Immunogenic characteristics of p113/p126 family in Mycoplasma ovipneumoniae

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Abstract

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Immunogenic characteristics of p113/p126 family in *Mycoplasma ovipneumoniae*

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Abstract

In order to obtain the dominant immunogen of the protein encoded by p113/p126 family members of*Mycoplasma ovipneumoniae* . In this study, p113/p126 family recombinant proteins were prepared. We evaluated the antibody level induced by the recombinant protein of each member in the family and its binding ability with the whole bacterial protein. The binding ability of recombinant protein to whole bacterial protein was also evaluated. p113/p126 family members were rich in antigen epitopes. The recombinant proteins (rP128, rP126, and rP113) have the strongest binding ability to the serum of infected animals and belonged to the dominant immunogen. Mice immunized with recombinant protein could produce specific antibodies. The antibody produced by immunizing mice with recombinant proteins (rP113, rP109, and rP130) can effectively bind to bacterial protein. In summary, these results provided a basis for further research on the development of subunit vaccine and the establishment of serological diagnosis methods.

Keywords: *Mycoplasma ovipneumoniae* , p113/p126 family; recombinant protein; immunogenicity

1. INTRODUCTION

Mycoplasma ovipneumoniae(*M. ovipneumoniae*) is an important respiratory disease pathogen (T. Besser et al., 2012). It can cause non typical pneumonia in goats, sheep and bighorn (M. Jaÿ, C. Ambroset, A. Tricot, A. Colin, & F. Tardy, 2020; M. Jaÿ, C. Ambroset, A. Tricot, A. Colin, & F. J. V. m. Tardy, 2020; Nielsen et al., 2021), which sheep are the most sensitive *M. ovipneumoniae* . The sick sheep showed symptoms such as high fever, asthma, cough and losing weight. The disease spreads very fast and is widely prevalent all over the world, which has caused huge economic losses in the sheep feeding industry (Shen, Hu, Wei, Feng, & Yang, 2017). Because it is highly contagious, it brings great challenges to the prevention and control of epidemic diseases.

M. ovipneumoniae has the closest genetic relationship with*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) (Clampitt, Madsen, & Minion, 2021). In *M. hyopneumoniae* , P97 and P102 are not only adhesin molecules that mediate the adhesion, colonization and pathogenesis of host cells, but also the most important immunogen molecules, which can effectively induce the immune response of the body (Raymond et al., 2013; L. K. Woolley et al., 2014). Studies have shown that the genes p97 and p102 encoding P97 and P102 form operon structures, and there are multiple paralogous genes of p97 and p102 in the genome of *M. hyopneumoniae* , forming a p97/p102 paralogous gene family (Petersen, Clampitt, & Minion, 2019), whose members include p97, p102, p146 and p216 (Liu et al., 2013). Further research on p97/p102 family found that its members are mostly adhesion molecules, which are not only involved in the adhesion of *M. hyopneumoniae*to host cells, but also a good immunogen of *M. hyopneumoniae*(Bogema et al., 2012; Tacchi et al., 2016; L. K. Woolley et al., 2014). Therefore, it has become an important candidate protein for new vaccines (such as genetic engineering subunit vaccine and vector vaccine,) and serological diagnosis technology. It is very necessary to find the dominant immunogen protein of *M. ovipneumoniae* .

At present, there is a lack of in-depth understanding of the important functional proteins of *M. ovipneumoniae* . In particular, little is known about its important immunogen proteins. It has the closest genetic relationship with *Mycoplasma hyopneumoniae*(*M. hyopneumoniae*) (B. Zhang, Han, Yue, & Tang, 2013). Therefore, the research strategy for the related functional proteins of *M. hyopneumoniae* has a good reference significance for the discovery and study of *M. ovipneumoniae* immunogen. Previously, our laboratory has completed the whole genome sequencing, gene prediction and functional annotation of *M. ovipneumoniae* strain SC01 (Yang, Tang, Wang, Zhang, & Yue, 2011). p113 and p126 genes of *M. ovipneumoniae* are highly homologous with p97 and p102 of *M. hyopneumoniae* (X. Y. Zhang, Yang, Feng, & Wang, 2013), respectively. They have the similar structural characteristics and belong to lineal homologous genes. p97/p102 family members are mostly adhesion molecules, which are involved in the adhesion of *M. hyopneumoniae* to host cells (Kamminga, Benis, Martins Dos Santos, Bijlsma, & Schaap, 2020; Machado, Paes, Souza Dos Santos, & Ferreira, 2020). They are good immunogens and the important candidate proteins for vaccine and serological diagnosis.

In this study, the molecular characteristics of p113/p126 family encoded proteins of *M. ovipneumoniae* were analyzed. The members of p113/p126 gene family were cloned and prokaryotic expressed, and their immunological characteristics were analyzed. The dominant immunogen proteins that can bind with the serum of infected animals were screened. The aim is to provide useful candidate antigen proteins for the development of sensitive serological diagnostic techniques and new nucleic acid vaccines.

2. Materials and Methods

2.1. Bacterial strains and serum samples

This study was undertaken in strict accordance with the Institutional Animal Care and Use Committee of Southwest Minzu University and the Regulations for the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China.

The *M. ovipneumoniae* strains SC01, positive serum and negative serum of sheep infected with *M. ovipneumoniae* was isolated, identified and preserved by the veterinary medicine laboratory of Southwest Minzu University. Among them, the sera of infected animals were goat and sheep sera naturally infected with*M.*

ovipneumoniae in clinic. The negative serum was collected from the serum of newborn lambs without colostrum, and the whole bacteria ELSIA test showed that the antibody of *M. ovipneumoniae* was negative.

2.2 Bioinformatics analysis

Orthomcl software was used to self-compare the amino acid sequences of all predicted genes in Mycoplasma genome. The amino acid sequences of each predicted gene were compared with those of other genes. The certain threshold (E-value: $1e^{-5}$, Percent Identity Cutoff: 0, MarkovInflation26 Index: 1.5) for similarity clustering was selected. The antigenic epitopes of P113/P126 family proteins were predicted by Immunomedicine Group: Tools (http://imed.med.ucm.es/Tools/index.html).

2.3. Recombinant proteins of M. ovipneumoniae expressed in Escherichia coli (E.coli)

The whole genome DNA of *M. ovipneumoniae* strain SC01 was taken as template. PCR amplification primers for prokaryotic expression of p113/p126 family proteins were designed by Premier 5 software (Canada). The coding region of amplified gene fragment in primer design region contained abundant epitopes with high antigen index, without containing TGA codon. The specific information of primers was shown in Table 1. The primers and target fragments were synthesized by Shanghai Shenggong bioengineering company (China).

The PCR product was purified and recovered with gel extraction kit (Tiangen Biochemical Technology Co., Ltd, China). pET-32a (+) empty plasmid and purified target fragment were digested by restriction enzymes (BamH I and Xho I; BamH I and hind III). Cyclipure kit (Omega, USA) was used to purify, recover and connect the enzyme digestion products. Convert the linked product to*E.coli* DH5α. Competent cells were cultured at 37 . Single colonies were selected and cultured in LB medium containing ampicillin for overnight. Plasmids were extracted by Plasma Miniprep kit (Omega, USA). Which was identified by PCR amplification and double enzyme digestion. The samples identified as positive clones were sent to the sequencing company for sequencing confirmation. The correct recombinant expression vector was identified and named pET-32a (+) – X. The encoded recombinant protein was named rPX (X represents each member of the gene family).

The positive recombinant plasmids pET-32a (+) - X and pET-32a (+) were transformed into *E.coli* Rosetta (DE3) and *E.coli* BL21 (DE3), respectively. The single colony was selected and inoculated into LB culture medium for overnight culture. IPTG with final concentrations of 0.4 mM and 1 mM was induced at 2 h, 4 h, 6 h, 8 h and overnight (recorded as 9 h). The precipitates were collected by centrifugation and resuspended with PBS, and then mixed with the same volume of 2 × SDS-PAGE gel sample buffer mixture. After boiling water bath for 5 min, SDS-PAGE detection was carried out with 12% concentration of separation gel.

2.4. Antigenicity assays of recombinant proteins

To evaluate the antigenicity of *M. ovipneumoniae* proteins expressed in *E. coli* , Western blot and ELISA assays were carried out using sera from sera from goats immunized with *M. ovipneumoniae* strain SC01 inactivated whole cell (n = 5), clinically infected sheep (n = 50) and negative sera (n = 8).

2.4.1 Western blot with recombinant proteins

Purified recombinant P128 proteins were subjected to SDS-PAGE electrophoresis. The protein transferred to the nitrocellulose membrane was sealed with skimmed milk powder solution overnight. It was incubated with Goat anti *M. ovipneumoniae* strain SC01 positive serum as primary antibody. Horseradish peroxidase labeled Rabbit anti goat IgG secondary antibody was incubated.

Imaging was performed in the Immun-Star Western Chemiluminescence Kit (Bio-Rad, USA) and Versa Doc imaging system (Bio-Rad, USA). The goat serum with negative antibody to *M. ovipneumoniae* was used as the control for Western blot analysis. The determination of other proteins was the same as P128.

2.4.2 ELISA with recombinant proteins

The purified recombinant protein was used as the coating antigen. Indirect ELISA was performed with 50 clinically infected sheep sera and 8 negative animal sera as primary antibodies. The purified recombinant pro-

tein was coated with enzyme label plate. After adding enzyme labeled secondary antibody, OD_{450} absorbance value was measured by enzyme labeled instrument.

2.5. Immunogenicity assays of recombinant proteins

2.5.1. Immunization of mice

Sixty female BALB/c mice (Dashuo experimental animal Co., Ltd., China) aged 6 ˜ 8 weeks were divided into 10 groups with 6 mice in each group. Mice were immunized for 60 μg proteins (rP113, rP128, rP133, rP116, rP126, rP125, rP130, rP109, rP114) and 200 μL PBS as negative control. The second immunization was conducted 21 days after the first immunization, and the dose was the same as that of the first immunization.

2.5.2. Evaluation of the humoral immune response through ELISA with recombinant proteins

The serum of mice was collected at 0, 7, 21, 28, 42 and 63 days to evaluate the antibody level induced by recombinant protein. The recombinant protein was used as coating antigen. The serum titers of the same group of 6 sera mixed at the same time were detected by indirect ELISA.

2.5.3. Evaluation of the humoral immune response through extract ELISA

Ultrasonic fragmentation of *M. ovipneumoniae* strain SC01 was carried out in ice water bath. After the concentration was determined by BCA kit (Abcam, China), the bacterial protein was coated on the ELISA plate. The serum titers of 6 sera in the same group at the same time were detected. The PBS immunized group was the negative control group, which was detected by indirect ELISA.

2.6. Statistical analysis

GraphPad Prism (version 5.0, Graph Pad Software Inc., San Diego, CA, USA) was used to perform statistical analysis. The student's t-test was performed to analysis, as the data followed a standard normal distribution. Differences were considered significant when $P< 0.05$.

3. Results

3.1 Bioinformatics analysis

There are several gene families in the whole genome of *M. ovipneumoniae* strain SC01 strain. There are four paralogous genes of p113 and p126. According to the estimated molecular weight of the encoded protein, the homologous genes of p113 were named p128, p133, p141 and p116. The paralogous genes with p126 are named p125, p130, p109 and p114. The basic characteristics such as the size of each gene and the molecular weight of the coding protein are analyzed. The specific information is shown in Table 2. The G + C content (%) of each gene ranged from 27.16 to 33.92, which was consistent with the G + C content (%) of *M. ovipneumoniae* 28.85. In addition, except p109, each gene contained 2 ˜ 6 TGA codons, which encoded tryptophan in Mycoplasma. The 10 proteins of p113/p126 family contain at least 33 epitopes, suggesting that they have good immunogenicity. The results were shown in Table 2.

p113 and p126 and their paralogous gene members were arranged in pairs adjacent to each other in the genome. It was completely consistent with the arrangement of lineal homologous genes in *M. hyopneumoniae* , as shown in Fig. 1. At present, there is no complete map of the whole genome of *M. ovipneumoniae,* so the physical location of each gene in the genome cannot be determined.

3.2 Expression and purification of recombinant proteins

The amplifications of all 10 gene (p113, p128, p114, p133, p141, p116, p126, p125, p130, p109) fragments were consistent with the expected size, as shown in Fig. 2a. Double enzyme digestion identification showed that there were obvious bands at the target band and about 5800 bp, as shown in Fig. 2b. Therefore, 10 recombinant prokaryotic expression vectors were successfully constructed.

Induced by IPTG at the concentration of 0.4 mM and 1 mM, the bacterial solution was collected at the induction time points (0 h, 2 h, 4 h, 6 h, 8 h and overnight) for SDS-PAGE detection. The results showed that

except that p141 was not expressed successfully, the other members could express the target protein bands consistent with the expectation. The best induction time of rP113 and rP109 was 6 h; the best induction time of rP128, rP133, rP126 and rP125 was overnight; the best induction time of rP116 and rP114 was 8 h; the best induction time of rP130 was 4 h. The best induced IPTG concentration of rP114 was 1.0 mm; the other eight proteins were the best induced IPTG, and the concentration was 0.4 mM. The expression engineering bacteria transformed by no-load had no expression of related proteins. The results were shown in Fig. 3.

3.3 Antigenicity of recombinant proteins

The purified 9 recombinant proteins can bind to Goat anti *M. ovipneumoniae* whole bacteria antibody, as shown in Fig. 4. The results showed that p113/p126 family proteins had good antigenicity and could induce the body to produce specific antibodies, except for p141 protein.

The 9 recombinant proteins could react with the clinical infected sera, which were significantly higher than those in the negative control. According to the generated OD_{450} value, the order was rP128 > rP126 > rP113 > rP125 > rP130 > rP114 > rP116 > rP133 > rP109. Among them, rP128 can produce the highest OD_{450} value, followed by rP126 and rP113, as shown in Fig. 5.

3.4 Immunogenicity assays of recombinant proteins

The result of the humoral immune response induced by 9 recombinant proteins could induce mice to produce specific antibodies against the purified recombinant protein. Among them, the antibody levels of rP113, rP128, rP126, rP130 and rP109 increased continuously after twice immunization, and did not decrease until the 63^{rd} day after the first immunization. The antibody levels of rP114, rP133, rP125 and rP116 decreased at 28 and 42 days after the first immunization, respectively, as shown in Fig. 6.

The antibodies induced by rP113, rP109 and rP130 could efficiently bind to the whole bacterial protein, and their OD_{450} value was significantly higher than that of PBS control group. At 63 days after immunization, the induced antibody could still bind to the whole bacterial protein, as shown in Fig. 7.

4. Discussion

M. ovipneumoniae was first isolated in 1963 and is the main pathogen causing atypical pneumonia in sheep, goats and small ruminants (T. E. Besser et al., 2021; Rifatbegovic, Maksimovic, & Hulaj, 2011). The p113/P126 gene family of*M. ovipneumoniae* was highly homologous with p97/P102 of *M. hyopneumoniae* , and has similar structural characteristics (Falong, Huanrong, Cheng, & Hua, 2013). In this study, the remaining 9 proteins of p113/P126 family except p141 were successfully expressed, and the recombinant proteins were prepared. It was proved that these 9 proteins were good immunogens. Among them, rp128, rp126 and rp113 had the strongest binding ability with the serum of infected animals and belonged to the dominant immunogen; After immunizing mice with the above recombinant proteins, the mice can produce specific antibodies. Among them, the antibodies produced by immunizing the body with rp113, rp109 and rp130 can effectively bind to bacterial proteins.

Adhesin is an important component of Mycoplasma(Guo et al., 2017; Huang et al., 2019). It is correlated with pathogenicity, which plays an important role in the colonization of Mycoplasma on host cells(Deng et al., 2018). p113 and p126 of *M. ovipneumoniae* are homologous with p97 and P102 of *M. hyopneumoniae* . Therefore, there is also a p113/p126 paralogous gene family in *M. ovipneumoniae.* p113 belonged to the same family as p128, p133, p141 and p116, and p126 belonged to the same family as p125, p130, p109 and p114. The genes P97 and P102 encoding p97 and p102 form an operon structure, and there are multiple paralogous genes of p97 and p102 in the genome of *M. hyopneumoniae* , forming a p97/p102 paralogous gene family. Its members include p97, p102, p146 and p216. p97/p102 family members are mostly adhesion molecules, which are not only involved in the adhesion of*M. hyopneumoniae* to host cells, but also a good immunogen of*M. hyopneumoniae* (Gauthier, Babych, Segura, Bourgault, & Archambault, 2020; Kamminga et al., 2020). Therefore, it has become an important candidate protein for new vaccines and serological diagnostic techniques.

Serological detection of *M. ovipneumoniae* antibody is an important means for epidemiological investigation, detection and evaluation of vaccine immune effect. Serological methods need specific, sensitive, stable and reliable antigen proteins(Ayouba et al., 2017). Each member protein of p113/p126 family of *M. ovipneumoniae* is an immunogenic molecule of *M. ovipneumoniae* , which can induce immune response in infected animals. When establishing serological diagnostic methods such as ELISA, the coated antigen must be able to react with the serum of most infected animals and bind efficiently. Infected animal serum and negative serum was used to obtain dominant immunogens by immunizing the antibody level produced by recombinant protein in*M. hyopneumoniae* (S. Simionatto et al., 2012). In order to screen the coating antigen of dominant immunogen for the serological diagnosis of *M. ovipneumoniae* , we took the recombinant protein as the coating antigen, and used 50 infected animal sera and 8 negative sera as the primary antibody for indirect ELISA analysis. rP128, rP126 and rP113 can efficiently combine with most clinical infection sera and are dominant immunogens, so they have the potential to develop serological diagnosis technology. In another study, serological detection was used to detection of *M. hyopneumoniae* on pigs by ELISA analysis (Qiu et al., 2017). In this study, the dominant immunogens were screened from p113/p126 family, which provided a basis for the establishment of serological diagnosis technology.

The recombinant proteins of p113/p126 family have the potential value of developing recombinant subunit vaccine and genetic engineering vector vaccine. 9 recombinant proteins immunized mice could induce effective humoral immunity. Among them, the antibody level of rP113, rP128, rP126, rP130 and rP109 increased continuously after twice immunization and continued to the 63rd day after the first immunization at the end of the experiment. The antibody levels of rP114, rP133, rP125 and rP116 decreased 28 and 42 days after the first immunization, respectively. rP128, rP113, rP109, rP133, rP130 and rP126 protein immune sera can bind to whole bacterial protein to varying degrees, while the antibodies against rP113, rP109 and rP130 have the strongest binding ability to bacterial protein. Recombinant Proteins (P102, P95, P46, P97) were used to diagnose the infection of *M. hyopneumoniae* (S Simionatto et al., 2022). The antibodies produced after immunization with rP113, rP109 and rP130 can effectively bind to the bacteria, which may play an immune protective role, but it still needs further research by immunization and virus attack protection test. However, this study provides useful candidate antigen molecules for the subsequent development of recombinant subunit vaccine and genetic engineering vector vaccine.

In *M. hyopneumoniae* , p97/p102 family is adhesin family(L. Woolley et al., 2014). Therefore, antibodies against these proteins can block the adhesion of *M. hyopneumoniae* to the host, resulting in immune protection. The high homology between *M. ovipneumoniae* p113/p126 and *M. hyopneumoniae* p97/p102 family suggests that p113/p126 family may have adhesion characteristics, but whether these protein molecules are adhesin molecules of *M. ovipneumoniae* and participate in the adhesion process of *M. ovipneumoniae* still need to be verified by further experiments.

5. Conclusion

Members of P113/P126 family have high homology with members of P97/P102 family of *M. hyopneumoniae* . This study proved that P97/P102 family proteins were immunogens (except P141, which was not successfully expressed), and the dominant immunogens were obtained. The ability of recombinant protein as subunit vaccine was evaluated. It provides an experimental basis for the establishment of serological diagnostic technology and the development of new nucleic acid vaccines.

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Conflict of interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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FigureLegend

Fig.1 The position of each gene in the whole genome

Fig.2 Gene amplification and digestion of recombinant plasmid fragments

A: Amplification of p113/p126 gene family by PCR (M: DNA Marker; 1˜10: p113, p128, p114, p133, p141, p116, p126, p125, p130 and p109). B: Identification of recombinant plasmids digested (M1, M2: DNA Marker; 1˜10: p113, p128, p114, p133, p141, p116, p126, p125, p130, p109).

Fig. 3 Expression of pET32a (+)-P113/P126 in *E.coli* BL21 and E.coli Rosetta

A˜I: rP113、rP128、rP133、rP116、rP126、rP125、rP130、rP109 and rP114; M: Protein Marker; 0a˜9a: Expression products of pET32a (+) after 0, 2, 4, 6, 8 h and overnight; 0b˜9b: Expression products after 0, 2, 4, 6, 8 h and overnight with 0.4 mM IPTG; 0c˜9c: Expression products after 0, 2, 4, 6, 8 h and overnight with 1 mM IPTG

Fig. 4 Western blot analysis of rP113/rP126(A˜I: rP113, rP128, rP133, rP116, rP126, rP125, rP130, rP109, and rP114; M: Protein Marker; 1: Purified protein P113/P126)

Fig. 5 Screening for recombinant immunogens by ELISA

Fig. 6 Results of antibody response of mice immunized with rP113/rP126 by ELISA

Fig. 7 The binding ability between P113/P126 and whole bacterial protein of M. ovipneumoniae