ORIGINAL ARTICLE

Development of an ELISA for distinguishing convalescent sera with *Mycoplasma hyopneumoniae* infection from hyperimmune sera responses to bacterin vaccination in pigs

Honglei Ding[1,2] | Yukang Wen[1,2] | Zuobo Xu[1,2] | Bingqian Zhou[1,2] | Chaker Tlili[3] | Yaqin Tian[1,2] | Zhaodi Wang[1,2] | Yaru Ning[1,2] | Jiuqing Xin[4]

[1]Laboratory of Veterinary Mycoplasmology, College of Veterinary Science, Southwest University, Chongqing, China

[2]Immunology Research Center, Medical Research Institute, Southwest University, Chongqing, China

[3]Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing, China

[4]State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China

Correspondence
Honglei Ding, Laboratory of Veterinary Mycoplasmology, College of Veterinary Science, Southwest University, No.2, Tiansheng Road, Beibei District, Chongqing 400715, China.
Email: hongleiding@swu.edu.cn

Jiuqing Xin, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No.678 Haping Road, Xiangfang District, Harbin 150069, China
Email: xinjiuqing@caas.cn

Abstract

Vaccination with inactivated bacterin is the most popular and practical measure to control enzootic pneumonia. After immunisation with inactivated bacterin, *Mycoplasma hyopneumoniae* colonised on the respiratory tract and lung stimulates the humoural immune responses and produces IgG and IgA antibodies. ELISA is a widely used serological method to detect *M. hyopneumoniae* antibodies. However, commercial IgG-ELISA kit cannot distinguish between inactivated bacterin-induced hyperimmune sera and convalescent sera stimulated by natural infection. SIgA-ELISA method needs to collect nasal swabs, but collecting nasal swabs is not easy to operate. Establishment of a discriminative ELISA detecting humoural IgG from convalescent sera but not hyperimmune sera facilitates to evaluate the natural infection of *M. hyopneumoniae* after inactivated bacterin vaccination. We expressed and purified a recombinant protein named Mhp366-N which contains an epitope recognised by the convalescent sera but not hyperimmune sera. The developed discriminative IgG-ELISA could discriminate between inactivated bacterin-induced hyperimmune sera and convalescent sera and was reproducible, sensitive and specific to *M. hyopneumoniae* antibody produced by natural infection. Compared to SIgA-ELISA method, discriminative IgG-ELISA was more convenient to detect IgG antibody from sera than IgA from nasal swabs, although it has limited sensitivity in the early stages of infection. Additionally, to some extent, it has a potential to avoid the interference of maternally derived IgG antibodies. The established discriminative IgG-ELISA was efficient to judge the serological IgG antibodies induced from natural infection or inactivated vaccine stimulation and provided a useful method to investigate and evaluate the live organism infection after the application of inactivated bacterin.

KEYWORDS

convalescent sera, hyperimmune sera, IgG, indirect ELISA, *Mycoplasma hyopneumoniae*

1 | INTRODUCTION

Enzootic pneumonia (EP), caused by *Mycoplasma hyopneumoniae*, is one of the most common and significant economically infectious diseases in pig husbandry worldwide (Maes et al., 2018). Control of this disease can be achieved by applying several techniques, such as the optimisation of management practices and housing conditions, and the application of antimicrobials and vaccination (Maes et al., 2018; Simionatto et al., 2013). Nowadays, two types of vaccines are used clinically. One is inactivated, adjuvanted whole-cell bacterins applied worldwide, whereas the other one is attenuated live vaccine which has been licensed and used clinically in China (Feng et al., 2013). Vaccination with an inactivated bacterin is the most popular and practical measure to control EP. However, these commercial vaccines have a limited effect on the transmission of microorganism and cannot prevent colonisation (Matthijs et al., 2019). Therefore, after immunisation with inactivated bacterin, *M. hyopneumoniae* colonised on or subsequently adhered to the respiratory tract and lungs stimulates the humoural immune responses and produces IgG and IgA antibodies (Tao et al., 2019).

ELISA is a widely used serological method to detect *M. hyopneumoniae* antibodies. The indirect ELISA kit (IDEXX Laboratories) is the most frequently used serological tool that was implemented to detect *M. hyopneumoniae* IgG antibody. However, this commercial kit cannot distinguish between inactivated bacterin-induced hyperimmune sera and convalescent sera stimulated by natural infection. One research group developed an ELISA method for detection of *M. hyopneumoniae* in naturally infected pigs based on secretory IgA (sIgA) collected from nasal swab (Bai et al., 2018; Feng et al., 2010). Nevertheless, collecting nasal swabs is not easy to operate and standardise, and, in general, the amount of each swab sample obtained from the nasal cavity is less compared to the serum sample.

It was reported that porcine convalescent serum revealed a strong immunoreaction to Mhp366 protein which could not react with sera from bacterin-immunised pigs (Meens et al., 2010). In addition, Mhp366 from in vitro grown *M. hyopneumoniae* strains was not detected by using a polyclonal serum raised against Mhp366 (Meens et al., 2010). Based on these characteristics of Mhp366, we have developed an indirect ELISA for detecting humoural immunodominant proteins of *M. hyopneumoniae* which can discriminate between inactivated bacterin-induced hyperimmune sera and convalescent sera (Ding et al., 2019). Therefore, Mhp366 protein has the potential to be used as an antigen to develop an ELISA method to react with antibodies stimulated by natural infection but not by bacterin vaccination.

In this study, we develop an indirect ELISA based on Mhp366 protein for the detection of convalescent sera but not inactivated bacterin-induced hyperimmune sera, which could be highly beneficial to discriminate between IgG antibody raised in *M. hyopneumoniae* inactivated bacterin and natural infection.

2 | MATERIAL AND METHODS

2.1 | Cloning of *mhp366-N* gene fragment

Plasmid pGEX-6P-2-mhp366 was extracted from recombinant bacteria GST-Mhp366 (Zhou et al., 2018) using HiPure Plasmid Micro Kit (Magen). Nucleotide fragment *mhp366-N* which contains the corresponding peptide segment recognised by the convalescent serum but not by hyperimmue serum was amplified with two primers 5'-CGCGGATCCATGAAAAAAATGGTAAAATATTTTCTAG-3' (*Bam*H I) and 5'-CCGCTCGAGCCAAAATGGGCCACCGTT-3' (*Xho*I) using PrimeSTAR® Max DNA Polymerase (Takara, China). After that, the PCR product was ligated into vector pET-28a(+) to construct the recombinant plasmid. Finally, the ligation product was transformed into *E. coli* DH5α competent cells and was identified by double restriction enzyme digestion and sequencing.

2.2 | Expression and purification of recombinant protein Mhp366-N

Recombinant plasmids were transformed into *E. coli* BL21(DE3) competent cells. Transformed clone was grown at 16℃ for 20 hr with shaking supplemented with 50 µg/mL kanamycin and 1 mM IPTG. Recombinant Mhp366-N protein was purified by Ni affinity chromatography (GE Healthcare, USA) using a gradient of 0.1–1 M imidazole and identified by SDS-PAGE and Western blot. The concentration of Mhp366-N protein was determined by BCA protein assay kit (Beyotime).

2.3 | Animal source

Serum samples used in this study were collected from four farms. Pigs from farm A were *M. hyopneumoniae*-free and had no EP-like clinical syndromes occurred or pathological changes on lung. Pathogen and serology detection were carried out in recent 2 years. *M. hyopneumoniae* organism and nucleotide are free by bacterial culture and nested PCR. Also, the sera are negative by immunological diagnosis with a commercial ELISA kit (IDEXX Laboratories). While, pigs from farms B, C and D had a history of EP according to the clinical observation and serological surveillance in last 2 years. For the farm B, about one-quarter of pigs showed EP-like clinical syndromes. However, EP sporadically occurred at farms C and D. All pigs were weaned on day 21.

2.4 | Sample collection and preparation

Two hundred and seventy-five pigs from farm A were immunised with a commercial *M. hyopneumoniae* inactivated vaccine (MYPRAVAC SUIS, Hipra Lab) on day 7 and day 21 after their bearing. MYPRAVAC SUIS is a whole cell, inactivated bacterin based on J strain, with mineral oil and aluminium hydroxide as adjuvants. After

56 days of the last immunisation, serum samples were collected from the front cavity veins of immunised pigs from farm A. Meanwhile, laryngeal swabs were obtained from the laryngeal cartilages with the help of snares and mouth gags for pig restraint as described previously (Pieters et al., 2017). Pigs from farms C and D were also vaccinated with MYPRAVAC SUIS on day 7 and day 21. Twenty pigs of 21 weeks old and 297 pigs of 10–11 weeks old were chosen from farms B and C, respectively. Fifteen piglets of 7 days old before immunisation and other 10 piglets of 14 days old shot on day 7 from farm D were picked up randomly. Laryngeal swabs were collected from corresponding pigs at farm B (Pieters et al., 2017), whereas nasal and laryngeal swabs were collected from corresponding pigs at farms C and D, as previously described (Feng et al., 2010; Pieters et al., 2017).

Glycerol was added to the collected sera and the final concentration was 50%. Then, the sera were kept in aliquots at −20°C until further use. The contents of laryngeal swabs were concentrated by centrifugation at 12 000 g for 10 min after soaking into 1 ml sterile PBS at 4℃ overnight. *M. hyopneumoniae* was determined by nested PCR from laryngeal swabs as described previously (Feng et al., 2010). Each nasal swab was put into 1.5 ml microcentrifuge tube containing 1 ml sterile PBS and stored at 4℃ overnight. After centrifugation at 10 000 r/min for 10 min, the supernatant was collected for SIgA-ELISA to detect SIgA in nasal swab according to the kit's procedure (Feng et al., 2010). After centrifugation at 1,000 r/min for 10 min, the supernatant was collected for SIgA-ELISA to detect sIgA in nasal swab.

All pigs used in this study were released after sample collection.

2.5 | Optimisation of ELISA procedure and working condition

The 96-well microtiter plates (Corning Inc., USA) were coated with 100 μL Mhp366-N protein (from 0.25 μg/mL to 8 μg/mL) in 0.5 M carbonate buffer (pH 9.6) overnight at 4°C after 37℃ for 1 hr. Unbound antigen was discarded, and the wells were washed five times with PBS containing 0.05% Tween-20 (PBST). Non-specific bindings were blocked with 200 μL PBST, 1% BSA, 2.5% skim milk, 10% FBS, 1% gelatin or 1% ovalbumin at 37℃ for 0.5 hr, 1 hr or 2 hr. After five washes with PBST, 100 μL serum samples diluted from 1:50 to 1:8,000 were added and incubated at 37℃ for 0.5 hr, 1 hr or 2 hr. Following five washes with PBST, the plates were conjugated with 100 μL of HRP-conjugate rabbit anti-pig IgG (H + L) secondary antibody (Invitrogen, USA) diluted in blocking buffer (from 1:10,000 to 1:80,000) at 37℃ for different times (0.5 hr, 1 hr and 2 hr). The plates were washed as described above, 50 μL of substrate A (100 ml H_2O containing anhydrous sodium acetate 2.72 g, citric acid monohydrate 0.35 g, 30% hydrogen peroxide 0.06 ml) and substrate B (100 ml H_2O containing EDTA·Na_2 0.04 g, citric acid monohydrate 0.2078 g, glycerol 10 ml, TMB·2HCl 0.0391 g) were added, respectively. After incubation for different time periods (5 min, 10 min and 20 min) at RT, the reaction was terminated

by adding 50 μL 2 M H_2SO_4. The optical density at 450 nm (OD_{450}) was recorded using an automatic ELISA plate reader (Thermo Fisher Scientific, Ratastie 2, FI-01620 Vantaa). All samples were run in triplicate, and each experiment was performed at least twice. Each working condition was optimised and determined with the highest P/N ratio between convalescent serum samples (P) and hyperimmune serum samples (N).

2.6 | Calculation of cut-off value

The cut-off value was obtained by determining the OD_{450} calculated from the mean of hyperimmune serum control plus three standard deviations (*SD*), as described previously (Poolperm et al., 2017; Tankaew et al., 2017).

2.7 | Evaluation of reproducibility

Reproducibility of intra- and inter-assay variation between runs was performed as described by Feng et al., (2010) with minor modification. In brief, two hyperimmune and two convalescent sera were selected randomly for the reproducibility experiments. Five replicates of each sample in the same batch were chosen for intra-assay (within plate) reproducibility and three plates from different batches were chosen for inter-assay (between runs) reproducibility. Mean values, *SD* and coefficient of variations (CV) were calculated.

2.8 | Estimation of specificity and sensitivity

The specificity of this assay was investigated by using positive sera of *M. hyorhinis* (Mhr), *A. pleuropneumoniae* (App), *S. suis* serotype 2 (SS2), classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) and pseudorabies virus gB protein (gB-PRV). Two hyperimmune and two convalescent sera were used as negative and positive controls, respectively.

Five convalescent sera were diluted with blocking buffer as follows: 1:500, 1:1,000, 1:2000, 1:4,000, 1:8,000, 1:16,000, 1:32,000 and 1:64,000. Then, ELISA was carried out with the optimal working conditions except the optimal dilution of convalescent sera. The sensitivity of the ELISA assay was accessed according to the cut-off value.

2.9 | Proof of discriminative IgG-ELISA

Samples from farms C and D were processed for the detection of *M. hyopneumoniae* IgG and sIgA. Serum samples were used for the detection of IgG with both commercial IDEXX kit and our established ELISA method. sIgA-ELISA kit was applied to decide sIgA from nasal swabs. Each sample was conducted in duplicate. *M. hyopneumoniae*

DNA was tested by nested PCR from laryngeal swabs as described previously (Feng et al., 2010).

2.10 | SDS-PAGE and Western blot

Pretreated bacteria or purified protein were mixed with loading buffer and loaded onto SDS polyacrylamide gels. After electrophoresis, gel was used for staining with coomassie brilliant blue or transferred to polyvinylidene difluoride membrane (Roche Diagnostics) for 2 hr at 100 V using a transblotting apparatus (Bio-Rad). The membrane was blocked overnight at 4°C in 5% skimmed milk-TBST and was detected by His-tag (4C2) monoclonal antibody (Bioworld Technology) with a 1:8,000 dilution at RT for 1 hr. The primary antibody binding was incubated with a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Proteintech, China) at RT for 1 hr and visualised with an enhanced chemiluminescence kit (CWBio).

3 | RESULTS

3.1 | Expression and purification of Mhp366-N

The fragment of *mhp366* from 1 to 837 nucleotide was cloned into pET-28a(+) and expressed in *E. coli* BL21(DE3). The Mhp366-N protein was expressed as soluble form and inclusion body (insoluble form) with a band of 40 kDa in SDS-PAGE gel (Figure 1a). The results were also confirmed by Western blot analysis, as the target protein could react strongly with anti-His-tag antibody (Figure 1b). Purification of the soluble recombinant protein was achieved by Ni chelating affinity chromatography as C-terminal $6 \times$ His-tagged fusion (Figure 1c).

3.2 | Classification of sera for establishment of ELISA

The results of *M. hyopneumoniae* DNA amplification in laryngeal swabs by nested PCR and IgG detection from sera by IDEXX ELISA kit have been summarised in Table 1. Eight weeks after immunisation, 183 serum samples from farm A were considered positive to *M. hyopneumoniae* and no *P36* gene was detected by nested PCR for all 275 piglets. From farm B, nine positive samples were detected by nested PCR method, and the prevalence of *M. hyopneumoniae* was 60% (12/20) by IgG detection. The number of positive and negative diagnostic results confirmed by both molecular biology and anti-*M. hyopneumoniae* IgG antibody was 7 and 6, respectively. Finally, we randomly picked up 12 *M. hyopneumoniae* hyperimmune sera from farm A and five convalescent sera from farm B for optimising the ELISA procedure.

3.3 | Optimisation of ELISA procedure

First, we investigated the effect of the antigen concentration. As shown in Figure 2a, the OD_{450} increased gradually with the increase of the antigen concentration for both convalescent and hyperimmune sera. The highest P/N value was obtained at 0.25 μg/mL. Thus, 0.25 μg/mL was considered as the optimal antigen concentration for further experiments. PBST, 1% BSA, 2.5% skim milk, 10% FBS, 1% gelatin and 1% ovalbumin had been investigated for their blocking efficiency of the 96-well surface and the results were presented in Figure 2b. By comparison of the P/N ratio, as a result, 2.5% skimmed milk was the most efficient blocking agent for our ELISA assay. After the blocking agent was confirmed, we assessed the incubation time for blocking step. Figure 2c demonstrates that complete blocking saturation was obtained at 30 min. With further
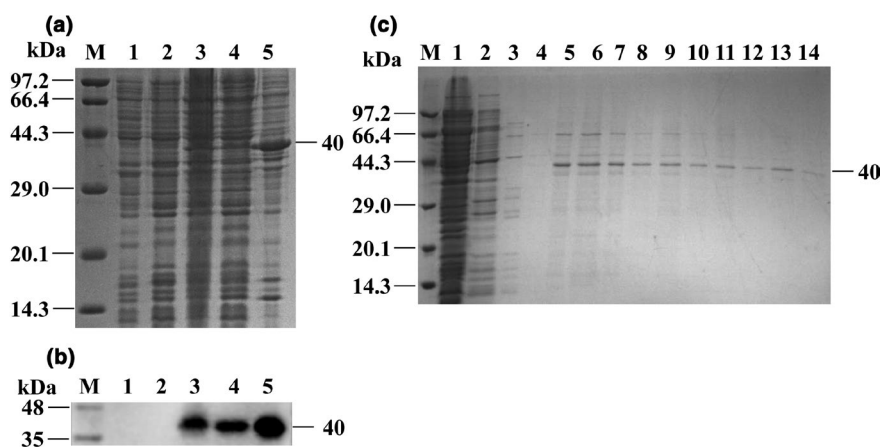

FIGURE 1 Expression and purification of Mhp366-N protein. Identification of the expression form of Mhp366-N in induced recombinant bacteria by SDS-PAGE (a) and Western blotting (b). Mhp366-N protein could be expressed as a soluble form (Lane 4) and inclusion body (Lane 5) with the induction of IPTG (Lane 3), but it did not express without IPTG induction in a small amount (Lane 2). No Mhp366-N could be detected in *E. coli* BL21(DE3) containing pET-28a(+) empty vector (Lane 1). (c) Mhp366-N protein was purified by Ni affinity chromatography. Crude supernatant was loaded onto the column (Lane 1). After other proteins were washed with a linear imidazole gradient of 0.1 M (Lane 2), 0.2 M (Lane 3) and 0.5 M (Lane 4), purified protein was collected (Lanes 8–14)

TABLE 1 Prevalence of *M. hyopneumoniae* infection and *M. hyopneumoniae* positive sera in selected pigs from two farms

Farm	No. of pigs	PCR result of LS		Commercial ELISA results of sera	
		+	−	+	−
A	275	0	275	183	92
B	20	9	11	12	8

Abbreviation: LS, laryngeal swabs.

increase of the incubation time, the P/N ratio did not go up anymore. Thus, we chose 30 min as the optimal incubation time.

The convalescence sera and the hyperimmune sera were diluted from 1:50 to 1:4,000. As seen in Figure 2d, the P/N ratio enhanced with the increase of the serum dilution and reached a maximum at 1:1,000 and then subsequently decreased upon further increase of the serum dilution to 1:4,000. Moreover, the incubation time of the serum with immobilised antigen could also affect the sensitivity of the final assay. In this regard, we have investigated the incubation time from 0.5 hr to 2 hr and we found that the highest P/N ratio was obtained at 0.5 hr (Figure 2e).

Finally, we investigated the effect of HRP-conjugate rabbit anti-pig IgG (H + L) secondary antibody by twofold serial dilution from 1:10 000 to 1:80 000. The P/N exhibited the highest ratio at 1:10 000, then it decreased with increasing the conjugated dilution as shown in Figure 2f. After that, we checked whether the conjugated incubation time can affect the sensitivity or not. We found that extended incubation times of the conjugated from 0.5 hr to 2 hr increased the assay sensitivity (Figure 2g). The optimal incubation time of the secondary antibody was 2 hr. After the above-mentioned conditions were checked, the colorimetric reaction time was optimised. As shown in Figure 2h, the highest P/N value was obtained when the enzyme reacted with the substrate for only 10 min.

3.4 | Calculation of the cut-off value

It has been suggested that to determine a cut-off value, at least 100 negative control sera should be applied (Gardner et al., 1996; Pruvot et al., 2013). To get a relatively precise result, we used 183 hyperimmune sera to calculate the cut-off value. As a result, the average OD_{450} value of 183 hyperimmune sera was 0.153, and the *SD* was 0.059. Therefore, the cut-off value of our indirect ELISA was calculated as 0.33 (mean ELISA value $+3SD = 0.33$). For better interpretation, any pig serum that had an OD_{450} value of 0.33 or higher than 0.33 was classified as convalescent serum. Serum with an OD_{450} value lower than 0.33 was classified as hyperimmune serum.

3.5 | Reproducibility, specificity and sensitivity

Reproducibility was measured by determining intra- and inter-assay variation. The intra-assay CV of two hyperimmune serum and two convalescent serum samples ranged from 0.88% to 6.01%, while the

inter-assay CV of these samples ranged between 3.18% and 6.44%. These data showed that this assay was reproducible and yielded a low and acceptable variation.

The specificity of the ELISA was tested by using seven porcine respiratory disease pathogens' antisera, including antisera of *M. hyorhinis*, *A. pleuropneumoniae*, *S. suis* serotype 2, classical swine fever virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and pseudorabies virus gB protein. As shown in Figure 3a, all the obtained results from these antisera used as primary antibodies were negative as the hyperimmune sera. It indicated that the ELISA was specific only to *M. hyopneumoniae* antibody produced by natural infection and there was no cross-reaction with other porcine respiratory disease pathogen's antisera.

The sensitivity of the ELISA was evaluated by maximum dilution of convalescent sera. With the increase of dilutions of five convalescent sera, the OD_{450} values decreased gradually. Five convalescent sera were still positive at 1:500, 1:1,000 and 1:2000 dilutions, four sera showed positive result at 1:4,000 dilution, two sera were positive at 1:8,000 dilution and five sera gave negative result at 1:16,000 or more dilutions (Figure 3b). As a result, the convalescent serum could be diluted up to 2000 times in this assay.

3.6 | Comparisons among different ELISA methods

Serum samples collected from farms C and D were detected by commercial ELISA kit and discriminative IgG-ELISA for convalescent and hyperimmune sera (Table 2). At farm C, 66 samples were positive and 231 were negative by using a commercial ELISA kit. Three samples which were determined as positive by commercial ELISA kit were judged as seropositive by the SIgA-ELISA kit and discriminative IgG-ELISA. On the other hand, nested PCR result showed that 32 laryngeal samples were positive for *M. hyopneumoniae* DNA and others were negative. All three sera tested positive by discriminative IgG-ELISA and their corresponding laryngeal swab samples were also confirmed positive for *M. hyopneumoniae* DNA by nested PCR. For farm D, 12 and 4 serum samples obtained from piglets of 7 and 14 days old, respectively, were positive for IgG detection by commercial ELISA kit. Nevertheless, no one was positively tested by SIgA-ELISA or discriminative IgG-ELISA, although one and two laryngeal samples were positive to nested PCR detection from each subgroup. Hence, the commercial ELISA results were not consistent with the results generated by two other ELISA methods.

4 | DISCUSSION

Detection of IgG antibodies by ELISA kits is the most widely used method for the determination of EP. Although *M. hyopneumoniae* culture is the "gold standard" method, it is time-consuming, and not easy to get the organism due to the overgrowth of *M. hyorhinis* and
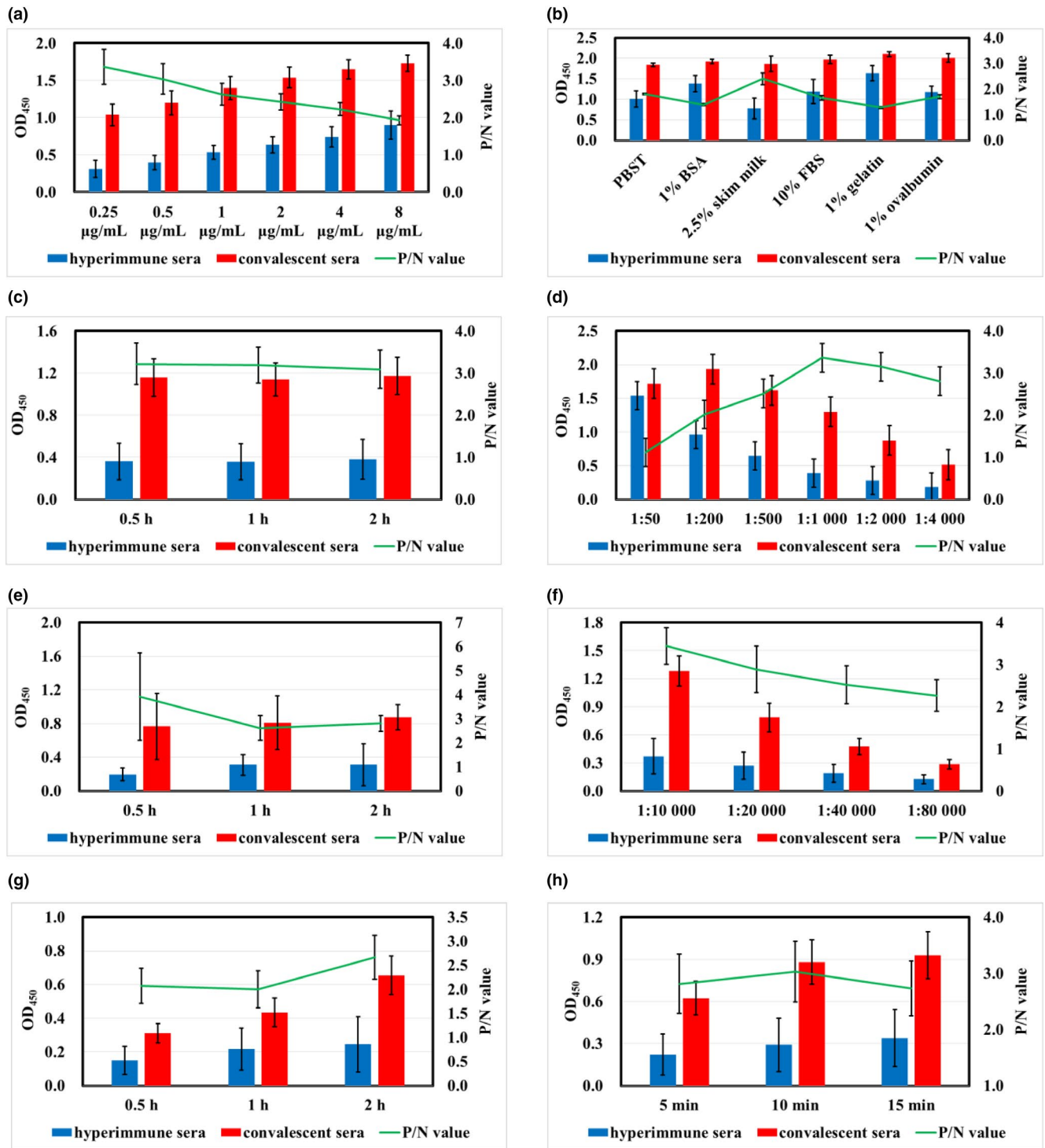
FIGURE 2 Optimisation of ELISA working conditions. The optimal antigen concentration was 0.25 μg/mL in coating buffer (a). The optimal blocking buffer was 2.5% skim milk dissolved in PBS (b), and the highest P/N value was got if the antigen was blocked for 0.5 hr (c). The optimal dilutions of serum and secondary antibody were 1:1,000 (d) and 1:10,000 (f) diluted in blocking buffer. The optimal incubation times of serum and secondary antibodies were 0.5 hr (e) and 2 hr (g), respectively. The optimal colorimetric reaction time was observed after exposing to substrate solution for 10 min (h)

M. flocculare (Maes et al., 1996). Tracheobronchial swabs, bronchoalveolar lavage fluid and lung tissue which are used to prepare the template for PCR are not easy to get, and nasal swabs, to some extent, are not reliable (Bai et al., 2018). Commercial inactivated vaccines are

the most popular strategy to control EP and are applied in more than 70% of the pig herds (Maes et al., 2018). Therefore, the stimulation of anti-*M. hyopneumoniae* IgG could be the result of natural infection or vaccination. The commercial ELISA kits cannot distinguish
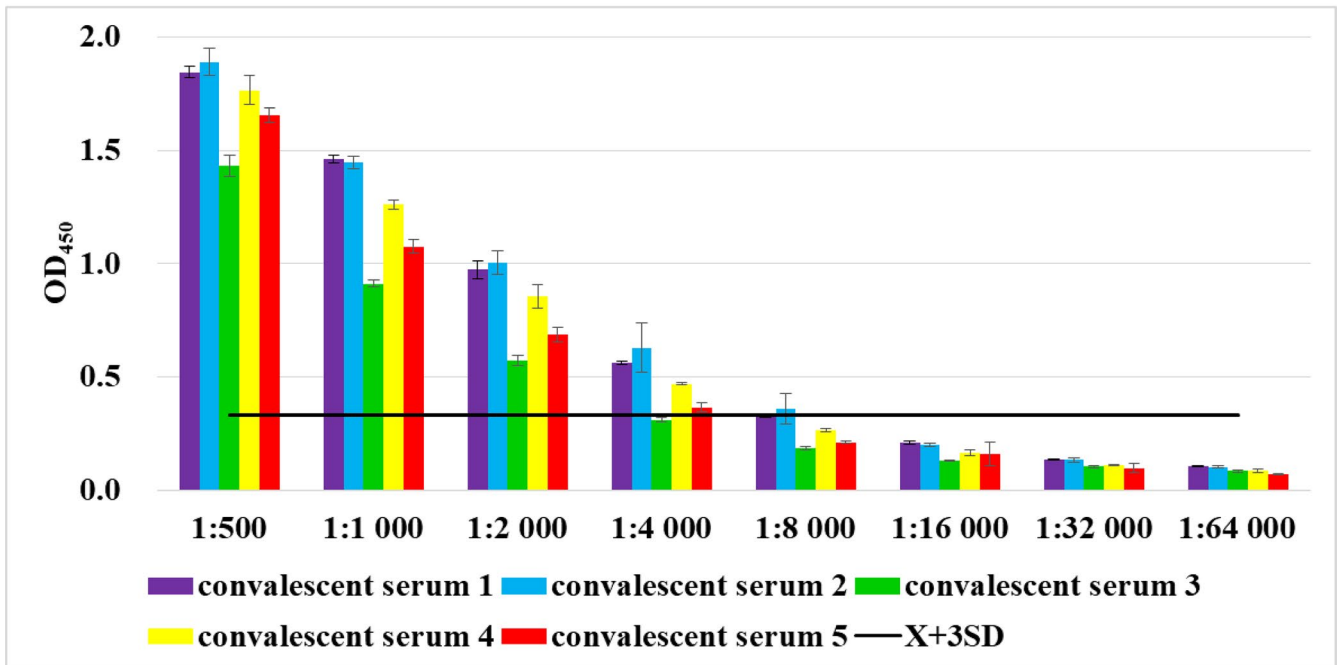
(a)
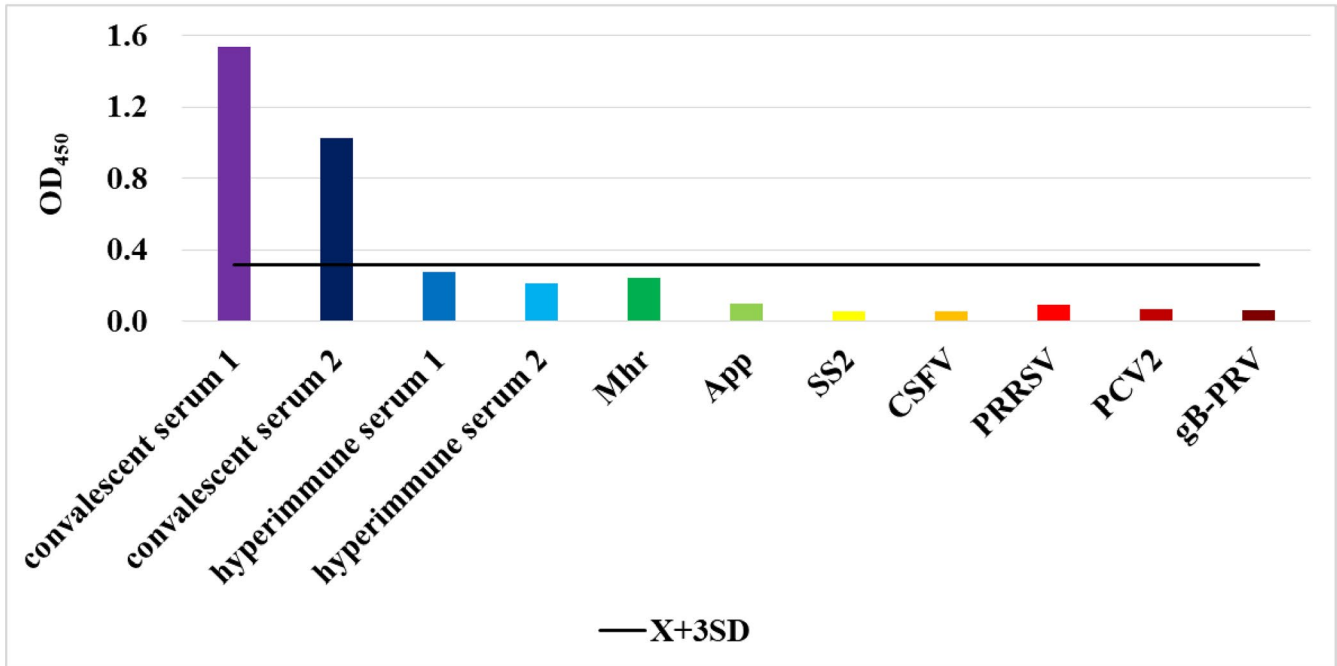
(b)

FIGURE 3 Specificity and sensitivity detection of ELISA. (a) The results were positive when used two convalescent sera. However, negative results were obtained that used antisera of *Mycoplasma hyorhinis*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis* serotype 2, classical swine fever virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and pseudorabies virus gB protein, two hyperimmune sera as primary antibodies. (Bb) Five convalescent sera were still positive at 1:500, 1:1,000 and 1:2000 dilutions, four sera were positive at 1:4,000 dilution, two sera were positive at 1:8,000 dilution and five sera were negative at 1:16,000 or more dilutions. The convalescent serum could be diluted up to 2000 times in this assay

TABLE 2 Comparisons of commercial IgG-ELISA, SIgA-ELISA and discriminative IgG-ELISA for convalescent and hyperimmune sera

Sera collection	Status	Commercial IgG-ELISA	SIgA-ELISA	Discriminative IgG-ELISA	Nested PCR
Pigs from farm C	+	66	3	3	32
	−	231	294	294	265
Sucking pigs of age 7 days from farm D	+	12	0	0	1
	−	3	15	15	14
Sucking pigs of age 14 days from farm D	+	4	0	0	2
	−	6	10	10	8
Total	+	82	3	3	35
	−	240	319	319	287

+: positive, −: negative.

between convalescent and hyperimmune sera. It is therefore necessary to develop a method to verify the two different antibodies. Feng and co-workers have developed an SIgA-ELISA method based on P97 protein to overcome the aforementioned issues (Feng et al., 2010). This method used SIgA collected from nasal fluid by swab. However, the nasal swab could only be stored at −20℃ for a short time. Based on our experience, SIgA lost its activity in 3 months. It is hard to carry out retrospective experiments when the nasal swabs are stored for a long time. Furthermore, nasal swab sample collection is inconvenient in live pigs for their curved nasal cavities, and swabs were found to be not reliable at individual pig level (Fablet et al., 2010; Pieters et al., 2017; Vangroenweghe et al., 2015). Therefore, development of an IgG-ELISA method which can differentiate convalescent and hyperimmune sera is easy to get serum samples clinically. Also, it is labour-saving for sample collection.

In our experiment, we used strongly immunoreactive protein Mhp366 as the coating antigen which did not react with sera from bacterin-immunised pigs. Although Mhp366 has a length of 555 amino acid residues with a calculated molecular weight of 64.4 kDa, its epitope recognised by the convalescent sera (Meens et al., 2010). Therefore, we amplified 637 bp fragment of *mhp366* gene which covered the differential diagnostic region from the stating site.

Determination of a cut-off value is always a difficult step in the standardisation of an ELISA. Multiple criteria may be used to determine a cut-off value, including ROC curve analysis or the mean of the negative control serum samples plus two standard deviations. The method chosen to determine a cut-off value should be considered in the context of the test that is under investigation. For ROC analysis, we could have used a commercial IgG-ELISA to screen clinically confirmed *M. hyopneumoniae* positive and negative sera. However, as we had abundant negative sera (*n* = 183 samples), we used the mean OD_{450} of the negative sera plus three SDs. The number of sera used to calculate the cut-off value by mean OD_{450}+ 3SDs was larger than that available for ROC analysis; therefore, mean OD_{450}+ 3SD was considered the best approach to calculate the cut-off value for our ELISA.

The intra-assay CV ranged from 0.88% to 6.01%, and the inter-assay CV varied from 3.18% to 6.44%. Based on these results, the proposed method revealed good reproducibility. In addition to that,

our ELISA test was able to discriminate between *M. hyopneumoniae* and other seven porcine respiratory disease pathogens' antisera. Generally, the sera applied on porcine pathogenic diagnostic ELISA diluted from 1:40 to 1:200. However, in our method, the optimum dilution of sera was 1:1,000, and the maximum dilution was 1:2000. Therefore, a small volume of serum will be sufficient for antibody detection.

Some studies indicated seropositive pigs were observed at 6 weeks (Sibila et al., 2007) or even 98 days of age (Martelli et al., 2006) after application of vaccine. Based on our finding, after 7 weeks immunisation only 15% of serum samples collected from farm C was positively detected by commercial ELISA kit. Delayed seroconversion could contribute to the low seropositive rate. What cannot be ignored is the limited sensitivity of the IgG-ELISA kit and it was inefficient at detecting serum antibodies at the early stages of immunisation or infection (Erlandson et al., 2005).

Inactivated vaccines reduce the number of pathogens in the respiratory tract (Meyns et al., 2006). However, some studies indicate that vaccination does not significantly reduce the transmission of this respiratory pathogen in vaccinated herds compared to unvaccinated ones (Meyns et al., 2006; Villarreal, Maes, et al., 2011; e Villarreal, Meyns, et al., 2011). In 100 bacterin-vaccinated pigs of 10–11 weeks old, *M. hyopneumoniae* genetic material from 15 pigs was amplified.

The pathogens localised on the upper respiratory tract can stimulate the production of mucosal antibodies and serum antibodies. Mucosal response could be identified as early as 6 days postinfection (Feng et al., 2014), whereas seroconversion due to natural *M. hyopneumoniae* infection occurred in pigs within 8–24 weeks of old (Djordjevic et al., 1994; Leon et al., 2001). That was the explanation for the existence of IgA but not IgG antibody tested by discriminative IgG-ELISA. These results indicate that, in the early stage of the infection, the sensitivity of discriminative IgG-ELISA was less than IgA-ELISA. Exploring early diagnostic antigen which can discriminate between convalescent and hyperimmune sera is the further task for mycoplasmologists.

The detection rate of IgG against *M. hyopneumoniae* by using IgG-ELISA kit in serum was high in suckling pigs and this might be the result of colostral IgG that was transferred from sows to their

offspring. The low prevalence of mucosal antibody detected by IgA-ELISA and serum antibody detected by discriminative IgG-ELISA showed that the antibodies were produced by natural infection but not by inactivated bacterin. Interestingly, the IgG antibody derived from sucking pigs could not be recognised by discriminative IgG-ELISA. This indicated that, to some extent, the discriminative IgG-ELISA assay for *M. hyopneumoniae* detection was certified for detecting *M. hyopneumoniae* infections in sucking piglets without the interference with maternal antibodies and the antibodies stimulated by the application of inactivated vaccines. However, more piglet serum samples are needed to further prove this phenomenon.

We did not evaluate this method to identify negative sera and convalescent sera. The optimal working condition to detect convalescent sera from unvaccinated pig herds might be different from this procedure. We are establishing other protocols to identify positive sera induced by live *M. hyopneumoniae* infection from vaccine-free pig farms. We are still using Mhp366-N as the coating protein for the new ELISA method. But some parameters, such as the blocking time, dilutions of sera, incubation time of the secondary antibody and chromogenic time, are different from the ones established in this study.

5 | CONCLUSION

In this study, we have established a reproducible, sensitive and selective indirect ELISA assay to discriminate natural induced but not inactivated vaccine stimulated serum IgG antibody.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Honglei Ding: Conceptualization; Data curation; Methodology; Writing-original draft; Writing-review & editing. **Yukang Wen:** Methodology. **Zuobo Xu:** Investigation; Methodology. **Bingqian Zhou:** Methodology. **Chaker Tlili:** Writing-original draft. **Yaqin Tian:** Methodology. **Zhaodi Wang:** Methodology. **Yaru Ning:** Methodology. **Jiuqing Xin:** Supervision.

ETHICAL STATEMENT
The experiment was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. All experimental protocols were approved by the Institutional Animal Ethics Committee of Southwest University (Approval no. IAECSWU20170921) 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.

ORCID
Honglei Ding http://orcid.org/0000-0001-6978-0277

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