Original article

A new strategy: high level expression and immunogenicity analysis of triplicate repeated multigenes in *Mycoplasma hyopneumoniae*

J. Li, G. Wang

College of Veterinary Medicine,
Hunan Agricultural University, Nongda road 1#, Changsha, Hunan, 410128, China

Abstract

Highly immunogenic nucleotide fragments from 3 genes of *Mycoplasma hyopneumoniae* strain 232 were selected using information software technology. After repeating each fragment three times, a total of 9 nucleotide fragments were joined together to form a new nucleotide sequence called $Mhp232_{1092bp}$. $Mhp232_{1092bp}$ was directly synthesized and cloned into a pET100 vector and expressed in *Escherichia coli*. After purification, the proteins were successfully validated by SDS-PAGE and Western blotting using mouse His-tag antibody and pig anti-Mhp serum. BALB/c mice were intraperitoneally injected with purified proteins in the high-dose (100 μg), medium-dose group (50 μg) and low-dose (10 μg) groups. Mice in each group were injected on day 1, day 8 and day 15 of feeding, respectively. Serum samples were collected from all mice on the day before immunization and on day 22 after immunization. The antibody level in the mouse serum was detected using western blotting using purified expressed proteins as antigens. IL-2, TNF-α and IFN-γ were simultaneously detected in the mouse serum by ELISA. The results showed that the 60 kDa protein was successfully expressed and reacted specifically with the specific serum Mhp His-Tag mouse monoclonal antibody and pig anti-Mhp serum. From day 0 to day 22 of immunization, IFN-γ increased from 269.52 to 467.74 pg/mL, IL-2 increased from 14.03 to 145.16 pg/mL, and TNF-α increased from 6.86 to 12.37 pg/mL. The IgG antibody in mice increased significantly from 0 day to day 22 after immunization. This study suggests that the expressed recombinant protein may serve as one of the novel vaccine candidates for Mhp.

Keywords: multi-epitope fusion protein, Mycoplasma hyopneumoniae, new generation vaccines

Correspondence to: G. Wang, e-mail: wanggp003@hunau.edu.cn

Introduction

Mycoplasma pneumoniae of swine is a chronic disease of the respiratory system with *Mycoplasma hyopneumoniae* as the pathogen (Liu et al. 2022). It is a common infectious disease on pig farms and has caused major economic losses to the pig industry worldwide (Biebaut et al. 2021). An effective way to control Mhp infection is vaccination (Virginio et al. 2014, Xu et al. 2021). At present, Mhp inactivated and attenuated vaccines have a certain immune effect, which can reduce lung damage, alleviate clinical symptoms and improve the productivity of sick pigs, but only provide partial protection (Wang et al. 2019). In addition, *M. hyopneumoniae* is difficult to culture in vitro, which makes existing vaccines more expensive (Chen et al. 2008). In pursuit of low cost and reliable protection, Mhp genetic engineering vaccine is a key research direction. P97 adhesion protein is an important adhesion element of *M. hyopneumoniae*. It is responsible for binding to the host ciliary epithelium and can stimulate the body to produce a specific immune response during early Mhp infection (Lu et al. 2010). It is generally believed that the R1 repeat sequence of P97, which mediates ciliary adhesion in pigs, could serve as a potential Mhp protective antigen fragment (Chen et al. 2006, Feng et al. 2014); the P46 protein is a surface antigen that induces an early immune response. It is highly conserved within the gene species and is highly immunogenic (Wang et al. 2019). DnaK protein has the ability to act as a molecular chaperone and immune adjuvant, which can enhance the body's immune response (Chen et al. 2003). In view of this, this study used bioinformatics technology to screen and recombine epitope fragments in the above three proteins and express them in the *E. coli* system (Li et al. 2008). The immune effect of the expressed protein was evaluated using animal experiments, providing a theoretical basis for exploring Mhp genetically engineered multi-epitope vaccines.

Materials and Methods

Bioinformatics analysis software for this study

Materials used for this study include: IEDB immune epitope database (http://tools.iedb.org/bcell/); ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/), TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM); SignalP5.0 (http://www.cbs._dtu.dk/services/SignalP/); Expasy protein light hydrophobicity analysis software (https://web.expasy.org/protscale/)

Gene selection and design

The bioinformatics software TMHMM2.0 and SignalP5.0 were used to analyze the transmembrane regions and signal peptides of the P46 protein, Dnak protein and P97 protein in *Mycoplasma hyopneumoniae* strain 232 (NC_006360). The immunogenic amino acid fragments in the three proteins were then predicted using the IEDB immune epitope database and ABCpred software. Two software-predicted identical or similar fragments were selected and each fragment was repeated three times and then connected end-to-end with a connector (amino acid GGGS), and a flexible connecting peptide (amino acid GGGGS) was used to connect the different protein fragments end-to-end. The final multi-epitope fusion gene of size 1092bp, named $Mhp232_{1092bp}$, was designed and the hydrophobicity of the target protein was predicted using Expasy analysis software.

Synthesis of the target gene

The target gene $Mhp232_{1092bp}$ was synthesized by Hunan Qingke Biotechnology Co., Ltd. The synthesized target gene was ligated with pCold II to construct the plasmid pCold II-$Mhp232_{1092bp.}$

Transformation of BL21 cells with recombinant plasmid *pCold II-Mhp232$_{1092bp}$*

The constructed recombinant plasmid was transformed into *E. coli* DH5α, and the plasmid was extracted from the transformed *E. coli* DH5α and then transformed into *E. coli* BL21 (DE3).

Expression, purification and identification of recombinant protein

Expression was induced using 0.8 mM IPTG at 15°C for 20 h. The expression bacteria precipitates were collected and the supernatant proteins were identified using SDS-PAGE by adding lysate and disrupting them ultrasonically. After the recombinant protein was purified with nickel ions, the purification effect was analyzed using SDS-PAGE, and the immunological activity of the purified recombinant protein was verified using Western-blot assay with mouse His-tag antibody and pig Mhp-specific serum.

Animal trials

28 BALB/c mice were randomly divided into 4 groups (7 in each group), namely 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 (sterile PBS). The first immunization was carried out on the first day. Purified pro-

teins of different concentrations and Freund's complete adjuvant were emulsified at a ratio of 1:1 and injected into mice intraperitoneally, each with 0.25 ml following the requirements of the Animal Ethics Committee, College of Veterinary Medicine, Hunan Agricultural University. The control group was injected with an equal volume of PBS solution. The second immunization and the third immunization were performed on day 8 of the test and day 15 of the test, respectively. Blood was collected from all test mice 0 days before the first immunization and 22 days after the test, and the serum was separated and used directly for testing or stored at -80°C for later use.

Development of an indirect ELISA method to detect anti-Mhp mouse serum from animal trials using expressed protein as antigen

The optimal protein coating concentration and the dilution ratio of the serum to be tested (primary antibody) were determined. Purified fusion proteins were diluted to 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/mL with carbonate buffer; the mouse-positive and mouse-negative sera were diluted at 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800, respectively. The diluted proteins and sera were used for the square matrix titration determination. Optimal time of action and enzyme--labeled antibody concentrations were carried out according to the recommended ratio according to the instructions manual for the reagents.

The criterion for determining the sample to be tested as positive was established at a level of 99.9% for a sample to be tested when the sample OD exceeds the mean OD and 3 times the standard variance of the negative serum (Lu et al. 2010). Twelve mouse anti-Mhp--negative sera by western-blot, obtained from the animal assay, were used for this assay.

A total of 28 mice blood samples were collected from all 28 mice at 22 days after the first immunization. IgG antibodies in these sera were detected using the newly established indirect ELISA method.

For the IgG content obtained from the OD values of the mice blood sample, standard curves were prepared. The brief steps for the standard curve preparation are as follows. Standard proteins (Zhengsibai, China) were diluted to 400, 300, 200, 100, 50, 25, 0 of protein solutions (µg/ml). Each concentration of the protein was measured for its OD value. In this way, the standard curve preparation takes the OD value as the abscissa and the protein concentration in the sample as the ordinate. The regression equation between the protein concentration (Y) and the OD (X) of the sample was also calculated.

Detection of anti-Mhp mouse serum from animal trials by Western-blot

Purified protein was used as antigen and the mouse serum to be tested was diluted 1:500 as primary antibody. So that the Western-blot test was performed.

Detection of the content of IFN-γ, IL-2 and TNF-α in mouse serum from animal trials

Fifty-six isolated test mouse sera were tested using an ELISA kit (Zhengsibai, China), and the OD values and concentrations of the standard were plotted as a standard curve to obtain a regression equation. The content of IFN-γ, IL-2 and TNF-α in the mouse serum was calculated, and the data were analyzed using GraphPad Prism software (v8.0.0.2) to evaluate the level of cellular immunity in mice.

Results

Bioinformatics analysis, gene selection and design

The transmembrane region of the P46 protein is located at amino acids 9- 31, and the transmembrane region of the P97 protein is located at amino acids 7- 29. The Dnak protein has no transmembrane region, and all the sequences located in the outer membrane region. The probability of the P46 protein containing a signal peptide is 99.040%, with the cleavage site at amino acids 30- 31. The probability of the P97 protein containing signal peptide is 33.792% and the cleavage site is defined. Dnak protein has 0.602% probability of containing a signal peptide and the cleavage site position is undefined. Based on analysis of the epitope, the selected target amino acid sequence of the P46 protein is ESDPKADDSNTIDAE, and AAKPVAAKPETTKPV AAKPEAAKPEAAKPVAAKPEAAKPVAAK PEAAKPVAAKPEAAKPVAAKPE for the P97 protein. The results of epitope analysis of Dnak proteins are not listed in this article due to patent application. The final multi-epitope fusion gene of size 1092bp, named Mhp232$_{1092bp}$, was designed and the corresponding amino acid sequence is shown in Fig. 1.

Expression, purification and identification of recombinant protein

After the pCold II vector containing the target gene Mhp2321092bp was transformed into the DH$_{5α}$ cloned strain, the two clear bands were verified by double enzyme digestion to prove the success of the recombinant plasmid construction (Fig. 2). The target protein expression was successfully induced at 0.8 mM IPTG at 15°C for 20 h with the expressed target protein and

NTSESDPKADDSNTIDAEIKQ GGGGS NTSESDPKADDSNTIDAEIKQ GGGS NT

SESDPKADDSNTIDAEIKQ GGGGS AAKPVAAKPETTKPVAAKPEAAKPEAAKP

VAAKPEAAKPVAAKPEAAKPVAAKPEAAKPVAAKPE GGGGS AAKPVAAKPE

TTKPVAAKPEAAKPEAAKPVAAKPEAAKPVAAKPEAAKPVAAKPEAAKPVAA

KPE GGGGS AAKPVAAKPETTKPVAAKPEAAKPEAAKPVAAKPEAAKPVAAKP

EAAKPVAAKPEAAKPVAAKPE

Fig. 1. Partial amino acid sequence of Mhp2321092bp.

    All full-length 364 aa consists of a 3-fold partial of the P46 protein sequence (NTSESDPKADDSNTIDAEIKQ), 3-fold partial of the P97 protein (AAKPVAAKPET TKPVAAKPEAAKPEAAKPVAAKPEAAKPVAAKPEAAKPVAAKPEAAK-PVAAKPE) and 3-fold partial of the Dnak protein sequence (not showed due to the patent application). GGGGS : Peptides used for the links

Fig. 2. Identification of expression strain by double enzyme digestion and purification and identification map of target protein.

    Left M: DL5000 Marker; 1-6: Expression strain plasmid double digestion

    Right M: protein molecular mass standard; 1: protein flow-through solution; 2: protein washing solution; 3: protein eluent

anti-Mhp mouse positive serum (1:500) and anti-Mhp pig positive serum (1:512, Jiangsu Academy of Agricultural Sciences, China) by SDS-PAGE (Fig. 3).

Development of an indirect ELISA method to detect anti-Mhp mouse serum from animal trials using expressed protein as antigen

    The square matrix titration test (Table 1) showed that the optimal coated concentration of purified protein was 1 μ g/mL, and the optimal dilution ratio of mouse serum was 1:200. According to these parameters, the ELISA kit was successfully prepared. It was then used to detect anti-Mhp antibodies from mouse sera derived from animal tests. The mean OD for these 12 negative sera was 0.1002 with a standard deviation of 0.0156. Therefore, the OD of the sample to be tested as positive was 0.147. A sample OD value greater than 0.15 was used as a positive criterion.

    The developed indirect ELISA method was used to detect specific IgG antibodies in mouse serum.

The results show that the 21 mouse sera in each immunization dose group were all positive (Fig. 4, Table 2) and the control group was negative. The average OD values were 0.0751 (control group), 1.5920 (high-dose group), 1.2094 (medium-dose group), and 0.6151 (low-dose group). Among these, the high-dose and medium-dose groups had extremely significant differences compared to the low-dose group ($p<0.01$), and the high-dose group had no significant differences compared to the medium-dose group ($p>0.05$).

    During the preparation of the standard curves, when the standard protein concentrations (μg/ml) were 400, 300, 200, 100, 50, 25 and 0, their corresponding OD values were 0.499, 0.419, 0.336, 0.216, 0.147 and 0.099, respectively. The standard curve is listed in Fig. 5. The regression equation between the protein concentration (Y) and the OD (X) of the sample was also calculated (Fig. 5). The average OD values were 0.0751 (control group), 1.5920 (high-dose group), 1.2094 (medium-dose group), and 0.6151 (low-dose group). Correspondingly, the antibody concentrations
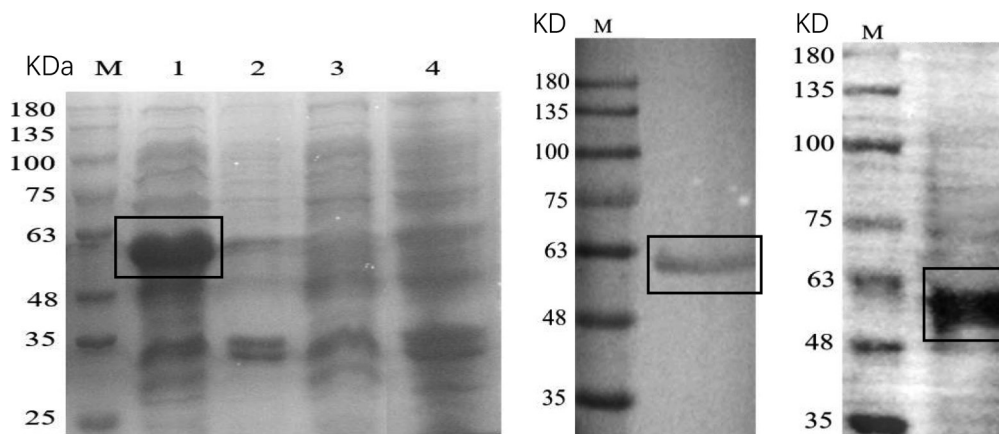
Fig. 3. SDS-PAGE electrophoresis diagram of protein expression analysis and Western-Blot identification map of the target protein.
Left M: Protein molecular quality standard; 1: Superalbumin after induction of expression; 2: Precipitation protein after induction of expression 3: Superalbumin before induction of expression; 4: Precipitation protein before induction of expression
Right M: protein molecular quality standard; left image: identification of purified protein using mouse His-tag antibody as primary antibody; right image: identification of purified protein using pig Mhp specific serum as primary antibody

Table 1. ELISA test results of different protein coating concentration and serum dilution ratio.

Mouse serum dilution ratio		Protein coating concentration (µg/mL)							
		8	4	2	1	0.5	0.25	0.125	0.0625
1:25	+	2.5212	2.5207	2.4622	2.4120	2.3737	2.0058	1.7070	1.1818
	-	0.0978	0.0961	0.0915	0.0910	0.0905	0.0891	0.0884	0.0813
	P/N	25.78	26.23	26.91	26.51	26.23	22.51	19.31	14.54
1:50	+	2.2666	2.2050	2.1510	2.0340	1.9134	1.8693	1.3915	0.9550
	-	0.0849	0.0825	0.0808	0.0831	0.0788	0.0782	0.0743	0.0722
	P/N	26.70	26.73	26.62	24.48	24.28	23.90	18.73	13.23
1:100	+	1.9339	1.8877	1.8009	1.6614	1.6172	1.5506	1.1708	0.7810
	-	0.0751	0.0713	0.0707	0.0660	0.0687	0.0627	0.0614	0.0578
	P/N	25.75	26.48	25.47	25.17	23.54	24.73	19.07	13.51
1:200	+	1.7699	1.7370	1.6871	**1.6514**	1.4650	1.2707	0.8715	0.6235
	-	0.0736	0.0679	0.0707	**0.0610**	0.0609	0.0586	0.0576	0.0571
	P/N	24.05	25.58	23.86	**27.07**	24.06	21.68	15.13	10.92
1:400	+	1.1616	1.0617	0.9978	0.9960	0.9485	0.6505	0.4876	0.3706
	-	0.0730	0.0646	0.0661	0.0624	0.0639	0.0583	0.0555	0.0510
	P/N	15.91	16.43	15.10	15.96	14.84	11.16	8.79	7.27
1:800	+	0.7351	0.7109	0.6933	0.6758	0.6625	0.4227	0.2713	0.2515
	-	0.0657	0.0608	0.0579	0.0565	0.0559	0.0558	0.0524	0.0505
	P/N	11.19	11.69	11.97	11.96	11.85	7.58	5.18	4.98

(µg/ml) in the serum of these mice were 0 (control group), 2581.11 (high-dose group), 1601.16 (medium-dose group) and 523.56 (low-dose group).

Detection of anti-Mhp mouse sera from animal trials by Western-blot

The western-blot test was performed to detect the anti-Mhp antibodies of mouse sera from animal trials. The results (Fig. 6) show that the intended band was in the expected position in each immunization group, which indicated a positive result. The results show

no intended band in the control group, which indicated a negative result. This suggested that the purified protein successfully stimulated the mice to produce specific antibodies and this protein is immunogenic.

Detection of the content of IFN-γ, IL-2 and TNF-α in mouse serum

The results (Fig. 7, Table 3-1) show that from day 0 to day 22 after immunization the serum levels of IFN-γ in mice increased by 423.16 pg/mL (high-dose group), 467.74 pg/mL (medium-dose group), and 269.52 pg/mL
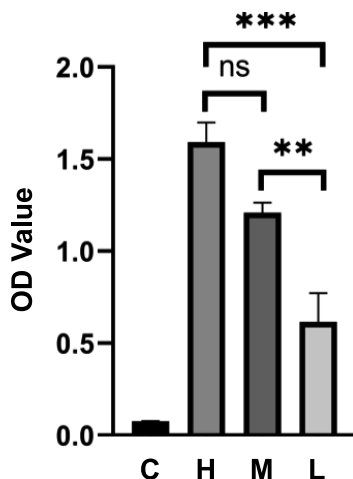
Fig. 4. Histogram of OD value of mouse serum specific IgG antibody.
Note: ns (p>0.05); ** (p<0.01); *** (p<0.001), the smaller the P value, the more significant

Table 2. ELISA test results of specific IgG antibody in mouse serum.

Group	OD_{450nm} measured value							OD_{450nm} average value
	1	2	3	4	5	6	7	
Control	0.0716	0.0795	0.0652	0.0849	0.0812	0.0737	0.0697	0.0751±0.0070
High	1.5445	1.5996	1.3083	1.2001	1.6067	1.8833	2.0018	1.5920±0.2856
Medium	1.3281	1.3361	1.1613	1.0895	1.0815	1.0598	1.4099	1.2095±0.1448
Low	0.6310	0.1819	0.2494	0.1782	1.1179	0.9076	1.0394	0.6151±0.4145
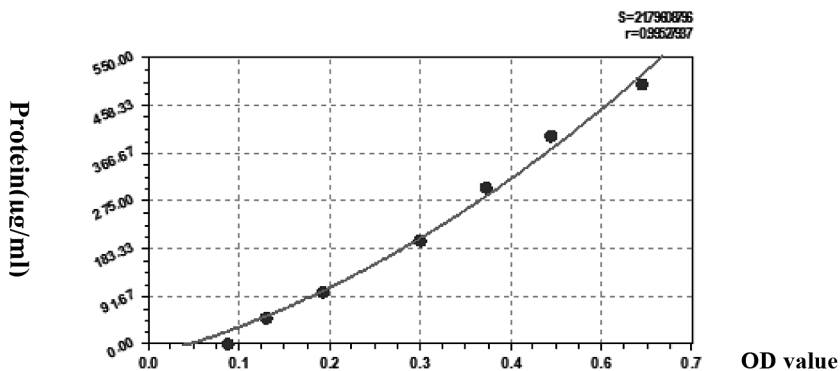
Fig. 5. The standard curve.
The regression equation: $Y = a + bX + cX2$
a = -22.1018139274, b = 416.091252506, c = 765.759599931

(low-dose group). Except for the control group, the differences were extremely significant (p<0.01) in each dose group compared to the pre-immunization period. Among them, the middle-dose group differed significantly from the high-dose group (p<0.05), and the middle-dose group differed significantly from the low-dose group. Compared to the dose groups, the differences were highly significant (p<0.01).

The results (Fig. 7, Table 3-2) show that from day 0 to day 22 after immunization the serum levels of IL-2 in mice after immunization increased by 145.16 pg/mL (high-dose group), 115.97 pg/mL (medium-dose group),

and 14.03 pg/mL (low-dose group). Among them, the high-dose group differed significantly from the pre-immunization (p<0.01), the middle-dose group differed significantly from the pre-immunization (p<0.05), and the low-dose group did not differ significantly from the pre-immunization (p>0.05).

The results (Fig. 7, Table 3-3) show that from day 0 to day 22 after immunization the serum TNF-α content of mice after immunization in each dose group increased by 10.04 pg/mL (high-dose group), 6.86 pg/mL (medium-dose group), and 12.37 pg/mL (low-dose group), respectively. There was no statistically signifi-
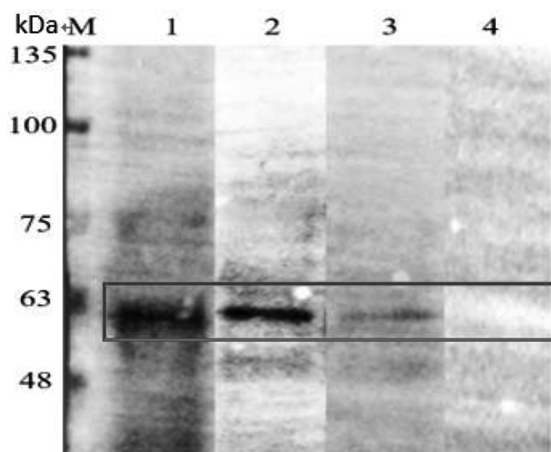
Fig. 6. Anti-Mhp mouse sera from animal trials were tested by SDS-PAGE.
1. Results from mouse serum from the high dose group
2. Results from mouse serum from the middle dose group
3. Results from mouse serum from the low dose group
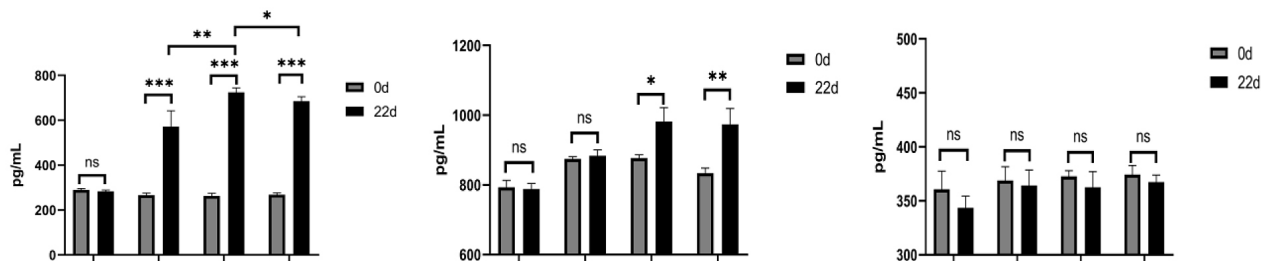4. Results from mouse serum from the control group

Fig. 7. Cytokines IFN-γ, IL-2, TNF-α detection histogram.
Leift: Histogram of serum IFN-γ content in each group
Note: ns (p>0.05); * (P<0.05); ** (p<0.01);*** (p<0.001), the smaller the P value, the more significant
Middle: Histogram of serum IL-2 content in each group
Note: ns (p>0.05); * (p<0.05); ** (p<0.01), the smaller the P value, the more significant
Left Histogram of serum TNF-α content in each group
Note: ns means the difference is not significant (p>0.05)

Table 3-1. ELISA test results of serum IFN-γ levels in each mouse group.

Date	Group	OD$_{450nm}$ value							Average	Level (pg/mL)
		1	2	3	4	5	6	7		
0 day	Control	0.3462	0.3427	0.3798	0.3483	0.3433	0.3598	0.3648	0.3550±0.0138	289.83
	High	0.3282	0.3301	0.3113	0.3334	0.3523	0.3643	0.3363	0.3366±0.0172	268.56
	Middle	0.3339	0.3210	0.3091	0.3887	0.3091	0.3265	0.3322	0.3315±0.0271	263.08
	Low	0.3426	0.3073	0.3530	0.3399	0.3364	0.3613	0.3019	0.3346±0.0222	266.47

cant difference between each dose group compared to pre-immunization (p>0.05).

Discussion

Mycoplasma porcine pneumonia is one of the serious respiratory diseases in the pig industry, and the conventional Mhp vaccine still has certain defects (Marchioro et al. 2014, Fisch et al. 2016). At present, the immune mechanism of *M. hyopneumoniae* vaccine is still not fully understood (Ogawa et al. 2009, Sette et al. 2010, Yang et al. 2021). According to the research of Thacker (2004), the commercial Mhp vaccine can effectively stimulate the body to produce specific antibodies and IFN-γ factors, which indicates that the body produces when responding to Mhp infection (Li et al. 2010). Humoral immunity and cellular immunity play an important role in Mhp infection (Trueeb et al. 2020, Mei et al. 2023). Therefore, in this stduy the ELISA method was used to detect the levels of IFN-γ, IL-2, TNF-α and specific IgG antibodies for assessing the immunogenicity of the fusion protein. The results showed

Table 3-2. ELISA test results of serum IL2 levels in each mouse group.

Date	Group	OD$_{450nm}$ Value							Average	Level (pg/mL)
		1	2	3	4	5	6	7		
0 day	Control	0.6983	0.6913	0.6738	0.6607	0.7653	0.6644	0.6743	0.6897±0.0360	793.43
	High	0.7133	0.7154	0.7631	0.7283	0.7258	0.6753	0.7041	0.7179±0.0266	833.93
	Middle	0.7425	0.7317	0.7717	0.7692	0.7325	0.7523	0.7342	0.7477±0.0171	871.15
	Low	0.7600	0.7590	0.7353	0.7409	0.7440	0.7524	0.7312	0.7461±0.0113	874.83
22day	Control	0.6393	0.7182	0.7210	0.6983	0.6913	0.6738	0.6607	0.6861±0.0300	788.14
	High	0.9072	0.8814	0.8789	0.8328	0.7490	0.7182	0.7210	0.8126±0.0815	973.80
	Middle	0.7724	0.8055	0.6999	0.9301	0.8463	0.8490	0.8243	0.8182±0.0714	981.84
	Low	0.7114	0.7872	0.7940	0.7423	0.7565	0.7521	0.7205	0.7520±0.0310	883.56

Note: A pre-test (data not showed) results suggested that standard curve formula is $Y = a + bX + cX^2 + d X^3 + e X^4$. Y refers to the level of the protein IL2 level (pg/ml) and the X refers to the IFN OD value. a = -0.342548695188, b = 142.729558265, c = 182.921229446, d = -95.7124134466, e = 21.7244774963

Table 3-3. ELISA test results of serum TNF-α levels in each mouse group.

Date	Group	OD$_{450nm}$ Value							Average	Level (pg/mL)
		1	2	3	4	5	6	7		
0 day	Control	0.5003	0.4516	0.5303	0.5603	0.5664	0.4403	0.4322	0.4973±0.0569	360.70
	High	0.5465	0.5406	0.5233	0.5033	0.5324	0.4843	0.4743	0.5150±0.0282	374.36
	Middle	0.4846	0.5262	0.5062	0.5112	0.5364	0.5026	0.5236	0.5130±0.0173	372.73
	Low	0.4514	0.5241	0.5445	0.5635	0.5240	0.4614	0.4874	0.5080±0.0423	368.99
22day	Control	0.4634	0.4447	0.4606	0.5112	0.4645	0.5412	0.4457	0.4759±0.0363	343.65
	High	0.5041	0.5027	0.5153	0.5147	0.5147	0.5312	0.4605	0.5062±0.0222	367.36
	Middle	0.4565	0.5005	0.5046	0.4441	0.4661	0.5664	0.5604	0.4998±0.0487	362.54
	Low	0.4622	0.5444	0.5446	0.5102	0.5544	0.4440	0.4546	0.5021±0.0476	364.31

Note: A pre-test (data not showed) results suggested that standard curve formula is $Y = a + bX + cX^2 + d X^3 + e X^4$. Y refers to the level of the protein TNF-α level (pg/ml) and the X refers to the IFN OD value. a = -1.25819299729, b = 139.693272118, c = 11.5273492313, d = 6.93875229399, e = 2.47878037319.

that the IFN-γ content of mice after immunization with the fusion protein was significantly higher than that before immunization. The best effect was observed in the 50 μg protein dose group, with an increase of 467.74 pg/mL; the content of IL-2 also increased significantly. The best effect was observed in the 100 μg protein dose group, with an increase of 145.16 pg/mL; TNF-α content was not significantly different from that before immunization. This indicates that the vaccine using this fusion protein as the immunogen can safely and effectively improve the cellular immunity of the mouse body, enhance the body's ability to resist infection, and prevent Mhp damage to the body.

In addition, the test results of mouse-specific IgG antibodies showed that mice in each immunization dose group could produce extremely high levels of specific IgG antibodies induced by the fusion protein, with the best effect in the 100 μg protein dose group, indicating that the vaccine using the fusion protein as the immunogen can induce good humoral immunity in the mouse body. However, although the low-dose group (10 μg protein) could induce the production of IFN-γ and specific IgG antibodies in mice, the overall immune effect was lower than that of the high- and medium-dose groups. Among them, mice 2, 3 and 4 induced the production of IFN-γ and specific IgG antibodies. The amount of IFN-γ and the value of specific IgG antibodies were lower than those of other mice in the same group. This may be due to the small dose of 10 μg protein and individual differences in the mice, resulting in an insufficient protein dose to induce an immune response. Therefore, an increase in the dose of immune

protein may be considered for further follow-up investigations.

In this study, the purified multi-epitope fusion protein was preliminarily explored in mice. It successfully induced mice to produce higher humoral immunity, and it obtained good immunogenicity verification. It helps to explore the multi-epitope of Mhp genetic engineering. This vaccine has laid the foundation, but the exact immune effect in pigs needs to be further studied.

Conclusion

The multi-epitope fusion protein constructed in this experiment has good immunogenicity, can effectively enhance the cellular immunity of mice and induce the production of specific IgG antibodies, and can be used as a new candidate vaccine for Mhp.

Acknowledgements

References

Biebaut E, Beuckelaere L, Boyen F, Haesebrouck F, Gomez-Duran CO, Devriendt B, Maes D **(2021)** Transfer of *Mycoplasma hyopneumoniae*-specific cell mediated immunity to neonatal piglets. Vet Res 52: 96.

Chen AY, Fry SR, Daggard GE, Mukkur TK **(2008)** Evaluation of immune response to recombinant potential protective antigens of *Mycoplasma hyopneumoniae* delivered as cocktail DNA and/or recombinant protein vaccines in mice. Vaccine 26: 4372-4378.

Chen YL, Wang SN, Yang WJ, Chen YJ, Lin HH, Shiuan D **(2003)** Expression and immunogenicity of *Mycoplasma hyopneumoniae* heat shock protein antigen P42 by DNA vaccination. Infect Immun 71: 1155-1160.

Chen AY, Fry SR, Forbes-Faulkner J, Daggard G, Mukkur TK **(2006)** Evaluation of the immunogenicity of the P97R1 adhesin of *Mycoplasma hyopneumoniae* as a mucosal vaccine in mice. J Med Microbiol 55: 923-929.

Feng ZX, Bai Y, Yao JT, Pharr GT, Wan XF, Xiao SB, Chi LZ, Gan Y, Wang HY, Wei YN, Liu MJ, Xiong QY, Bai FF, Li B, Wu XS, Shao G **(2014)** Use of serological and mucosal immune responses to *Mycoplasma hyopneumoniae* antigens P97R1, P46 and P36 in the diagnosis of infection. Vet J 202 : 128-133.

Fisch A, Marchioro SB, Gomes CK, Galli V, de Oliveira NR, Simionatto S, Dellagostin OA, Mendonça M, Moreira AN, Conceição FR **(2016)** Commercial bacterins did not induce detectable levels of antibodies in mice against *Mycoplasma hyopneumoniae* antigens strongly recognized by swine immune system. Trials Vaccinol 5: 32-37.

Li G, Zeng Y, Yin J, Lillehoj HS, Ren X **(2010)** Cloning, pro-

karyotic expression, and biological analysis of recombinant chicken IFN-gamma. Hybridoma (Larchmt) 29: 1-6.

Li Yuan, Zhang Jianhua, Wang Liang **(2008)** Whole-gene cloning and C-terminal gene expression and immune activity analysis of heat shock protein Hsp 70 (Dnak) of *M. hyopneumoniae* Chinese isolate. Chin J Prev Vet Med 03: 169-173.

Liu W, Jiang P, Yang K, Song Q, Yuan F, Liu Z, Gao T, Zhou D, Guo R, Li C, Sun P, Tian Y **(2022)** *Mycoplasma hyopneumoniae* Infection Activates the NOD1 Signaling Pathway to Modulate Inflammation. Front Cell Infect Microbiol 12: 927840.

Lu Huiying, Shen Qingchun, Ning Yibao **(2010)** Recombination and expression of the p97 R1 region gene of *M. hyopneumoniae* and the *Escherichia coli* LTB gene. Chin J Vet Med 46: 3-6.

Marchioro SB, Fisch A, Gomes CK, Jorge S, Galli V, Haesebrouck F, Maes D, Dellagostin O, Conceição FR **(2014)** Local and systemic immune responses induced by a recombinant chimeric protein containing *Mycoplasma hyopneumoniae* antigens fused to the B subunit of *Escherichia coli* heat-labile enterotoxin LTB. Vet Microbiol 173: 166-171.

Mei X, Wang J, Zhang C, Zhu J, Liu B, Xie Q, Yuan T, Wu Y, Chen R, Xie X, Wei Y, Wang L, Shao G, Xiong Q, Xu Y, Feng Z, Zhang Z **(2023)** Apigenin suppresses mycoplasma-induced alveolar macrophages necroptosis via enhancing the methylation of TNF-α promoter by PPARγ-Uhrf1 axis. Phytomedicine 108: 154504.

Ogawa Y, Oishi E, Muneta Y, Sano A, Hikono H, Shibahara T, Yagi Y, Shimoji Y **(2009)** Oral vaccination against *Mycoplasmal pneumonia* of swine using a live *Erysipelothrix rhusiopathiae* vaccine strain as a vector. Vaccine 27: 4543-4550.

Sette A, Rappuoli R **(2010)** Reverse vaccinology: developing vaccines in the era of genomics. Immunity 33: 530-541.

Thacker EL **(2004)** Diagnosis of *Mycoplasma hyopneumoniae*. Anim Health Res Rev 5: 317-320.

Trueeb BS, Braun RO, Auray G, Kuhnert P, Summerfield A **(2020)** Differential innate immune responses induced by *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* in various types of antigen presenting cells. Vet Microbiol 240: 108541.

Virginio VG, Gonchoroski T, Paes JA, Schuck DC, Zaha A, Ferreira HB **(2014)** Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential for use in vaccination against porcine enzootic pneumonia. Vaccine 32: 5832-5838.

Wang Y, Wang J, Zhou M, Liu P, Zhang E, Li Y, Lin J, Feng Z, Yang Q **(2019)** Mucosal and systemic immune responses induced by intranasal immunization of recombinant Bacillus subtilis expressing the P97R1, P46 antigens of *Mycoplasma hyopneumoniae*. Biosci Rep 39: BSR 20191126.

Xu M, Zheng J, Hu S, Wang G **(2021)** Expression and immunogenicity study of a novel mhp183 gene fragment of *Mycoplasma hyopneumoniae*. Pol J Vet Sci 24: 553-561.

Yang X, Xing F, Wang L, Zhao W, Fu Y, Tu F, Li B, Fang X, Ren S **(2021)** Effect of pregnane X receptor on *CYP3A29* expression in porcine alveolar macrophages during *Mycoplasma hyopneumoniae* infection. Animals (Basel) 11: 349.