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Porcine antibody profiles of 33 *Mycoplasma hyopneumoniae* fusion proteins from *M. hyopneumoniae* natural infection but not vaccination

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Abstract

Background: *Mycoplasma hyopneumoniae*, the primary pathogen responsible for porcine enzootic pneumonia, reduces average daily weight gain and causes substantial economic losses to the pig industry worldwide. Vaccination is the most common strategy to control this disease but offers partial protection. Therefore, developing next-generation vaccines by screening protective antigens is crucial.

Objectives: The aim of this study was to evaluate the antibody response to 33 recombinant proteins in pigs naturally infected with *M. hyopneumoniae*.

Methods: The genes encoding 33 (hypothetical) membrane proteins or secretory proteins were ligated into pGEX-6P-1, pGEX-6P-2, pGEX-5X-3 or pGEX-4T-3 vectors and transformed into *Escherichia coli* BL21(DE3) or *E. coli* XL-1 Blue to construct recombinant bacteria and to express the recombinant proteins. The recombinant bacteria expressing the target proteins reacted with porcine convalescent sera and negative sera to screen immunodominant proteins by ELISA. Then, recombinant bacteria expressing immunodominant proteins were used to identify the discriminating immunodominant proteins that were recognised by convalescent sera nut not hyperimmune sera.

Results: All recombinant bacteria could express the target recombinant proteins in soluble form. Twenty-one proteins were shown to present immunodominant antigens, and four proteins were not recognised by convalescent sera. Moreover, six proteins were considered discriminating and reacted with convalescent sera but not with hyperimmune sera.

Conclusions: The identified immunodominant proteins were antigenic and expressed during bacterial infection, suggesting that these proteins, especially those capable of

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discriminating between sera, can be used to identify protective antigens with the view to develop more effective vaccines against *M. hyopneumoniae* infection.

KEYWORDS
convalescent sera, ELISA, GST fusion protein, hyperimmune sera, immunodominant protein

1 | INTRODUCTION

Mycoplasma hyopneumoniae is the respiratory pathogen responsible for porcine enzootic pneumonia (EP), a chronic infectious disease characterised by dry nonproductive cough, dyspnoea and tachypnea. EP decreases feed conversion and reduces average daily weight gain, causing substantial economic losses to the pig industry (Maes et al., 2018). Moreover, infected animals are more sensitive to secondary bacterial or viral infections, leading to severe disease known as porcine respiratory disease complex (Chae, 2016).

Control of clinical symptoms and lung lesions in EP can be achieved by improving biosecurity precautions, using antibacterial agents, and performing vaccine immunisation (Maes et al., 2008). Vaccination with inactivated whole-cell bacteria (bacterins) is the most commonly used strategy in the clinical setting. Commercial inactivated vaccines improve clinical symptoms, reduce lung damage and increase average daily weight gain (Michiels et al., 2017; Villarreal et al., 2011a) but provide partial protection against *M. hyopneumoniae* and do not significantly reduce pathogen load in infected pigs (Villarreal et al., 2011a; Villarreal et al., 2011b). Furthermore, immunisation with inactivated vaccines does not significantly reduce pathogen transmission (Meyns et al., 2006; Villarreal et al., 2011b). Therefore, finding new vaccine development strategies and candidate antigens is essential to develop next-generation vaccines (Fagan et al., 2001; Okamba et al., 2010; Shimoji et al., 2003).

The genome of *M. hyopneumoniae* strain 232 contains 692 annotated open reading frames (ORFs) (Minion et al., 2004). Of these, 483 ORFs were identified, including 171 hypothetical proteins (Pendarvis et al., 2014), indicating that some proteins were not expressed or were poorly expressed in cell-free media. In turn, some proteins are highly expressed and immunogenic, inducing the production of IgG antibodies in pigs naturally infected with *M. hyopneumoniae* (Meens et al., 2010; Petersen et al., 2019). Some proteins not present in bacterins may stimulate protective humoral immunity, although the correlation between immune protection and antibody production by injecting inactivated vaccines is unclear. In addition, it is known that the systemic humoral immune response reduces clinical symptoms, lung lesions, and the number of pathogens in the respiratory tract (Meyns et al., 2006; Villarreal et al., 2011a). Therefore, screening for immunodominant proteins, especially those that stimulate the production of IgG antibodies in the sera of pigs naturally infected with *M. hyopneumoniae* but not in vaccinated pigs, is crucial. Two ELISA methods were used in previous studies to identify *M. hyopneumoniae* immunodominant proteins. One method involved screening for immunodominant antigens in convalescent sera (CS) from pigs naturally infected with *M. hyopneumoniae* (Zhou et al., 2018), and the other approach involved identifying immunodominant proteins that could discriminate between CS and inactivated vaccine-induced hyperimmune sera (HS) (Ding et al., 2019).

This study used two ELISA methods to evaluate antibody responses to 33 *M. hyopneumoniae* fusion proteins in CS and HS and identified 21 immunodominant antigens and six discriminating proteins.

2 | MATERIALS AND METHODS

2.1 | Prediction of the subcellular localisation of *M. hyopneumoniae* proteins

Twenty-four *M. hyopneumoniae* proteins, Mhp024, Mhp067, Mhp144, Mhp170, Mhp246, Mhp263, Mhp274, Mhp275, Mhp303, Mhp320, Mhp326, Mhp347, Mhp353, Mhp354, Mhp435, Mhp440, Mhp444, Mhp460, Mhp482, Mhp520, Mhp565, Mhp586, Mhp641 and Mhp681, were selected according to the protein sequences of strain 232. Homologous proteins were searched in the genome of *M. hyopneumoniae* strains 7448, J, 168 and 7422 using BLAST. The subcellular localisation of proteins found in at least three sequenced strains was predicted.

Mycoplasmas do not contain cell walls and are stained as Gram-negative. However, the structure of their cell membranes is similar to that of Gram-positive bacteria with one lipid bilayer. *Mycoplasma* secretes proteins using the secretion (Sec) and twin-arginine translocation (Tat) pathways. The subcellular localisation of 24 proteins from *M. hyopneumoniae*, considered as Gram-positive bacterium, was predicted using online bioinformatics tools (Table 1). The localisation of membrane proteins whose secondary structures contain alpha helices was predicted using Phobius (Käll et al., 2004) and TMHMM Server version 2.0 (Krogh et al., 2001). In addition, subcellular localisation was also predicted using those two online servers. N-terminal signal peptides of lipoproteins cleaved by signal peptidase II (LspA) and transported by the Sec translocon were identified using LipoP 1.0 (Juncker et al., 2003; Rahman et al., 2008). Signal peptides present in substrates of the Tat exporter, which was responsible for translocating folded proteins, were recognised by TatP 1.0 (Bendtsen et al., 2005). Secretory signal peptides cleaved by signal peptidase I (LepB) and transported by the Sec translocon, signal peptides of lipoproteins cleaved by LspA and transported by the Sec translocon, and Tat signal peptides cleaved by LepB were predicted using SignalP 5.0 (Almagro Armenteros et al., 2019).

TABLE 1 Computational tools used in this study

Computational tool	URL	References
Phobius	http://phobius.sbc.su.se/	Käll et al. (2004)
TMHMM Server v. 2.0	https://services.healthtech.dtu.dk/service.php?TMHMM-2.0	Krogh et al. (2001)
LipoP 1.0	http://www.cbs.dtu.dk/services/LipoP/	Juncker et al. (2003); Rahman et al. (2008)
TatP 1.0	http://www.cbs.dtu.dk/services/TatP-1.0/	Bendtsen et al. (2005)
SignalP 5.0	http://www.cbs.dtu.dk/services/SignalP/	Almagro Armenteros et al. (2019)

Manual curation was performed after prediction. Some membrane and secretory proteins of *M. hyopneumoniae* were identified by liquid chromatography-tandem mass spectrometry, as described previously (Paes et al., 2017; Reolon et al., 2014; Tacchi et al., 2016). The subcellular localisation of these proteins was based on bioinformatics prediction and literature reports, and localisation data were included in the final analysis.

2.2 | Prokaryotic expression and purification of *M. hyopneumoniae* GST fusion proteins

Twenty-four ORFs that encode known and predicted membrane and secretory proteins based on the genome of strain 232 were synthesised. TGA codons encoding tryptophan were mutated to TGG (Sangon Biotech, Shanghai, China). Two ORFs, *mhp067* and *mhp565*, were truncated into five regions, producing nucleotide sequences with lengths varying from 500 to 1500 bp. *Bam*H I and *Xho*I restriction sites, the ATG start codon and the TAA stop codon were added to the 5′-ends or 3′-ends of the synthesised genes as needed. Recombinant plasmids were obtained by ligating each synthesised gene into the expression vector pGEX-6P-1 digested with *Bam*HI and *Xho*I (Takara, Dalian, China). The 5'-end of *mhp246* (nucleotides 1 to 756), 3′-end of *mhp246* (nucleotides 757 to 1455) and 3′-end of *mhp353* (nucleotides 859 to 1728) from recombinant plasmids pGEX-6P-1-mhp246 and pGEX-6P-1-mhp353 were generated using PrimeSTAR® Max DNA polymerase (Takara, Dalian, China). *Bam*H I and *Xho*I restriction sites and the codons ATG and TAA were added to the corresponding primers (Table 2). PCR products were digested with *Bam*H I and *Xho*I and cloned into pGEX-6P-1 or pGEX-4T-3. For the soluble expression of recombinant proteins, some genes were ligated into the expression vectors pGEX-6P-2, pGEX-5X-3 or pGEX-4T-3 digested with *Bam*HI and *Xho*I. Recombinant plasmids were transformed into *E. coli* BL21(DE3) or *E. coli* XL-1 Blue by heat shock. Positive colonies were cultured in Luria-Bertani broth supplemented with 100 μg/ml ampicillin, and protein expression was induced with or without 1 mmol/L isopropyl-β-D-thiogalactoside (IPTG) at different temperatures. Some parameters, such as gene length, sites of mutation, protein molecular weight, expression vector, recipient strain, and induction temperature, were summarised in Table 3. Recombinant bacteria were processed as described previously (Ding et al., 2019), and an aliquot of each bacterial supernatant was purified using glutathione-conjugated agarose beads (GE Healthcare, Uppsala, Sweden). The purified proteins were cleaved from the beads using PreScission protease. The fusion proteins and cleaved proteins were analysed by 12% SDS-PAGE. *E. coli* BL21(DE3) harbouring empty pGEX-6P-1 and expressing GST served as controls, as reported previously (Ding et al., 2019).

2.3 | Porcine serum samples

Serum samples were collected from two pig farms. Pigs from farm A were *M. hyopneumoniae*-free and presented no clinical symptoms or lung lesions due to EP. Pathogen and serology detection were carried out in the last 2 years. Animals were shown to be free of *M. hyopneumoniae* organisms and nucleotides by culture and nested PCR (Feng et al., 2010). Additionally, the sera were negative by immunological diagnosis with a commercial ELISA kit (IDEXX laboratories, Westbrook, Maine, USA). At farm A, seven negative serum samples were collected from pigs aged 11–15 weeks, and seven hyperimmune serum samples were obtained from pigs aged 11 weeks immunised with an inactivated vaccine (MYPRAVAC SUIS, Laboratorios Hipra, La Selva, Spain) on Days 7 and 21 after birth. At farm B, 11 convalescent serum samples were obtained from naturally infected pigs with clinical signs or a history of EP.

M. hyopneumoniae IgG antibodies were detected by ELISA. Pathogens from bronchoalveolar lavage fluids (BALFs) collected by fibreoptic bronchoscopy were detected by nested PCR (Feng et al., 2010), as described in previous studies (Ganter & Hensel, 1997; Hensel et al., 1994). Negative sera and BALFs from each animal at farm A were negative for *M. hyopneumoniae*. HS were positive for IgG antibodies; however, the corresponding BALFs were negative for *M. hyopneumoniae* DNA. CS and the corresponding BALFs were positive for anti-*M. hyopneumoniae* IgG antibodies by nested PCR and ELISA.

2.4 | Screening for immunodominant proteins

The immunodominant proteins that reacted with CS were screened by ELISA, as previously described (Zhou et al., 2018), with minor modifications. Bacterial lysates of recombinant and control bacteria (200 μl/well without dilution) were transferred to glutathione-coated 96-well microplates (ThermoFisher Scientific, Rockford, IL, USA). The plates were incubated at 4°C overnight, washed five times with PBS containing 0.05% Tween-20 (PBST), blocked with 200 μl PBS + 10% NBS + 2.5% skimmed milk at room temperature (RT) for 1 h, and washed five times with PBST. One hundred microlitres of CS or negative serum diluted at 1:500 in blocking solution was added to each plate and incubated at RT for 2 h. Serum samples were preincubated with the lysate of the control bacterium to minimise cross-reactivity with the

TABLE 2 Parameters for PCR amplification of *mhp246-N*, *mhp246-C* and *mhp353-C*

Target gene	Primer	Sequence (5′–3′)[a]	Product size	Annealing temperature (°C)
mhp246-N	mhp246-N-F	CGCGGATCCATGAAAAAAAATTTTATTTTAAACTTC	759 bp	52
	mhp246-N-R	CCGCTCGAGTTACCAATACCAAGTATCTTTTTGT		
mhp246-C	mhp246-C-F	CGCGGATCCATGACAATTTTATATGGTTCTTGGC	702 bp	52
	mhp246-C-R	CCGCTCGAGTTATTTTTTGATTCGTTCAATTTCAG		
mhp353-C	mhp353-C-F	CGCGGATCCATGAAAAAATGGGATAACCATTTT	873 bp	52
	mhp353-C-R	CCGCTCGAGTTAAATATACCAAAAAAGATGTTCTAAAAA		

[a]Protected bases were wavy underlined; *Bam*H I and *Xho*I restriction sites were single underlined; the start codon ATG and the stop codon TAA were dot underlined.

antigens in the bacterial lysate and improve the accuracy of the results before dilution and addition to the plate. The plates were washed five times with PBST and developed with 100 μl HRP-labelled rabbit anti-pig IgG (Invitrogen, Rockford, IL, USA) in blocking solution (1:40,000) at 37°C for 1 h. The plates were washed as described above, and a colorimetric reaction was performed at RT using chromogenic substrate A (0.2078 g of citric acid monohydrate, 2.72 g of anhydrous sodium acetate, and 60 μl of 30% hydrogen peroxide in 100 ml of water) and substrate B (0.2078 g of citric acid monohydrate, 0.04 g of EDTA·Na_2, 0.0391 g of TMB·2HCl, and 10 ml glycerol in 100 ml of H_2O) for 10 min. The reaction was terminated with 50 μl of 2 M H_2SO_4, and absorbance was measured at 450 nm using a microplate reader (ThermoFisher Scientific, Vantaa, Finland). Seven negative sera and 11 CS were used in ELISA.

Seven negative sera reacted with lysates of recombinant bacteria and lysate of control bacterium, respectively. The difference in antibody titres in negative sera that reacted with test or control lysates was calculated. The cut-off value was shown as X + 2SD, where X was the average difference in titres, and SD was the standard deviation. Eleven CS reacted with these lysates. The difference in titres in CS that reacted with test or control lysates was calculated and designated A. The serum was considered positive when $A \geq X + 2SD$.

2.5 | Identification of discriminating immunodominant proteins recognised by CS but not by HS

The identified proteins were used to search for discriminating immunodominant proteins that reacted with CS but not with HS. The parameters used in this study were described previously (Ding et al., 2019), with minor modifications. All bacterial lysates of recombinant bacteria and control bacterium were diluted 1:5. CS or HS were diluted at 1:500 in blocking solution. All serum samples were incubated with the lysate of control bacterium and treated with HRP-labelled rabbit anti-pig IgG (1:40,000).

The average difference in antibody titres in HS that reacted with test or control lysates was determined. The cut-off value was calculated as $\bar{X} + 2\bar{S}\bar{D}$, where $\bar{X}$ was the average difference in titre, and $\bar{S}\bar{D}$ was the standard deviation. Eleven CS reacted with these lysates. The difference in titres in CS that reacted with test or control lysates was calculated and designated $\bar{A}$. The serum was considered positive when $\bar{A} \geq \bar{X} + 2\bar{S}\bar{D}$.

2.6 | Western blotting analysis

Recombinant bacteria that were preliminarily proven to express the target proteins were treated at 105°C for 10 min to make them soluble and loaded onto SDS polyacrylamide gels. After electrophoresis, samples were stained with Coomassie brilliant blue, or transferred (100 min at 100 V) to a polyvinylidene difluoride membrane (Roche Diagnostics, German) using a transblotting apparatus (Bio-Rad, USA). The membrane was blocked in 5% skimmed milk-TBST at RT for 2 h. The membrane was incubated with GST-tagged mouse monoclonal antibody (1:5000; Beyotime Biotechnology, Shanghai, China) at 4°C overnight followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) (1:10,000; ABclonal, Wuhan, China) at RT for 1 h and visualised with enhanced chemiluminescence (CWBio, China).

3 | RESULTS

3.1 | Sequence conservation and subcellular localisation of proteins

Sequence conservation analysis was carried out by BLAST sequence alignment of 24 proteins from *M. hyopneumoniae* strains 232, 7448, J, 168 and 7422, whose genomes were fully sequenced and annotated (Table 4). Twenty-one proteins were found in all five strains, whereas Mhp303, Mhp320 and Mhp444 were found in four strains, demonstrating that most proteins were shared by the five strains.

The prediction results using online bioinformatics tools and the manually curated results were shown in Table 5. A predictive decision tree was generated according to the computational tools used in this study. The subcellular localisation was considered accurate if the prediction results from both Phobius and TMHMM were identical. The results showed that Mhp024, Mhp326, Mhp353, Mhp354 and

TABLE 3 Data on 33 *M. hyopneumoniae* recombinant proteins expressed in this study

Gene	Gene size	Mutation site	MW[a] of pure protein	MW of recombinant protein	Vector	Host strain	Induction temperature (°C)
mhp024	822 bp	352–354, 445–447	31 kDa	57 kDa	pGEX-6P-1	BL21(DE3)	16
mhp067-1	720 bp	37–39, 442–444	28 kDa	54 kDa	pGEX-6P-1	BL21(DE3)	16
mhp067-2	828 bp	760–762	32 kDa	58 kDa	pGEX-6P-2	XL-1 Blue	30
mhp067-3	1044 bp	592–594, 751–753	42 kDa	68 kDa	pGEX-5X-3	XL-1 Blue	16
mhp067-4	1440 bp		56 kDa	82 kDa	pGEX-6P-1	BL21(DE3)	16
mhp067-5	681 bp	301–303	27 kDa	53 kDa	pGEX-6P-1	BL21(DE3)	16
mhp144	1044 bp	148–150, 598–600, 640–642	40 kDa	66 kDa	pGEX-6P-1	BL21(DE3)	16
mhp170	1053 bp	79–81, 229–231, 754–756	41 kDa	67 kDa	pGEX-6P-1	BL21(DE3)	30
mhp246-N	759 bp	82–84, 124–126, 283–285, 589–591, 748–750, 754–756	28 kDa	54 kDa	pGEX-6P-1	BL21(DE3)	30
mhp246-C	702 bp	22–24, 316–318, 322–324, 364–366	26 kDa	52 kDa	pGEX-4T-3	XL-1 Blue	16
mhp263	828 bp	154–156, 625–627	32 kDa	58 kDa	pGEX-4T-3	BL21(DE3)	16
mhp274	1356 bp	34–36, 241–243, 610–612, 877–879, 967–969	51 kDa	77 kDa	pGEX-6P-1	BL21(DE3)	16
mhp275	1383 bp	280–282, 715–717, 925–927	52 kDa	78 kDa	pGEX-6P-1	XL-1 Blue	16
mhp303	543 bp	118–120, 142–144, 238–240, 412–414, 454–456	20 kDa	46 kDa	pGEX-6P-1	BL21(DE3)	16
mhp320	894 bp	196–198, 391–393, 394–396, 448–450, 637–639, 766–768	34 kDa	60 kDa	pGEX-5X-3	XL-1 Blue	30
mhp326	894 bp	220–222, 238–240, 262–264, 451–453, 547–549	35 kDa	61 kDa	pGEX-6P-1	BL21(DE3)	16
mhp347	648 bp	112–114	25 kDa	51 kDa	pGEX-6P-1	BL21(DE3)	16
mhp353-C	873 bp	10–12	35 kDa	61 kDa	pGEX-6P-1	BL21(DE3)	30
mhp354	669 bp	250–252, 274–276, 358–360, 538–540, 643–645	25 kDa	51 kDa	pGEX-6P-1	BL21(DE3)	16
mhp435	1404 bp	19–21, 1249–1251, 1258–1260	53 kDa	79 kDa	pGEX-6P-1	XL-1 Blue	16
mhp440	930 bp	805–807	36 kDa	62 kDa	pGEX-6P-1	BL21(DE3)	16
mhp444	1773 bp	40–42, 808–810, 928–930, 1174–1176	69 kDa	95 kDa	pGEX-6P-1	BL21(DE3)	16
mhp460	1395 bp	1255–1257	53 kDa	79 kDa	pGEX-6P-1	BL21(DE3)	16
mhp482	1542 bp	388–390, 403–405, 472–474, 1123–1125, 1201–1203	60 kDa	86 kDa	pGEX-6P-1	XL-1 Blue	16
mhp520	1824 bp	367–369, 901–903, 1036–1038,1384–1386, 1687–1689	71 kDa	97 kDa	pGEX-6P-1	BL21(DE3)	30
mhp565-1	552 bp	337–339	21 kDa	47 kDa	pGEX-6P-1	BL21(DE3)	16
mhp565-2	1101 bp		43 kDa	69 kDa	pGEX-6P-1	BL21(DE3)	16
mhp565-3	549 bp	223–225	21 kDa	47 kDa	pGEX-6P-2	XL-1 Blue	16

(Continues)

TABLE 3 (Continued)

Gene	Gene size	Mutation site	MW[a] of pure protein	MW of recombinant protein	Vector	Host strain	Induction temperature (°C)
mhp565-4	1047 bp		40 kDa	66 kDa	pGEX-6P-1	BL21(DE3)	16
mhp565-5	837 bp	99–102, 127–129, 436–438, 586–558, 589–591	33 kDa	59 kDa	pGEX-6P-1	BL21(DE3)	16
mhp586	663 bp	97–99	25 kDa	51 kDa	pGEX-4T-3	XL-1 Blue	30
mhp641	1650 bp	676–678, 913–915, 1132–1134, 1516–1518	65 kDa	91 kDa	pGEX-6P-1	BL21(DE3)	30
mhp681	1686 bp	1162–1164, 1456–1458, 1549–1551	66 kDa	92 kDa	pGEX-6P-1	BL21(DE3)	16

[a]MW, molecular weight.

TABLE 4 Conservation analysis of 24 proteins from five sequenced and annotated *M. hyopneumoniae* strains

232	7448	J	168	7422
Mhp024	MHP7448_0022	MHJ_0021	MHP168_022	MHL_2644
Mhp067	MHP7448_0064	MHJ_0060	MHP168_064	MHL_3162
Mhp144	MHP7448_0235	MHJ_0228	MHP168_253	MHL_3371
Mhp170	MHP7448_0211	MHJ_0207	MHP168_210	MHL_2635
Mhp246	MHP7448_0136	MHJ_0132	MHP168_168	MHL_3026
Mhp263	MHP7448_0117	MHJ_0113	MHP168_184	MHL_3587
Mhp274	MHP7448_0199	MHJ_0195	MHP168_198	MHL_3599/3601
Mhp275	MHP7448_0199	MHJ_0195	MHP168_198	MHL_3599/3601
Mhp303		MHJ_0282	MHP168_310	MHL_2866
Mhp320		MHJ_0299	MHP168_327	MHL_2852
Mhp326	MHP7448_0313	MHJ_0305	MHP168_322	MHL_2637
Mhp347	MHP7448_0335	MHJ_0326	MHP168_358	MHL_2941
Mhp353	MHP7448_0341	MHJ_0334	MHP168_365	MHL_3060
Mhp354	MHP7448_0342	MHJ_0335	MHP168_366	MHL_2866
Mhp435	MHP7448_0432	MHJ_0430	MHP168_444	MHL_3413
Mhp440	MHP7448_0437	MHJ_0435	MHP168_449	MHL_1568
Mhp444	MHP7448_0440	MHJ_0438		MHL_3193
Mhp460	MHP7448_0461/0462	MHJ_0458/0459	MHP168_472	MHL_2652/3259
Mhp482	MHP7448_0484	MHJ_0481	MHP168_494	MHL_3238
Mhp520	MHP7448_0521	MHJ_0522	MHP168_530	MHL_3064
Mhp565	MHP7448_0547	MHJ_0551	MHP168_559	MHL_3005
Mhp586	MHP7448_0570	MHJ_0568	MHP168_577	MHL_3185
Mhp641	MHP7448_0622	MHJ_0623	MHP168_630	MHL_2964
Mhp681	MHP7448_0660	MHJ_0660	MHP168_672	MHL_3380

Mhp460 were localised on the bacterial surface. In Mhp347, a small region (less than ten amino acids) was embedded in the membrane, and most domains were localised in the cytoplasm. Mhp246, Mhp263 and Mhp482 had multiple transmembrane regions, and most domains were embedded in the membrane. Mhp067, Mhp144, Mhp170, Mhp275, Mhp303, Mhp320, Mhp444, Mhp565, Mhp641 and Mhp681 were predicted to be secreted proteins. Mhp274, Mhp435, Mhp440, Mhp520 and Mhp586 were predicted to be membrane-bound or extracellular according to Phobius or TMHMM, respectively. Proteins with signal peptides predicted by TMHMM, LipoP 1.0, TatP 1.0, or SignalP 5.0 and transmembrane domains localised in the signal peptide regions were considered to be extracellular, whereas other proteins were

TABLE 5 Predicted protein signal peptide and localisation using computational tools and manual curation

Protein name	Phobius[a]	TMHMM 2.0	LipoP 1.0[b]	TatP 1.0[c]	SignalP 5.0[d]	Predicted localisation	Reported localisation	Manual curation
Mhp024	1–26 (M[e])	M	1–31		1–41	M		M (surface)
Mhp067	E[f]	E				E	M [24]	M (identified)
Mhp144	1–21 (E)	E	1–21			E		E
Mhp170	1–28 (E)	E	1–25		1–25	E		E
Mhp246	M	M	1–34			M		M (multiple transmembrane)
Mhp263	M	M				M		M (multiple transmembrane)
Mhp274	1–27 (E)	M	1–26		1–33	M	M [25]	M (identified)
Mhp275	E	E				E	M [25]	M (identified)
Mhp303	E	E				E		E
Mhp320	1–27 (E)	E	1–27		1–27	E		E
Mhp326	M	M	1–45			M		M (surface)
Mhp347	M	M				M	M [25]	M (identified)
Mhp353	M	M				M	E [23]	E (identified)
Mhp354	1–32 (M)	M	1–30		1–31	M		M (surface)
Mhp435	1–20 (E)	M	1–21	1–18	1–17	M		M (surface)
Mhp440	Cytoplasm	E		1–19		E		E
Mhp444	1–31 (E)	E	1–29		1–31	E		E
Mhp460	1–20 (M)	M	1–21	1–18	1–17	M		M (surface)
Mhp482	M	M	1–39			M		M (multiple transmembrane)
Mhp520	M	E				M	M [25]	M (identified)
Mhp565	E	E				E	E [23]	E (identified)
Mhp586	1–29 (E)	M	1–29			E		E
Mhp641	E	E				E	E [23]	E (identified)
Mhp681	1–25 (E)	E	1–25		1–25	E	E [23]	E (identified)

[a–d] Position of the signal peptide in the protein.
[e] M: membrane.
[f] E: extracellular.

considered to be membrane-bound. Therefore, Mhp274, Mhp435 and Mhp520 were considered to be membrane proteins, and Mhp586 was secreted. Mhp440 was predicted to be cytoplasmic by Phobius but extracellular by TMHMM. Moreover, TatP 1.0 predicted that this protein contained a signal peptide cleavage site between positions 18 and 19. The results of Phobius, TMHMM and TatP 1.0 indicated that Mhp440 was secreted.

Mhp067, Mhp274, Mhp275, Mhp347 and Mhp520 were identified as membrane-bound (Reolon et al., 2014; Tacchi et al., 2016), and Mhp353, Mhp565, Mhp641 and Mhp681 were identified as secreted (Paes et al., 2017).

Literature search results and bioinformatics localisation prediction demonstrated that proteins were membrane-bound or secreted. We identified known membrane proteins (Mhp067, Mhp274, Mhp275, Mhp347 and Mhp520), predicted membrane proteins (Mhp024, Mhp326, Mhp354, Mhp435 and Mhp460), predicted proteins with multiple transmembrane domains (Mhp246, Mhp263 and Mhp482), known secretory proteins (Mhp353, Mhp565, Mhp641 and Mhp681) and predicted secretory proteins (Mhp144, Mhp170, Mhp303, Mhp320, Mhp440, Mhp444 and Mhp586).

3.2 | Expression and purification of recombinant fusion proteins

Recombinant plasmids were identified by restriction enzyme digestion with *Bam*H I and *Xho*I (Figure 1) and direct sequencing, and plasmid insert sequences were identical to those found in the genome of 232 strain. Lysates from 33 recombinant bacteria were purified using glutathione Sepharose beads, and GST fusion proteins were visualised by SDS-PAGE and Western blotting. Although the purification process was crude and purified products contained a few nonspecific proteins, the target proteins could be seen clearly in Figure 2a and b. From Figure 2a and b, we can see that all recombinant proteins were expressed in soluble form in *E. coli* BL21(DE3) or XL-1 Blue, and most proteins had the expected molecular weights. Moreover,

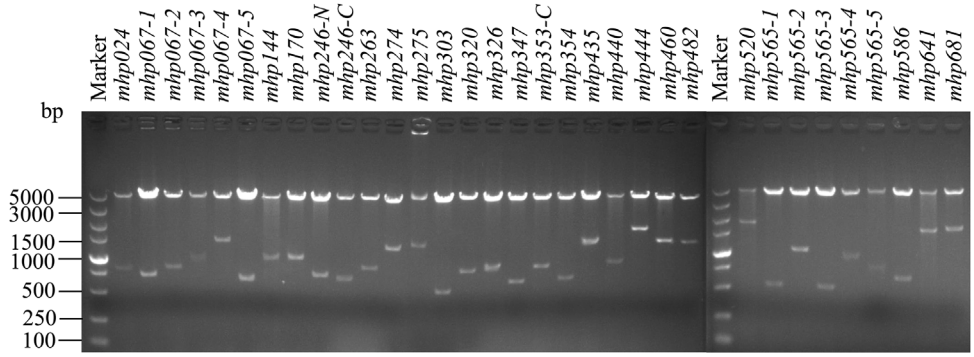

FIGURE 1 Identification of recombinant plasmids by double restriction digestion. The recombinant plasmids using pGEX expression vectors as backbones were digested with *Bam*H I and *Xho*I, and digestion products were separated by agarose gel electrophoresis

Figure S1 showed that the expression of target proteins needed induction with IPTG for most recombinant bacteria, except GST-Mhp067-4, GST-Mhp263 and GST-Mhp641. Mhp067-4, Mhp144, Mhp275, Mhp520, Mhp565-2, Mhp565-5 and Mhp681 were cleaved from GST beads using PreScission protease and had the expected molecular weights (Figure 2c). However, this protease did not remove the GST moiety from the other fusion proteins.

3.3 | Identification of immunodominant antigens recognised by CS

Eleven CS from pigs naturally infected with *M. hyopneumoniae* were used to determine the antigenicity and reactivity of 33 fusion proteins in microplate assays. The fusion proteins recognised by each serum sample were shown in Figure 3a. Serum 5, 6/8, 3, 4, 2/10/11, 7, 9 and 1 recognised 25, 23, 22, 20, 19, 15, 13 and 12 out of 33 proteins, respectively. The set of proteins recognised by each serum was unique. Moreover, several serum samples reacted with the same fusion proteins. The recognition frequency was defined as the total number of sera that recognised a specific fusion protein (Figure 3b). Proteins that reacted with more than 50% of sera were considered immunodominant during *M. hyopneumoniae* infection. Based on the criterion used in previous study (Zhou et al., 2018), proteins recognised by at least six of 11 CS were considered immunodominant. Twenty-one proteins met this criterion and were recognised by 11 (Mhp170, Mhp263, Mhp274, Mhp275, Mhp444 and Mhp460), 10 (Mhp067-4 and Mhp303), 9 (Mhp144, Mhp440, Mhp520 and Mhp565-1), 8 (Mhp246-N, Mhp347, Mhp482, Mhp565-2 and Mhp641), 7 (Mhp354 and Mhp681) and 6 (Mhp067-1 and Mhp565-5) CS. One CS reacted with Mhp067-2, and no sera reacted with Mhp024, Mhp067-3, Mhp067-5, Mhp326 or Mhp565-3. Immunodominance was represented by high antibody titres in some samples or the recognition frequency of CS. The titres of antibodies that reacted with fusion proteins were compared. Differences in total and average titres between CS and negative sera (Figure 3c and d) were calculated. Titre values tended to agree with recognition frequencies.

3.4 | Identification of immunodominant antigens recognised by porcine CS but not by HS

Immunodominant proteins that stimulated the production of antibodies by natural infection with *M. hyopneumoniae* but not by bacteria cultured in vitro were searched by identifying proteins that strongly reacted with CS but not with HS. There was high variability in antibody recognition frequencies using HS as controls (Figure 4a). Serum samples 1, 8, 4, 5, 3, 2/9/11, 6/10 and 7 recognised 13, 12, 10, 9, 8, 7, 6 and 4 out of 21 fusion proteins, respectively. Antigens that reacted with at least six of 11 CS but not with HS were considered immunodominant (Figure 4b). Mhp067-4, Mhp170, Mhp275, Mhp347, Mhp444 and Mhp565-2 met this criterion and were recognised by 9, 11, 11, 7, 10 and 10 CS, respectively. Other proteins were recognised by fewer than 6 CS, and no CS reacted with Mhp067-1, Mhp354 or Mhp440. The titres of antibodies that reacted with each fusion protein were measured. Differences in total and average antibody titres between CS and HS were calculated (Figure 4c and d). Mhp170 and Mhp275 had the highest total and average antibody titres. The total and average titres against Mhp354 and Mhp565-1 were very low or negative.

4 | DISCUSSION

The most common strategy currently adopted to control EP is immunisation against *M. hyopneumoniae*, especially with bacterins. Commercial inactivated vaccines partially reduce clinical symptoms and lung lesions but do not prevent the colonisation of *M. hyopneumoniae* in the respiratory tract and transmission of this organism in the herd and between pig farms (Meyns et al., 2006; Villarreal et al., 2011a). The search and identification of candidate antigens are the first steps to develop novel vaccines and diagnostic reagents. The release of the genome of several *M. hyopneumoniae* isolates (Liu et al., 2011; Minion et al., 2004; Qiu et al., 2019; Siqueira et al., 2013; Vasconcelos et al., 2005) has allowed searching for additional candidate antigens.

Vaccine antigens are often exposed to the surface of organisms or secreted into host cells, and membrane and secretory proteins

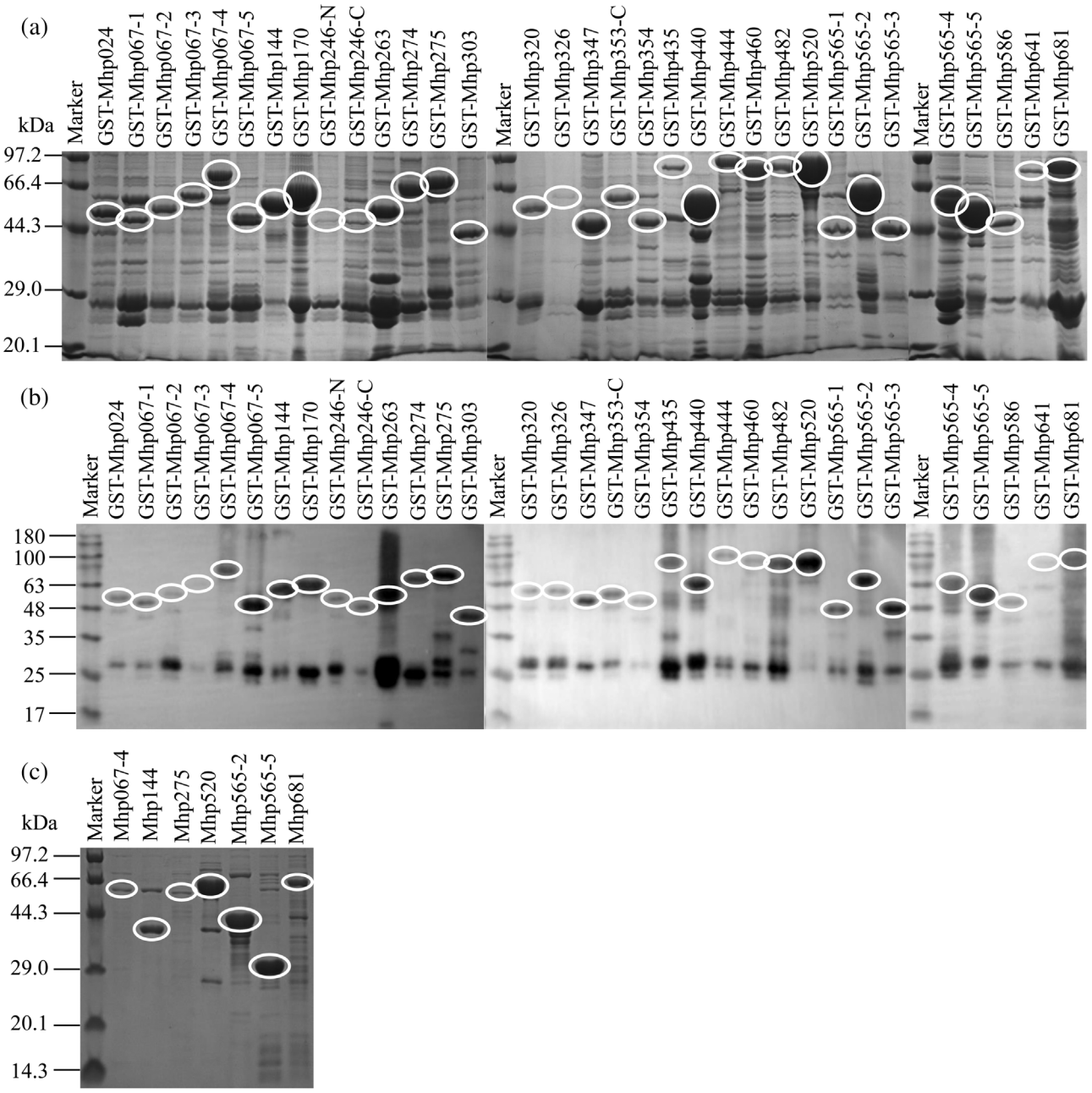

FIGURE 2 GST-*M. hyopneumoniae* fusion proteins purified from bacterial extracts and cleaved from GST beads with PreScission protease. (a) Identification of the expression of GST fusion proteins purified using glutathione Sepharose beads by SDS-PAGE. (b) Identification of the expression of GST fusion proteins purified using glutathione Sepharose beads by Western blotting. (c) Seven proteins were cleaved from beads using PreScission protease. The gel was stained with Coomassie blue. The expected full-length fusion proteins were circled

accessible to antigen-presenting cells stimulate the immune response. In this study, the localisation of 24 proteins was predicted using online computational tools and literature search. These proteins were proven or predicted to be localised in the cell membrane or extracellularly. Therefore, all proteins have the potential to be immunodominant antigens.

The genes encoding the identified proteins presented 100 stop codons (TGA), including 10 stop codons in *mhp067* and 9 in *mhp565*, and performing site-directed mutagenesis in all these genes would be laborious and time-consuming. For this reason, we synthesised all genes with the modification of TGA codons to TGGs to improve protein expression. The heterologous expression of long genes (>3000 bp) is challenging (Hunt, 2005). Therefore, protein expression was improved by truncating the genes *mhp067* and *mhp565* into five segments with nucleotide lengths varying from 500 to 1500 bp. Recombinant membrane proteins, which have hydrophobic regions, are usually expressed in insoluble form. However, the target proteins were expressed in soluble form by inserting a GST tag at the N-terminus. GST

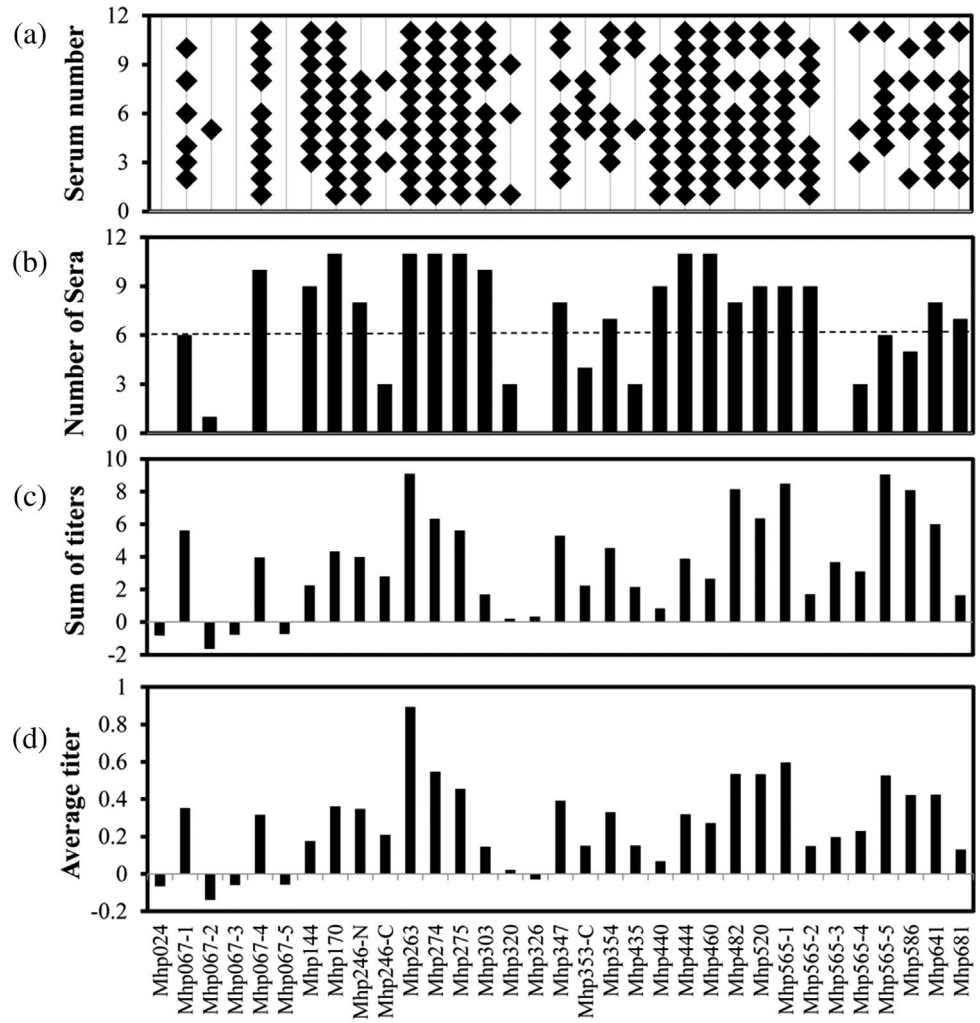

FIGURE 3 Reactivity of porcine convalescent sera to 33 GST-*M. hyopneumoniae* fusion proteins using negative sera as negative controls. (a) Positivity of 11 serum samples from pigs naturally infected with *M. hyopneumoniae* (*y* axis) against recombinant proteins immobilised on microtitre plates (*x* axis). Each diamond represents a positive reaction. (b) Number of sera that reacted with each fusion protein. (c) Differences in total titres between convalescent and negative sera. (d) Differences in average titres between convalescent and negative sera

is more hydrophilic and much larger than 6 consecutive His tags and increases protein hydrophilicity. The soluble expression of Mhp246 and Mhp353 failed because of the presence of several highly hydrophobic transmembrane domains according to bioinformatics models. For this reason, we attempted to express the N-terminus and C-terminus of Mhp246 and Mhp353. However, the soluble expression of the N-terminus of Mhp353 failed.

In this study, 21 immunodominant proteins, including dehydrogenase (Mhp144), L-xylulose 5-phosphate 3-epimerase (Mhp440), oligoendopeptidase F (Mhp520), DNA adenine methylase (Mhp641) and 17 hypothetical proteins, were identified as immunodominant, whereas Mhp024, Mhp320, Mhp326, Mhp353-C, Mhp435 and Mhp586 were not. Previous studies identified several hypothetical proteins with immunodominant epitopes (Pendarvis et al., 2014; Tacchi et al., 2016). The present study identified Mhp263, Mhp303, Mhp354, Mhp460 and Mhp482 for the first time. Moreover, Mhp320 was shown to be expressed in *M. hyopneumoniae* during infection in vivo and was recognised by three CS.

Proteins with putative immunodominant regions that were strongly recognised by CS may interact with host cells and provide protection by stimulating adaptive humoral immune responses, although the role of IgG antibodies in protective immunity needs to be further explored. No sera reacted with full-length proteins Mhp024 and Mhp326, which might be because they were not expressed during bacterial infection, their expression did not stimulate humoral immune responses, or they were localised in the cytoplasm and therefore were not accessible to antigen-presenting cells. In addition, CS did not recognise Mhp067-3, Mhp067-5 and Mhp565-3, which might be because these peptides were localised in protein cores and were not presented to immune cells.

ELISA results showed that CS strongly reacted with Mhp 681 (MHP_0660 in the 7448 strain), which agrees with a previous study

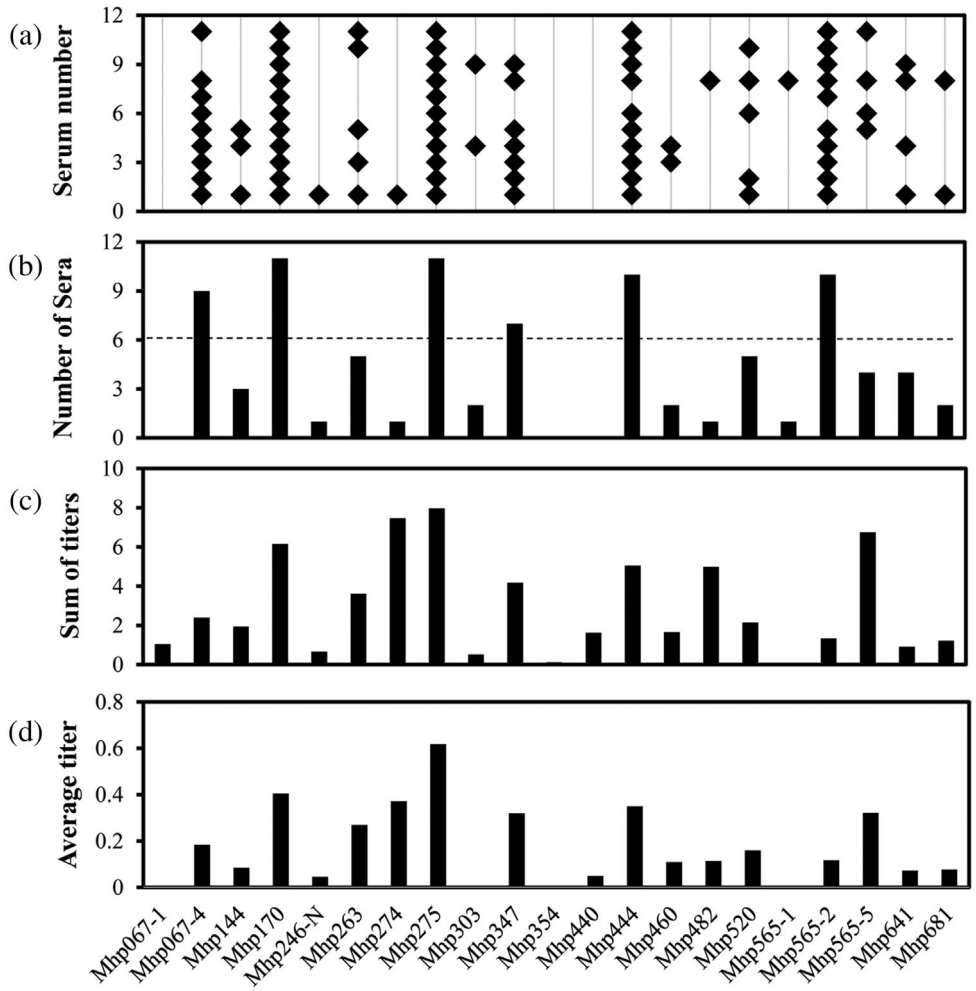

FIGURE 4 Reactivity of porcine convalescent sera to 21 GST-*M. hyopneumoniae* fusion proteins using hyperimmune sera as negative controls. (a) Positivity of 11 serum antibodies from pigs naturally infected with *M. hyopneumoniae* (*y* axis) against recombinant proteins immobilised on microplate plates (*x* axis). Each diamond represents a positive reaction. (b) Number of sera that reacted with each fusion protein. (c) Differences in total titres between convalescent and hyperimmune sera. (d) Differences in average titres between convalescent and hyperimmune sera

(Simionatto et al., 2012) and proves that the ELISA method we established previously (Zhou et al., 2018) was accurate and reproducible.

All membrane and secretory proteins expressed in this study, except for Mhp353, were immunodominant. Full-length Mhp353 was not adequately expressed, and its immunodominance was not assessed. The C-terminus of this protein was expressed in soluble form and was shown not to be immunodominant, although it was recognised by three CS. Moreover, the immunodominance of the N-terminus of this protein was not assessed because this region was not expressed in soluble form.

Six hypothetical proteins were discriminating, including Mhp275, a member of the P102 family. Other immunodominant proteins were nonreactive or reacted with less than 50% CS using HS as negative controls. In this respect, it was shown that these proteins did not stimulate humoral immune responses in pigs because their expression was low in in vitro cell-free culture conditions (Madsen et al., 2008). Moreover, several *M. hyopneumoniae* proteins do not evoke immune responses because they are secreted from the bacterins in cell-free media (Paes et al., 2017). The limited effectiveness of inactivated whole-cell vaccines in pigs could be partially due to the absence or limited amount of membrane and secretory proteins in *M. hyopneumoniae*.

Mhp274, a member of the P102 family, was not discriminating and reacted with one CS when HS were used as controls. Conversely, Mhp275 discriminated and recognised all CS, although both were localised on the bacterial surface and there genes located in the same operon (Minion et al., 2004). The reason for this discrepancy is unknown.

Compared with the sera obtained from *M. hyopneumoniae*-negative pigs, germ-free pig sera may be a better strict negative serum control. We used sera from germ-free pigs provided by Animal Husbandry Engineering Research Institute of Chongqing Academy of Animal Sciences as the control. However, there was no significant difference in the final results by using sera from germ-free pigs and sera from *M. hyopneumoniae*-negative pigs. Considering that sera from germ-free pigs are difficult to obtain and expensive, we chose sera from *M. hyopneumoniae*-negative pigs as controls according to previous results.

The total and average titres of some immunodominant proteins were lower than those of nonimmunodominant proteins. For instance, titres against Mhp274, which was recognised by one CS, were higher than titres against Mhp170, which was recognised by all CS. This result might be because the expression of Mhp170 was lower than that of Mhp274 in naturally infected pigs. Nonetheless, the immune response might be stronger if a higher concentration of recombinant proteins reactive to CS was used. Therefore, immunodominant proteins with low titres, especially discriminating proteins, may play a critical role in protective immunity.

Immunodominant proteins were not identified by Western blot by using *M. hyopneumoniae*-positive porcine sera as the primary antibodies, although secondary antibodies from different companies were tested. Therefore, we believe that pig sera cannot be used as primary antibodies.

Many recombinant subunit vaccines against *M. hyopneumoniae* are under development, and some of them are effective to some extent (Chen et al., 2003; Fagan et al., 2001; Okamba et al., 2010; Shimoji et al., 2003). However, only proteins P97 and NrdF, which are administered by the intranasal and oral routes, respectively, provide partial protection in pigs, which may be because these proteins contain a single antigen. In this respect, chimeric proteins may provide higher protection. Therefore, additional studies are necessary to identify highly immunogenic epitopes whose antigenicity is similar to that of full-length immunodominant proteins and evaluate the efficacy of chimeric proteins containing unique immunodominant epitopes in animal models.

5 | CONCLUSIONS

Twenty-one immunodominant proteins were recognised by CS and 6 of these proteins were reactive to CS but not to HS. Most immunodominant proteins and all discriminating proteins were hypothetical and were characterised for the first time. Immunodominant proteins were antigenic and expressed during infection with *M. hyopneumoniae*, suggesting that these proteins, especially those capable of discriminating between sera, are vaccine candidates against this bacterial pathogen. Notwithstanding, additional studies are necessary to identify and characterise immunodominant domains and evaluate the degree of protective immunity of chimeric proteins.

AUTHOR CONTRIBUTIONS

Yaru Ning: data curation; methodology. Yujiao Yang: data curation; methodology. Yaqin Tian: methodology. Yun Zhang: methodology. Wenyi Luo: methodology. Yukang Wen: methodology. Yaoqin Zhou: methodology. Honglei Ding: conceptualisation; writing – original draft; writing – review & editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The experiment was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Health of China. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Southwest University (Approval No. IACUC-20190305-01) and performed accordingly. The objectives, protocols and potential risks were clearly explained to all participating farm owners. Written informed consents were obtained from all participating farm owners.

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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