycoplasma ovipneumoniae is a respiratory pathogen causing interstitial pneumonia in sheep and goats [1-4], which is characterized by chronic non-progressive pneumonia with clinical manifestations of cough, wheezing, runny nose, anemia, weight loss and growth retardation [5,6]. The disease is widely prevalent in sheep farming countries and has caused tremendous economic losses. In recent years, with the rapid development of China's sheep industry, sheep pneumonia caused by *M. ovipneumoniae* has been widely prevalent in western regions of China including Gansu, Ningxia, Sichuan, and Xinjiang provinces. At present, it becomes one of the main infectious diseases in sheep [7,8]. More importantly, *M. ovipneumoniae* infection can also cause immunosuppression, leading to the increased susceptibility of sheep to other pathogens, e.g., *Pasteurella*, *Mannheimia haemolytica* and *Parainfluenza-3 virus* [9-13].

Due to the high nutritional requirements of *M. ovipneumoniae* *in vitro* cultivation, the production cost of conventional inactivated vaccine based on whole-cells is very high. Therefore, the development of new vaccine is of great significance for the control of *M. ovipneumoniae* infection. However, identification of microbial components that give rise to a protective immune response is the key to the development of subunit vaccine. Over the last decade, the genome of three *M. ovipneumoniae* epidemic strains (SC01 strain, NM2010 strain and France 14811) has been fully sequenced, however, the antigenic proteins have not been identified [14,15], which has greatly limited the development of serological diagnostics and subunit vaccines to prevent *M. ovipneumoniae* infection in sheep. Nowadays, construction of genomic expression library is considered as one of the effective strategies to identify genes encoding pathogens' immunogenic antigens. Using genomic expression library, antigenic proteins and virulence factors have been identified in *Mycoplasma mycoides* subsp. *mycoides* and *Campylobacter jejuni*, respectively [16,17]. Compared to other *Mycoplasma* spp. (*M. gallisepticum*, *M. hyopneumoniae*, *M. bovis* and *M. suis*) [18], antigenic proteins and virulence factors of *M. ovipneumoniae* was still not extensively studied.

The aim of this study is to identify and characterize immune-associated proteins encoded by the genome of *M. ovipneumoniae*. We constructed *M. ovipneumoniae* genomic expression library and identified clones that could be recognized by *M. ovipneumoniae* positive serum. The genes encoding these immune-associated proteins were then identified by sequencing, and the immunogenicity of recombinant proteins were further confirmed by animal immunization test.

MATERIAL and METHODS

Ethical Approval

The experiments were carried out in accordance with the

guidelines issued by the Ethical Committee of Shihezi University.

Culture of *M. ovipneumoniae*

Mycoplasma ovipneumoniae shz-1 strain was isolated from nasal secretions of a sheep infected with *M. ovipneumoniae* in a farm in Xinjiang province. *M. ovipneumoniae* was confirmed by biochemical and molecular methods. *M. ovipneumoniae* was stored in the Animal Disease Prevention and Control Laboratory of Xinjiang. Purified *M. ovipneumoniae* was inoculated onto Brain Heart Infusion (BHI) containing 15% (v/v) heat-inactivated horse serum (Biotopped, China), 0.004% (w/v) phenol red (Sigma, USA), and 25 µg/mL ampicillin (Omega, USA) and cultured at 37°C incubator with 5% CO₂ for 4 days.

Preparation of Positive Serum Against *M. ovipneumoniae*

Mycoplasma ovipneumoniae shz-1 strain was cultured to reach the concentration of 10⁸ CCU/mL. Two 60-day-old lambs were infected with 500 µL culture (10⁸ CCU/mL) of *M. ovipneumoniae* by intratracheal inoculation, respectively. After 4 weeks of inoculation, blood was collected, and antibody titer was determined by indirect hemagglutination assay (IHA) kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) as described previously [7]. The individual sera were combined and used for the screening of immunogenic genes from genomic expression library of *M. ovipneumoniae*.

Adsorption and Removing the Antibodies Against *E. coli*

The serum samples were mixed with the lysate of *E. coli* to remove the antibodies that have cross-reactions with *E. coli*. Briefly, saturated 100 mL *E. coli* BL21 culture was centrifuged at 12000 r/min and 4°C for 10 min. The bacteria pellet was resuspended in 10 mL PBS buffer. Following three times of freezing and thawing, bacteria were lysed by sonication for 6 times (10 sec for each time and 10 sec interval). Subsequently, 500 µL of sonication positive serum was mixed with the *E. coli* cell lysate for 2 h. The supernatant serum was collected after 10 min of centrifugation at 12.000 r/min, and 4°C. Western blot was performed to analyze the reaction between the *E. coli* whole cell lysate and the adsorbed serum. Qualified serum samples were stored at -20°C.

Generation of Genomic Expression Library of *M. ovipneumoniae*

Mycoplasma ovipneumoniae genomic DNA (gDNA) was extracted using Mini Extraction Kit (BIOMIGA, USA). The gDNA was digested by restriction enzyme *Sau3A* I (TaKaRa, Japan). The digested fragments were ligated to the expression vector pET28 (a/b/c) (Invitrogen, USA) that was pre-digested with *BamH* I (TaKaRa, Japan) and dephosphorylated, respectively. The ligation products were respectively transformed to *E. coli* DH5α competent cells (TaKaRa, Japan) and incubated at 37°C for 12 h. The plasmids were extracted and

then transferred into *E. coli* BL21 competent cells. A total of 100 colonies were randomly picked and the inserts were analyzed by PCR using T7 (5'-TAATACGACTCACTATAGGG-3') and T7ter (5'-TGCTAGTTATTGCTCAGCGG-3') primers [19].

Screening of Immunogenic Genes from Genomic Expression Library

Screening of library was performed as described previously [18]. Briefly, the library was diluted and plated on LB plate containing kanamycin (OMEGA, USA). After 8 h of incubation at 37°C, the clones were transferred onto a nitrocellulose membrane, which was subsequently placed on a new LB plate containing kanamycin and incubated for 2-3 h. Subsequently, the nitrocellulose membrane was plated on LB agarose plate containing 1 mM IPTG and cultured for 4-6 h at 37°C. The membrane was exposed to the chloroform steam for 15-20 min and air dried. The membrane was then placed in the lysis buffer (100 mM Tris-HCl pH7.8, 150 mM MgCl₂, 1.5% BSA, 1ug/ml pancreatic RNAase, 40 ug/mL lysozyme) (Sigma, USA) and incubated at room temperature for 14 h. The membrane was incubated with the *M. ovipneumoniae* positive serum (1:3000) at room temperature for 1 h followed by incubation at HRP-conjugated rabbit anti-sheep IgG (1:5000) (Bethyl, USA) for 1 h. Finally, the membrane was added with the TMB substrate and the positive clones were verified for several times.

Sequence Analysis of Genes Encoding Immunogenic Proteins

Plasmids were isolated from the positive clones that had reactions with *M. ovipneumoniae* positive serum, and each plasmid was sequenced 3 times. The sequence of recombinant plasmids from identical sequencing results was used for sequence analysis. Then, these sequences were blasted against NCBI database using blast X (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). After verifying that the inserts were from *M. ovipneumoniae* genome, their molecular characteristics were analyzed.

Expression and Reactogenicity Analysis of Immunogenic Proteins

Based on the sequence of positive clones, specific primers (Table 1) were designed using Primer premier 5.0 software, respectively. Then, PCR-amplified fragments were cloned into the expression vector pET32a (+) to generate

pET32a-IF-1, pET32a-MAP, pET32a-P97-like recombinant plasmids, respectively. These three recombinant plasmids were then transformed into BL21 competent cells. The transformants were cultured in LB medium containing the appropriate antibiotic, and protein expression was induced by adding a final concentration of 1 mM IPTG (TaKaRa, Japan) when OD_{600nm} of the culture reached 0.6 ~ 0.8. Culture was collected after 4, 6, 8 and 10 h of IPTG induction, respectively. Bacterial cells were harvested by centrifugation and used for SDS-PAGE analysis. *M. ovipneumoniae* positive serum was used as the primary antibody and HRP-conjugated rabbit anti-sheep antibody (Bethyl, USA) was used as secondary antibody for Western blot analysis. Protein was purified according to the instruction of the Ni-NTA Purification System (Invitrogen, USA).

Immunogenicity Analysis of *M. ovipneumoniae* Immune-Related Antigens

Immunogenicity of purified proteins was evaluated in lambs. Briefly, a total of twenty 60-day-old lambs being antibody-negative were divided into 4 groups with 5 lambs in each group. Purified IF-1, MAP and P97-like protein were respectively mixed with Freund's complete adjuvant (Sigma, USA) to prepare different antigens with protein concentration at 0.05 µg/µL. PBS buffer was mixed with Freund's complete adjuvant at ratio of 1: 1 and was as the negative control. These antigens were intradermally injected at multiple sites at the dose of 500 µL/lambs, respectively. After 14 days, boost immunization was performed similarly. Seven days after the boost immunization, the blood samples from different groups were collected and sera were separated. IHA was performed to determinate the specific antibody against *M. ovipneumoniae*.

RESULTS

Identification of *M. ovipneumoniae* Genomic Library

According to the *M. ovipneumoniae* genome size and an average of 1500 bp-size insert, 3.2×10³ clones were needed to cover the entire genome. Because the DNA fragments were cloned into three different plasmids, and fragment ligation efficiency was over 90%, 10.436 clones from each vector were only needed to screen. In this study, a total of 2.5×10⁴ clones were obtained, which is in line with the coverage requirement of genomic library.

Table 1. List of primers used in this study

Primer Name	Target Gene	Nucleotide Sequence (5'-3')	Product Size (bp)
P1	IF-1	GAATTCATGCAAAATCTTCAAAAAG	240
P2		CTCGAGTTATTTAAAGCGGTAACA	
P3	MAP	GAATTCATGTCTCTAATTTAAACAGAAT	642
P4		CTCGAGTATTCGCGGAGAAGCTTT	
P5	P97-like	GAATTCATGAGTAAACCTAAACAAAATC	714
P6		CTCGAGTAAATTGAAAAACTTGATATGTC	

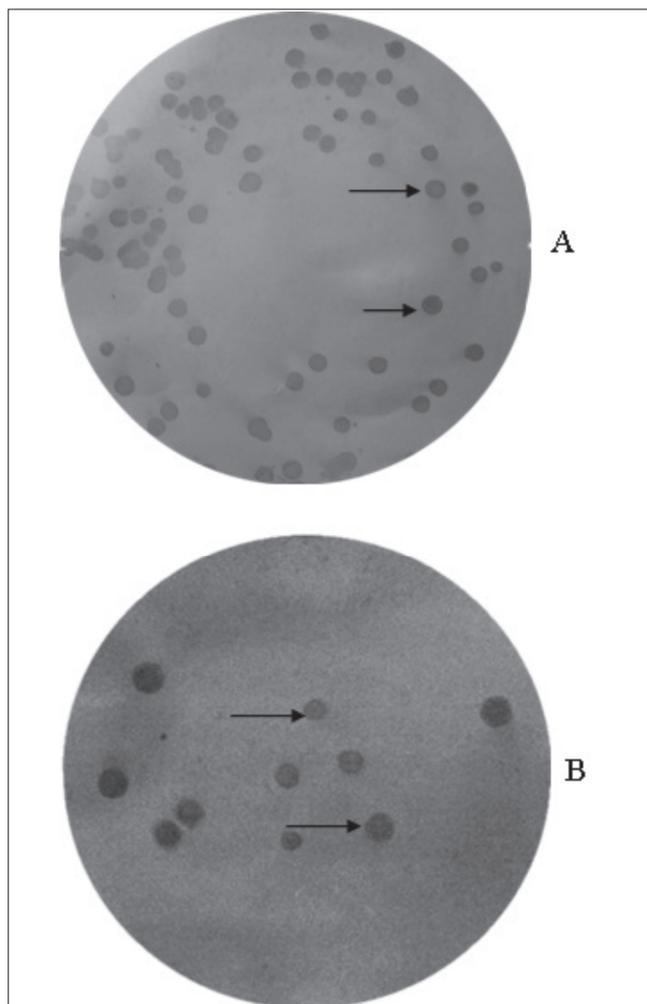
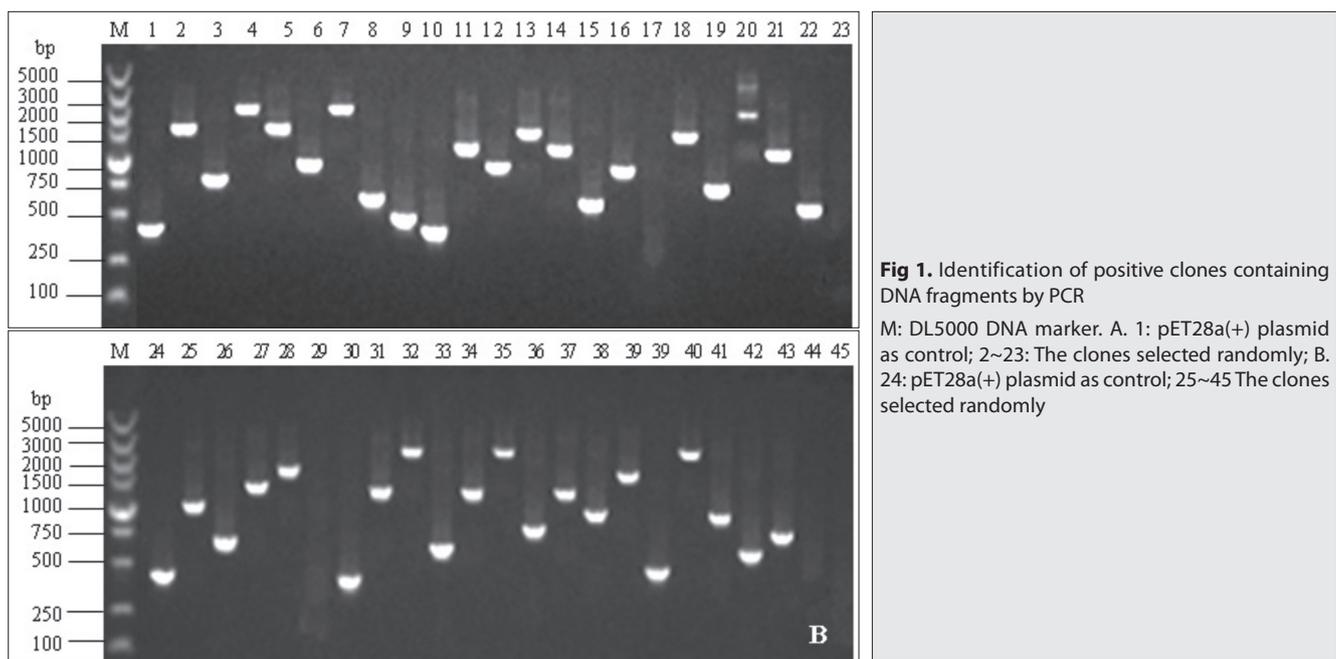


Fig 2. The screening of positive clones expressing DNA fragments of *M. ovipneumoniae* from whole genomic library. A: The preliminary screening of positive clones from whole genomic library; B: The re-screening of true positive clones from the positive clones

Screening of Positive Clones Containing Insert Sequences

Positive clones containing insert sequences were successfully screened by PCR from the genomic library (Fig. 1 A, B). The analysis of insert sequences showed that they shared high identities (from 90% to 100%) with *M. ovipneumoniae* epidemic strains SC01, NM2010 and France14811. Among positive clones, a total of 10 clones were screened by immunoblotting assay, which could react with the *E. coli*-adsorbed *M. ovipneumoniae* positive serum (Fig. 2A, B). Sequence analysis indicated that these 10 clones contained 5 different open reading frames of *M. ovipneumoniae* genome.

Sequence Analysis of Genes Encoding Immunogenic Proteins

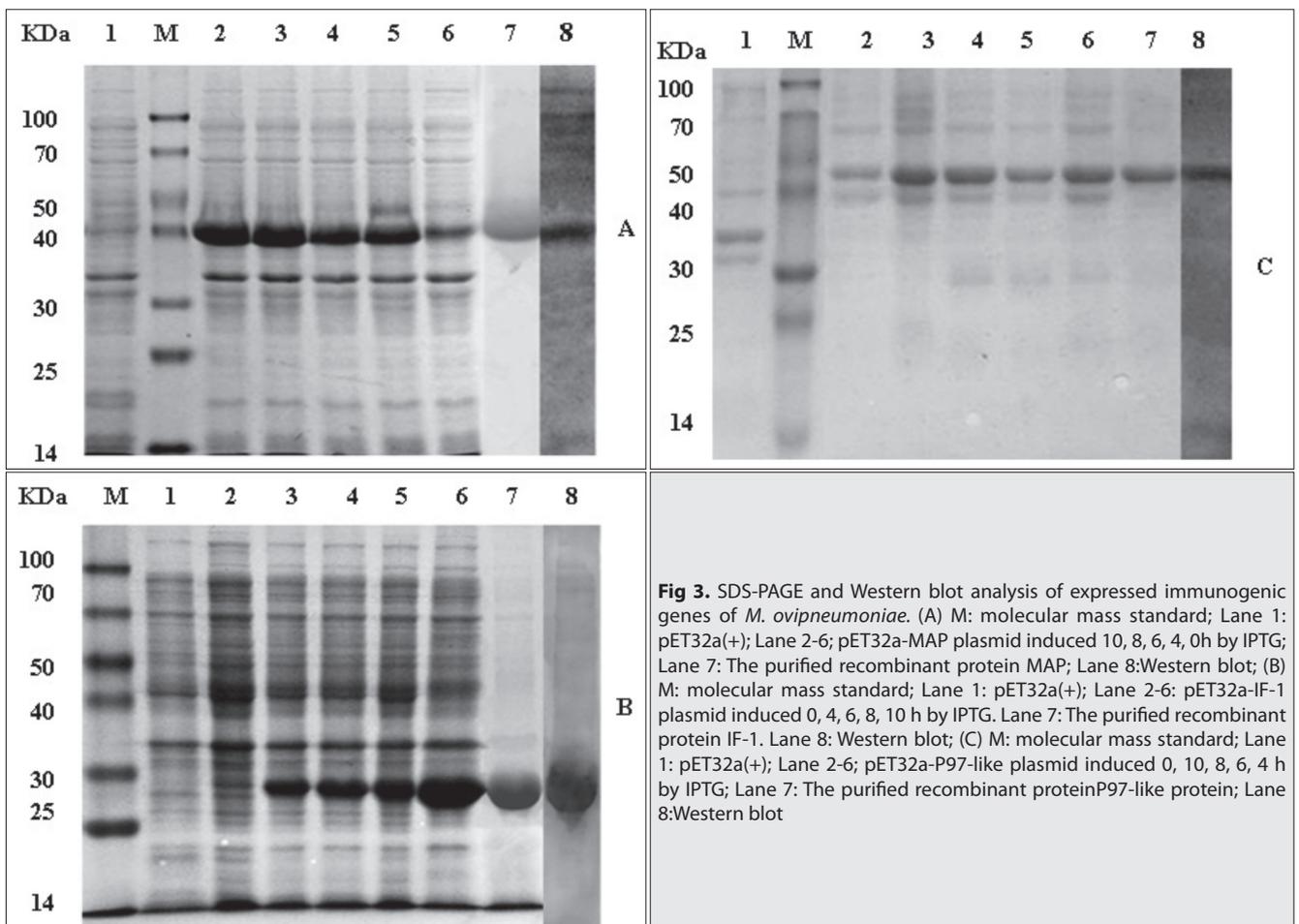
The identities of the 5 immune-associated proteins were shown in Table 2. Sequence analysis showed that P56 protein contains PotE conserved domain that is involved in amino acid transport and P97-like protein contains a transmembrane region at the position of 21-43 amino acids. Based on our knowledge, this is the first time that IF-1 was identified from *M. ovipneumoniae*. IF-1 contains an S1-like RNA-binding domain, which is found in a wide variety of RNA-associated proteins. These domains described above play an important role in relevant *M. ovipneumoniae* biological processes.

Immunogenicity Analysis of *M. ovipneumoniae* Immune-Related Antigens

MAP, IF-1 and P97-like proteins were successfully expressed in *E. coli* (Fig. 3), respectively. Western blot showed that all the three proteins have reactivity with *M. ovipneumoniae* positive serum (Fig. 3A, B, C). The results of immunogenicity analysis showed that MAP could stimulate strong immune response, inducing an antibody titer of 1:32. In contrast,

Table 2. Overview of the immunogenic proteins identified in *M. ovipneumoniae*

<i>M. ovipneumoniae</i> Strain shz-1					Identity to Other Strains		
Number	Insert (bp)	Genome Location	Gene Product	Predicted Function	Strain SC01 Locus Tag (% Identity)	Strain NM2010 Locus Tag (% Identity)	Strain 14811 Locus Tag (% Identity)
1	228	20098-20325	Translation initiation factor IF-1	Translation-related protein	MOSC01_RS0103275 (100%)	AG35_RS00360 (99%)	MOVI_1910 (97%)
2	630	19345-19974	Methionine aminopeptidase	Unknown	MOSC01_RS0103270 (97%)	AG35_RS00360 (99%)	MOVI_1910 (97%)
3	702	7677-8378	Hypothetical protein (P97-like protein)	Transmembrane protein	MOSC01_RS0103395 (93%)	AG35_RS03010 (96%)	MOVI_4210 (98%)
4	921	19813-20733	P56 membrane protein	Amino acid transporters-related protein	MOSC01_RS0103630 (99%)	AG35_RS03280 (95%)	MOVI_0270 (95%)
5	975	11471-12445	Hypothetical protein (P102-like protein)	Transmembrane protein	MOSC01_RS0103400 (100%)	AG35_RS03005 (90%)	MOVI_4200 (91%)



IF- 1 and P97-like protein produced relatively low antibody titer (Fig. 4A, B, C).

DISCUSSION

Identification of immune-associated proteins may contribute to the development of diagnostic reagents and vaccines for infectious agents. At present, genomic expression library has been frequently used to screen and identify pathogens' immunogenic proteins. Ron et al.^[20]

employed *in vivo* induced antigen technology (IVIAT) and identified 13 proteins from the genomic expression library of *M. gallisepticum*, among which five hypothetical virulence factors (GapA, PlpA, Hlp3, VlhA 1.07 and VlhA 4.01) that have been previously described and eight new virulence factors (transport protein PotE, MGA_0241, 0654, translation protein L2, L23, ValS, chaperon GroEL and MGA_0042, a protein with unknown function). Kügler et al.^[21] identified 6 immunogenic proteins from genomic library of *M. hypopneumoniae*. In the present study, we screened genomic

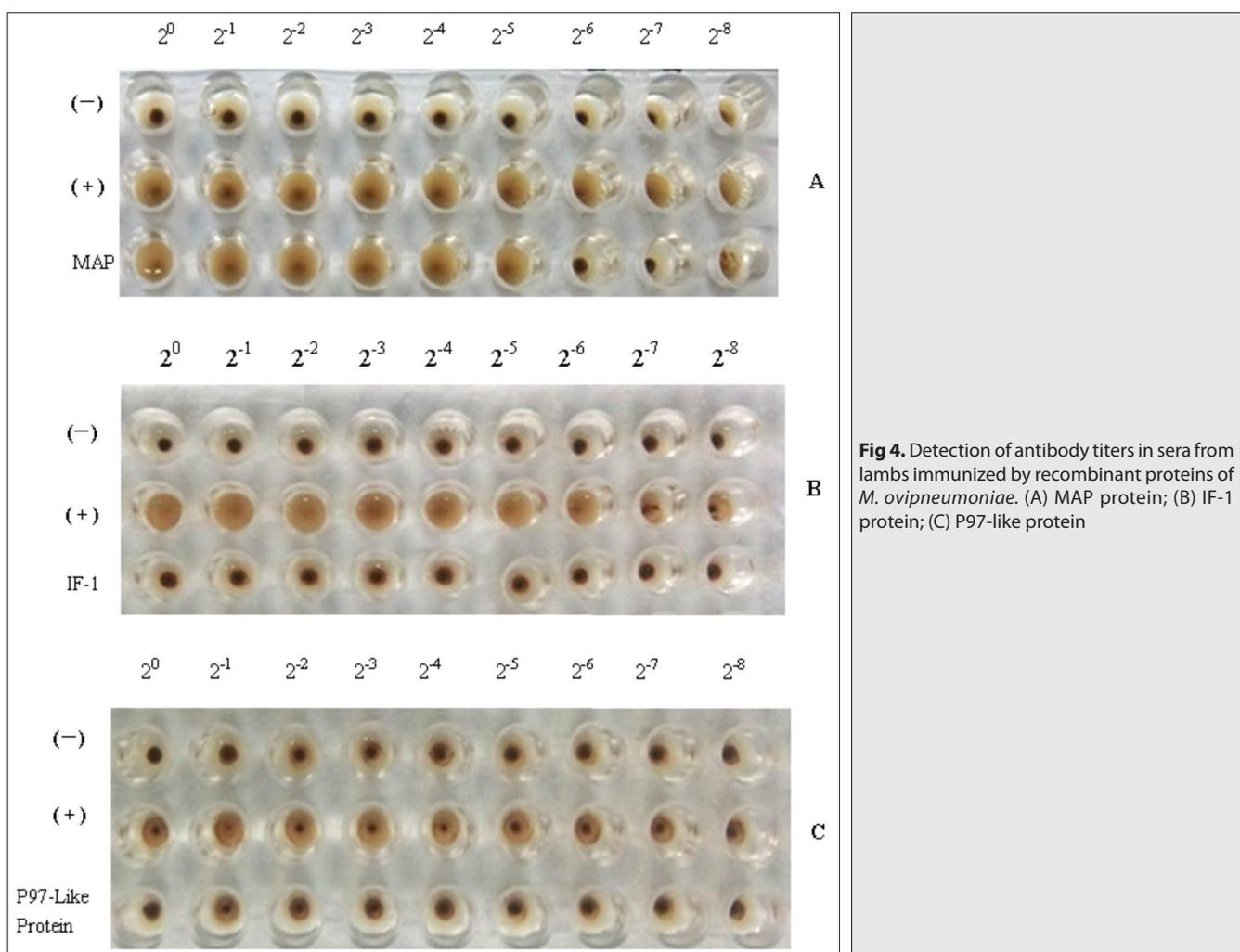


Fig 4. Detection of antibody titers in sera from lambs immunized by recombinant proteins of *M. ovipneumoniae*. (A) MAP protein; (B) IF-1 protein; (C) P97-like protein

expression library using *M. ovipneumoniae* positive serum and identified 5 proteins, including intracellular protein (Methionine Aminopeptidase), two hypothetical proteins (P97-like protein, P102-like protein), a membrane protein (P56) and the translation initiation factor IF-1. The sequence of these proteins shared 90-100% identities with MO strain SC01, NM2010 and France 14811.

Although the complete genome of the *M. ovipneumoniae* France 14811, SC01 and NM2010 strains have been sequenced^[14,15], the genes that encode protective antigens remain unclear. Given that *M. ovipneumoniae* lacks cell wall structure, antigenic proteins are mainly in the cell membrane. Yang et al.^[22] performed *in silico* analysis of MO genome and predicted virulence associated proteins including P146 adhesin like-protein, P97-like protein, adhesin, P76 and P113 protein^[14]. However, these proteins have not been immunologically validated. Some studies have confirmed P97 protein is an essential virulence factor, which is involved in invasion process of *Mycoplasma hyopneumoniae*. P56 membrane protein, identified in this study, is a permease containing multiple transmembrane domains. The PotE domain of P56 is presumed to be involved in the transport and metabolism of polyamines,

whereas polyamine is involved in gene expression, specific binding with proteins and cell permeability. The second protein identified in this study, MAP, is an important cytosolic enzyme, which is mainly involved in the N-terminal methionine cleavage and formation of the mature protein. It is worth noting that IF-1, which participates in protein translation^[23], displays strong reactivity with *M. ovipneumoniae* positive serum but weak immunogenicity in animal experiment.

EF-Tu, HSP70, PDHA and PDHB have been reported to be immune-associated proteins in *M. ovipneumoniae*^[24]; however, these proteins were not identified in this study. This is possibly due to the fact that *M. ovipneumoniae* uses UGA as the preference amino acid codon for tryptophan. In *E. coli*, UGA is a stop codon, which prevents gene expression in *E. coli* host during screening. Furthermore, some genes may be disrupted by the restriction endonuclease, which is responsible for the limitations of genomic expression library. Further studies (e.g., Immunization and challenges) are required to determine the protective antigens in lambs against *M. ovipneumoniae* infection.

In conclusion, the present study identified five immune-

associated proteins from *M. ovipneumoniae* genomic expression library. Among these proteins, MAP, a novel protein, showed strong immunogenicity in lambs, which displayed the potential value for the development of serological diagnostics and subunit vaccine against *M. ovipneumoniae* infection.

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CONFLICT OF INTERESTS STATEMENT

The authors declare that they have no conflict of interest.

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