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Original article

Expression and immunogenicity study of a novel mhp183 gene fragment of *Mycoplasma hyopneumoniae*

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Abstract

A highly immunogenic nucleotide fragment (195bp) was selected from the Mhp183 gene of *Mycoplasma hyopneumoniae* using information software technology and was named Mhp183195bp. Three Mhp183195bp were linked to form a new nucleotide sequence called Mhp183615bp. Mhp183615bp was directly synthesized and cloned into a pET100 vector and expressed in *Escherichia coli*. After purification, the proteins were successfully validated using SDS-PAGE and Western blot. BALB/c mice were injected with purified proteins on the first, eighth, and fifteenth days of feeding, respectively; serum samples were collected from mice on the day of immunization and on the 22nd day after immunization. The antibody level in mouse serum was detected by Western blotting using purified expressed proteins as antigens. IL-2, TNF-α and IFN-γ were simultaneously detected in mouse serum by ELISA. The 30 kDa protein was successfully expressed and reacted specifically with the specific serum Mhp His-Tag mouse monoclonal antibody and pig antibody. The expressed recombinant protein was immunogenic. The expression levels of IFN-γ, IL-2 and TNF-α were found to be significantly higher on day 22 than in the control group. This study suggests that the expressed recombinant protein could be used as one of the novel vaccine candidates for Mhp.

Key words: *Mycoplasma hyopneumoniae*, Mhp183 gene, new generation vaccines

Introduction

Mycoplasma pneumoniae of swine is a chronic respiratory disease caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*, Mhp), which can cause cough and asthma in diseased pigs under natural conditions (Zhu et al. 2017). *M. hyopneumoniae* vaccines include an inactivated vaccine, an attenuated vaccine, and a subunit vaccine that includes genetic engineering (Zhu et al. 2017). Genetically engineered vaccines are more advantageous in terms of yield, cost, safety and efficiency (Yu et al. 2018, Yu et al. 2019). Therefore, genetically engineered vaccines for *M. hyopneumoniae* have a broader prospect.

Mhp P97 protein is one of the antigens for the study of new genetically engineered vaccines. Zhang et al. (1995) first discovered the adhesion protein P97 and demonstrated that the protein has two repeat regions

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GCAGCAAAACCAGAAGCAGTTAAACCAGTCGCCGCAAAACCAGAGGCCG
CAAAACCGGAAGCAGCAAAACCGGTCGCAGCAAAACCTGAAGCAGCAAA
ACCGGTAGCAGCAAAACCGGAGGCAGCAAAACCGGAAGCCGCAAAACCT
GTGGCAGCAAAACCGGTTGCAGCAAAACCGGTTGGAGGCGGTGGAAGCG
CAGCAAAACCAGAAGCAGTTAAACCAGTCGCCGCAAAACCAGAGGCCGC
AAAACCGGAAGCAGCAAAACCGGTCGCAGCAAAACCTGAAGCAGCAAAA
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TGGCAGCAAAACCGGTTGCAGCAAAACCGGTTGGAGGCGGTGGAAGCGC
AGCAAAACCAGAAGCAGTTAAACCAGTCGCCGCAAAACCAGAGGCCGCA
AAACCGGAAGCAGCAAAACCGGTCGCAGCAAAACCTGAAGCAGCAAAAC
CGGTAGCAGCAAAACCGGAGGCAGCAAAACCGGAAGCCGCAAAACCTGT
GGCAGCAAAACCGGTTGCAGCAAAACCGGTT

Fig. 1. Nucleotide sequence of *Mhp183*$_{615bp}$

at the C-terminus. Later, Liu et al. (2005) expressed the R1 region of P97 protein by constructing a vector, which proved that this region has strong immunogenicity. Since then, the R1 region of P97 protein has been the main target of recombinant subunit vaccines. However, several studies have shown that these vaccines do not stimulate the production of large amounts of anti-Mhp antibodies in the organism of immunized pigs and provide only partial immune protection against Mps (Thaker et al. 1998, Peng et al. 2019). Therefore, scholars have sought to recombine P97R1 with other antigens. P97R1 can induce specific immunoglobulin G by recombination with toxins (Chen et al. 2001). They also recombine with the B subunit of Escherichia coli dengue enterotoxin, which also produces specific IgA and IgG and has a protective role (Lu et al. 2010, Cunha et al. 2017). A chimeric protein containing a single antigenic protein of P97R1 enhanced immune protection, indicating the feasibility of chimeric protein compositions. This suggests that even recombinant MhpP97R1 vaccines containing other antigens can only partially protect the body from damage. As a result, the search for better Mhp immune protection has become a new research hotspot. IM and SC injections of DNA or protein-mixed vaccines composed of potentially protective antigens P36, P46, NRDF, P97 and P97R1 antigens have been found to produce significant humoral immunity and Th1 responses in mice (Chen et al. 2008). Mixed vaccines also have some advantages. Ma et al. (2011) constructed and expressed four adhesion genes (p36, p46, p65 and p97R1-NRDF), and recombinantly injected the expression products intramuscularly into mice, thus showing good immune effects.

Nevertheless, polyantigenic vaccines are ineffective in the prevention of Mhp infections (Ma et al. 2011, Marchioro et al. 2014). Both single-antigen and multi-antigen Mhp genetically engineered vaccines struggle to provide adequate protection. Hence, new ideas for Mhp genetically engineered vaccine research need to be sought. Wang et al. (2016), while studying the multivalent nano-antibodies of dengue virus type 2 NS1 protein, found that the expression of polyated epitope polypeptide in E. coli BL21(DE3) was highly soluble and significant by repeating the antibody fragments two and three times, respectively, and the effect was good. Such a research concept provides a useful reference for this study.

The binding of Mhp to porcine cilia requires at least eight P97R1 repeaters; meanwhile, antibody recognition requires at least three repeaters (Minion et al. 2000). The R1 region can be used as the primary antigenic determinant to generate a robust immune response. However, it also can play the role of cilia adhesion. Increasing the number of repeats provides strong adhesion capacity (Minion et al. 2000). In this study, a 195bp nucleotide fragment from Mhp183 genes with strong immunogenicity was selected by informational software. These 65 amino acids expressed from the 195bp nucleotide fragment are the 13 repeats in the P97 protein. A new type of subunit vaccine with good immunity was obtained by linking three identical 195bp nucleotide sequences and expressing them in E. coli.

Materials and Methods

Identification of target genes and encoded amino acids

The R1 region gene fragment (195bp) of the Mhp183 gene obtained from the whole genome of the *M. hyopneumoniae* strain (GenBank accession number: NC_006360) was designed as a novel gene fragment (615bp) according to the method shown in Fig.1. As can be observed, the 615bp nucleotide fragment consists

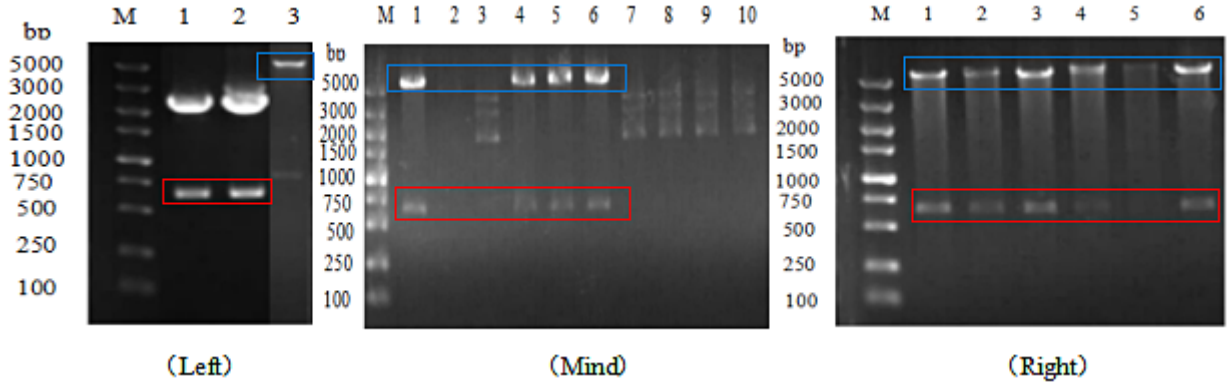


Fig. 2. Identification of *pUC57-Mhp183*$_{615bp}$ by *BamHI* and *NdeI*.
(Left) 1-2: *pUC57-Mhp183*$_{615bp}$ digested with *BamHI* and *NdeI* at 37°C for 2 h, where 615bp (red box) is the fragment to be recovered; 3: PET100 digested with *BamHI* and *NdeI* at 37°C for 2 h, where 5646bp (blue box) is the fragment to be recovered.
(Mid) 1-10: *pUC57-Mhp183*$_{615bp}$ from 10 strains of *E. coli* DH5α digested with *BamHI* and *NdeI* at 37°C for 2 h, where 5646bp (blue box) and 615bp (red box) were harvested, respectively.
(Right) 1-6: *pUC57-Mhp183*$_{615bp}$ from 6 strains of *E. coli* BL21(DE3) digested at 37°C

of three R1 region nucleotides (195bp, underlined) and two 15 bp linker genes (black frames).

Gene synthesis

The target gene (615bp) synthesized by Hunan Qingke Biotechnology Co., Ltd. ligated the synthesized target gene to the pUC57 of the vector. The constructed plasmid was named pUC57-Mhp183$_{615bp}$.

Construction and transformation of pET100-Mhp183$_{615bp}$ plasmids into BL21 cells

The pUC57-Mhp183$_{615bp}$ plasmid and pET100 vector were digested with BamHl and Ndel restriction endonucleases, respectively. The two target fragments were ligated and transformed into *E. coli* DH5α, and the plasmid was extracted from the transformed *E. coli* DH5α and transformed into BL21.

Expression and identification of recombinant plasmid

E. coli BL21 containing the target fragment were induced for expression by IPTG at 29°C for 5 h. *E. coli* BL21 was collected and identified by SDS-PAGE.

Purification and identification of recombinant protein

After the recombinant protein was purified by nickel column, the purification effect was confirmed by SDS-PAGE. Western blot identified the immunogenicity of the recombinant proteins.

Animal trial

Twenty BALB/c female mice were randomly divided into four groups, each with an average weight of about 21 g. The four groups were a high-dose group (100 μg purified protein), a medium-dose group (50 μg purified protein), a low-dose group (10 μg purified protein), and a control group (equal volume of PBS). Emulsification of purified protein and adjuvant was performed by intraperitoneal injection on d-1 (Freund s Adjuvant Complete), d-8 (Freund's incomplete adjuvant), and d-15 (Freund's incomplete adjuvant) of the trial, respectively. Blood samples were collected from mice before the test and on d-22. The level of IL-2, TNF-α, and IFN- γ in the serum was detected by ELISA (BD company, American). Purified protein was used as an antigen to detect antibodies in mouse serum by Western blot.

Results and Analysis

Cloning of Mhp183$_{615bp}$ gene fragments

Plasmid pUC57-Mhp183$_{615bp}$ and vector pET100 were simultaneously verified by BamHI and NdeI, and the target fragment (615bp) and vector fragment (5646bp) are consistent with expectations (Fig. 2, left). To obtain the connection product pET100-Mhp183$_{615bp}$, 10 strains of *E. coli* DH5α were extracted and verified by BamHI and NdeI. Four clones simultaneously showed 5646bp and 615 bands. Among them, 615 bp was sequenced and analyzed and found to be consistent with the expected size (Fig. 2, mid). The recombinant positive plasmid pET100-Mhp183$_{615bp}$ was transformed into *E. coli* BL21 (DE3), and six transformed BL21 clones were re-extracted and verified by BamHI and NdeI. All six clones were successfully transformed (Fig. 2, right). The plasmids extracted from *E. coli* BL21 clones were sequenced and analyzed, and the sequence is consistent with expectations (615 bp).

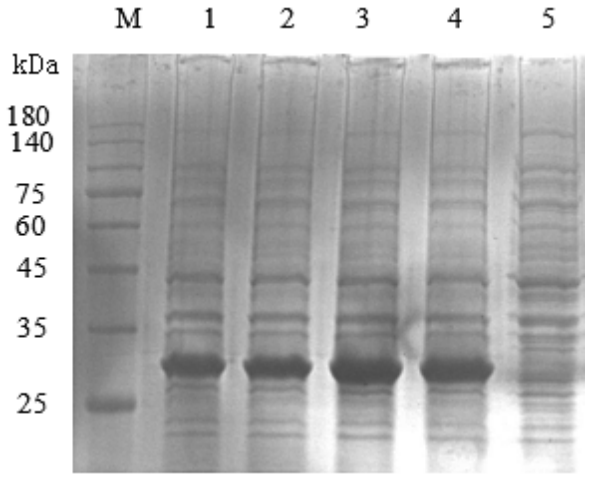


Fig. 3. *pUC57-Mhp183*$_{615bp}$ expressed and identified by SDS-PAGE in *E. coli* BL21(DE3).
M: molecular weight standard for PM2500 protein; 1-4: identification of expressed proteins of four recombinant *E. coli* BL21 (DE3) induced by 1 mM IPTG for 5 h; identification of a 30 KD protein (Red box); 5: identification of expressed proteins of one recombinant *E. coli* BL21 (DE3) without induction

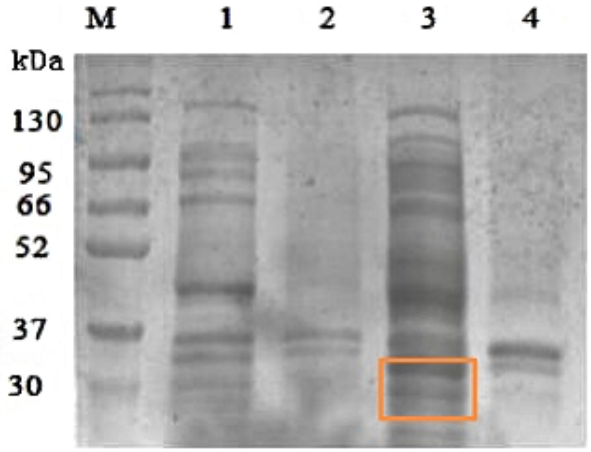


Fig. 4. Screening of high-level expression plasmids and SDS-PAGE identification results.
M: PM001-0500 Protein Molecular Quality Standard; 1: recombinant positive bacteria expressing supernatant before induction; 2: recombinant positive bacteria expressing precipitated protein before induction; 3: recombinant positive bacteria expressing supernatant after 5 h induction in 1 mM IPTG; 4: recombinant positive bacteria expressing precipitated protein after 5 h induction in 1 mM IPTG; SDS-PAGE simultaneous identification of the four proteins

Expression and SDS-PAGE identification of recombinant plasmid in BL21

After induction, whole protein electrophoresis was compared with that before induction (Fig. 3). There were apparent bands at the expected position of recombinant bacteria around 30 kDa, indicating a successful expression of pET100-Mhp183615bp under the action of IPTG. As can be seen from the figure, the highest expression of the target protein was observed in lane 3. In comparison with the supernatant and precipitate after ultrasonic cleavage of recombinant bacteria before and after induction (Fig. 4), lane 3 had a clear band in the expected position as compared to the other three lanes.

Protein purification and identification

The collected supernatant was purified using a nickel affinity chromatography column (Ni-). The target protein was washed with an imidazole at 20mM in lysis buffer and 50mM wash buffer, respectively. Finally, the target protein was eluted with an imidazole concentration of 500mM elution buffer (Fig. 5). The purified target protein was obtained after SDS-PAGE electrophoresis analysis.

Western blot identification of immunogenicity of purified protein

The purified protein was used as antigen and 6 His-tag mouse McAb was used as primary antibody; Western blot identification was successful (Fig. 6, left).

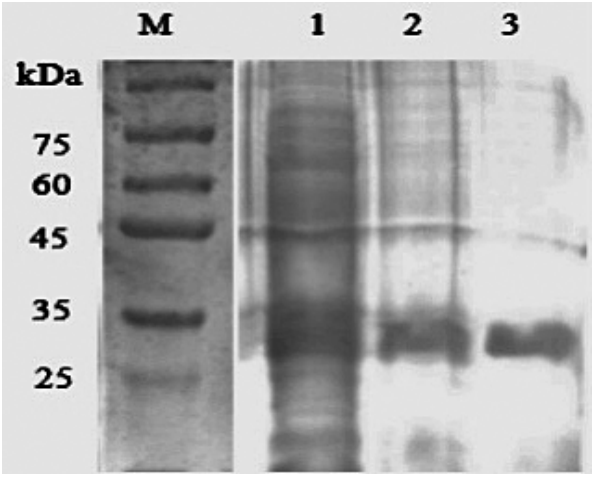


Fig. 5. Protein identification by SDS-PAGE.
M: PM2500 Protein Molecular Quality Standard; 1: identification of protein eluted by 20 mM lysis buffer; 2: identification of protein eluted by 50 mM wash buffer; 3: identification of protein eluted by 500 mM elution buffer

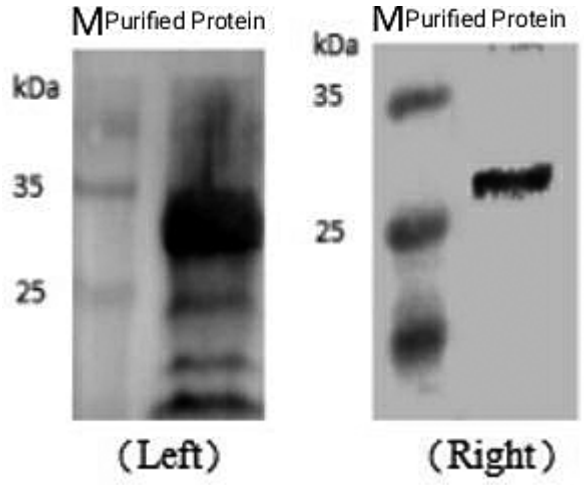


Fig. 6. Western blot identification of immunogenicity of purified protein.
M: Molecular weight standard of PM2500 protein
(Left): Purified protein used as antigen and mouse monoclonal antibody against His-tagged protein identified by western blot.
(Right): Purified protein used as antigen and primary antibody identified by western blot using porcine anti-Mhp specific serum

The purified protein was used as antigen and pig anti-Mhp specific serum as primary antibody; Western blot showed immunoreactivity (Fig. 6, right). The results show that the target gene expresses specific proteins.

Animal trial results

3.5.1 Assay of IFN- γ, IL-2, and TNF-α levels

Twenty mice were divided into four groups: high-dose group (5), medium-dose group (5), low-dose group (5), and control group (5). Twenty blood samples were collected before immunization and 20 blood samples were collected on d-22. The 40 sera were assayed with ELISA kits (Hua Mei, China) and the trends in IFN-γ (Table 1), IL-2 (Table 2), and TNF-α (Table 3) levels were analyzed. Fig. 8 shows the changing trend of cytokine doubling before and after the trial.

Taking the concentration of the standard as the ordinate and the OD value as the abscissa, the professional curve making software "Curve Expert" was used to analyze and make the standard curve according to the prompts. Based on the concentration and OD value of the standard sample, the regression equation of the standard curve is calculated. The OD value of the sample was substituted into the equation to calculate the concentration of the sample.

The results showed that after 22 days of immunization, the level of IFN-γ was 0.80 times higher than that 0 days before immunization (control group), 3.56 times in the high-dose group, 4.04 times in the medium-dose group, and 3.73 times in the low-dose group, suggesting that 50 μg (medium-dose group) is the optimal dose for immunized mice. The level of IL-2 was 0.71 times higher than that 0 days before immunization (control group), 1.17 times for the high-dose group, 0.98 times

Table 1. Detection of IFN - γ level in each group with ELISA Kits.

Time	Group	OD450nm measured value					average	IFN - γ level (pg/ml)
		1	2	3	4	5		
Day 0	Control	0.3207	0.541	0.2811	0.3822	0.3796	0.3809	251.8942
	High-dose	0.3755	0.3747	0.4597	0.3721	0.3802	0.3924	260.3654
	Medium-dose	0.4079	0.6260	0.4235	0.3716	0.2705	0.4199	277.5205
	Low-dose	0.1297	0.3595	0.2899	0.3687	1.3849	0.5065	328.0934
Day 22	Control	0.3033	0.3833	0.2200	0.3566	0.2478	0.3022	200.0493
	High-dose	1.0814	1.7806	0.7676	1.7427	1.6374	1.4019	927.8396
	Medium-dose	2.3018	2.2523	1.4485	0.7257	0.6772	1.4811	1120.740
	Low-dose	0.8266	1.844	1.8771	2.1256	1.9368	1.7220	1224.731

4th Degree Polynomial Fit: y = a + bx + cx^2 + dx^3 + ex^4, a = -3.50334185085, b = 346.265005529, c = 4.38242472254, d = -59.0103117946, e = 30.8414270076.

Table 2. Detection of IL-2 level in each group with ELISA Kits.

Time	Group	OD450nm measured value					average	IL-2 level (pg/ml)
		1	2	3	4	5		
Day 0	Control	0.1766	0.1529	0.1627	0.1452	0.1829	0.1640	119.9016
	High- dose	0.1324	0.2036	0.1275	0.1173	0.1289	0.1419	103.1871
	Medium -dose	0.1687	0.1500	0.1973	0.1317	0.1795	0.1654	120.9530
	Low dose	0.0995	0.1424	0.1772	0.1757	0.2082	0.1606	117.3092
Day 22	Control	0.1285	0.1111	0.1148	0.1058	0.1304	0.1181	85.17861
	High- dose	0.1599	0.1516	0.1453	0.1934	0.1758	0.1652	120.7656
	Medium- dose	0.1651	0.1658	0.1513	0.1635	0.162	0.1615	117.9872
	Low- dose	0.1436	0.1737	0.1712	0.1916	0.1973	0.1755	128.5528

3rd degree Polynomial Fit: y = a + bx + cx^2 + dx^3, a = -1.99482133899, b = 377.973902826, c = -10.7438132852, d = 49.9496419117.

Table 3. Detection of TNF-α level in each group with ELISA Kits.

Time	Group	OD450nm measured value					average	TNF-α level (pg/ml)
		1	2	3	4	5		
Day 0	Control	0.1105	0.0807	0.0686	0.0799	0.0933	0.0866	82.1861
	High- dose	0.0765	0.0679	0.0749	0.0463	0.0596	0.0650	59.1050
	Medium- dose	0.0849	0.0677	0.0844	0.063	0.1080	0.0816	76.7568
	Low- dose	0.0317	0.0429	0.054	0.0752	0.0608	0.0529	46.6344
Day 22	Control	0.0572	0.0624	0.0655	0.0612	0.0622	0.0617	55.5232
	High- dose	0.1393	0.1683	0.0775	0.1765	0.1980	0.1519	156.6730
	Medium- dose	0.2525	0.2658	0.1912	0.1164	0.1082	0.1868	199.2661
	Low- dose	0.1061	0.192	0.1669	0.1833	0.1854	0.16674	173.6335

4th Degree Polynomial Fit: y = a + bx + cx^2 + dx^3 +e x^4, a = -2.6004414581, b = 461.120970378, c = 539.658644582, d = -654.37639794, e = 354.741390321.

for the medium-dose group, and 1.10 times for the low-dose group, suggesting that 100 μg (high-dose group) may be the optimal dose for immunized mice. The level of TNF-α was 0.68 times higher than that 0 days before immunization (control group), 2.65 times for the high-dose group, 2.60 times for the medium-dose group, and 3.72 times for the low-dose group, suggesting that 10 μg (low-dose group) may be the optimal dose for immunized mice.

Identification of antibody against pure protein

Forty blood samples were collected in the experiment. The purified protein was used as an antigen

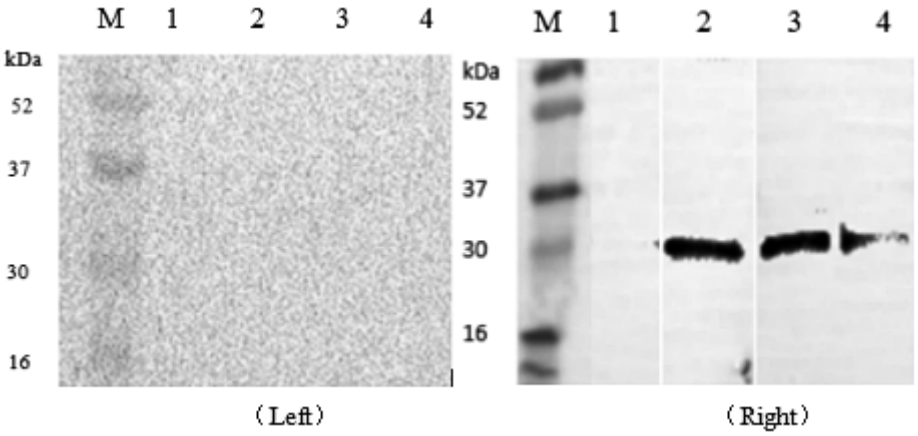
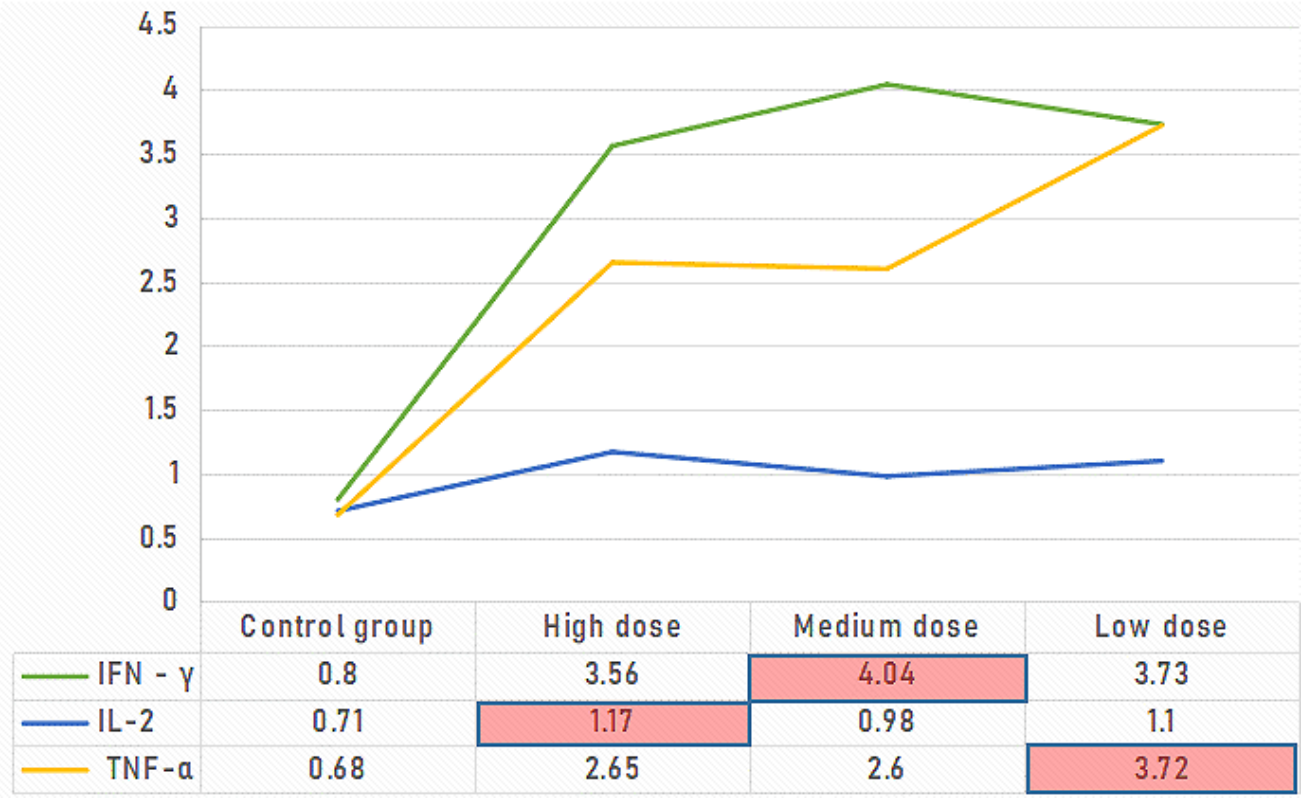


Fig. 7. Western blot identification in protein-immunized mouse sera.
(Left) M: molecular weight standard of PM001-0500 protein
(Left) 1-4: all four groups of mice were negative by western blot identification with purified protein as antigen 0 days before immunization
(Right) 1-4: Western blot identification of sera from control, high-dose, medium-dose and low-dose groups with purified protein as antigen 22 days after immunization, respectively



	Control group	High dose	Medium dose	Low dose
IFN - γ	0.8	3.56	4.04	3.73
IL-2	0.71	1.17	0.98	1.1
TNF-α	0.68	2.65	2.6	3.72

Fig. 8. The changing trend of cytokine doubling before and after the trial.

to identify anti-purified protein antibody content in mouse sera by Western blot. The results showed immunoreactivity in 20 mice 0 days before immunization (Fig. 7). On the 22nd day of immunization, 15 samples of the test group showed positive. In contrast, five sera from the control group showed negative, indicating an immunogenicity of the purified protein as an antigen.

The results showed high expression of purified protein, high antibody level in the medium-dose group, with no significant difference, and low expression in the low-dose group.

Discussion

Studies at home and abroad have shown that the ciliary adhesion factor P97 gene is a specific immunogenic gene for Mhp, and the R1 region located at the C-termi-

nus of the gene has been shown to be the antigenic determinant of P97, which is directly involved in adhesion (Li et al. 2018, Huo et al. 2019). As a result, 65 nucleotide sequences encoded by Mhp183 genes were used as research objects, and the recombinant gene Mhp183615bp was cloned into the prokaryotic expression vector pET100 to construct pET100-Mhp183$_{615bp}$. The correct position and orientation of the target gene insertion was determined by double restricted enzyme identification and sequencing, and named as pET100-Mhp183$_{615bp}$. After 5 h of induction at 29°C in its host strain BL21 (DE3), the final concentration of IPTG was 1.0 mmol/L, and a protein of about 30 kDa was expressed. The protein content was significantly increased compared to the bacterial protein before induction, indicating the expression of the recombinant gene. The recombinant protein being a fusion protein with a His-tag and was detected by Western blot using a His-tag mouse monoclonal antibody. The specific reaction between them indicated that the recombinant gene carrying six histidines was expressed. The recombinant protein was then isolated and purified by nickel affinity chromatography. The purified protein reacted specifically with porcine anti-Mhp-specific serum, demonstrating that the protein is highly immunogenic and can be further investigated as a vaccine protein.

The purified protein was used as the immunogen for the recombinant vaccine and the vaccine was administered to BALB/c mice. Commercial ELISA kits showed significantly increased serum levels of cytokines before and after immunization. The results showed that the ratio of IFN levels at day 22 to day 0 was 0.8 in control group. it were 3.56 in the high-dose group, 4.04 in the medium-dose group and 3.73 in the low-dose group, respectively. This suggested that 50 μg in medium-dose group may be the optimal dose for immunized mice. The results showed that the ratio of IL-2 levels at day 22 to day 0 was 0.71 in control group. it were 1.17 in the high-dose group, 0.98 in the medium-dose group and 1.10 in the low-dose group, respectively. This suggested that 100 μg in high-dose group may be the optimal dose for immunized mice. The results showed that the ratio of TNF-α levels at day 22 to day 0 was 0.68 in control group. it were 2.65 in the high-dose group, 2.60 in the medium-dose group and 3.72 in the low-dose group, respectively. This suggested that 10 μg in low-dose group may be the optimal dose for immunized mice. These showed that the vaccine can stimulate the body to produce cellular immunity and cytokines with different doses of purified proteins. On the day 22 after immunization, the serum of mice and recombinant protein in the Western blot group reacted with high specificity. These showed that the antibody levels were high in the high-dose group and in the medium-dose group, respectively, This suggested that 50-100 μg may be the optimal dose range for immunized mice. In this study, only the application of purified recombinant protein has been initially explored, so further studies are needed to prepare a commercial Mhp genetically engineered vaccine using recombinant protein.

The results showed that the P97R1 region of *M. hyopneumoniae* was highly antigenic and immunologically active (Liu et al. 2005). Scholars have constructed a recombinant Bacillus subtilis expressing *M. hyopneumoniae* P97R1 and evaluated its immunogenicity in mice. Western blot verified the expression of the antigenic protein, and the recombinant protein was then injected into the nasal cavity to immunize mice. ELISA analysis showed that cellular immunity were induced in serum and bronchoalveolar lavage fluid. Specific IL-4 and IFN-γ levels were increased in immunized mice, and the ability of lymphocytes to proliferate was enhanced (Wang et al. 2019). The protective effect of recombinant chimera rLTB-R1 has been tested in SPF piglets immunized by *M. hyopneumoniae* strains via the intranasal or intramuscular route. In this study, piglets injected intramuscularly with rLTB-R1 exhibited high levels of anti-R1 antibodies, but were not protected. On the other hand, piglets immunized intranasally with rLTB-R1 produced lower levels of anti-R1 antibodies, but were fully protected against experimental SMP (Ferreira et al. 2019). In contrast, the present study verified that this recombinant protein is highly immunogenic and can play a protective role through the construction of pET100-Mhp183615bp, intraperitoneal injection, mouse immunization, western blot and ELISA; however, it needs further research.

Conclusion

The recombinant protein expressed from a novel mhp183 gene fragment can be used as one of the novel vaccine candidates for Mhp.

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