Development and evaluation of an indirect ELISA for the detection of *Mycoplasma hyopneumoniae* natural infection but not inactivated bacterin vaccination

Honglei Ding (hongleiding@swu.edu.cn)
Southwest University https://orcid.org/0000-0001-6978-0277
Yukang Wen
Southwest University
Zuobo Xu
Southwest University
Chaker Tlili
Chongqing Institute of Green and Intelligent Technology
Yaqin Tian
Southwest University
Zhaodi Wang
Southwest University
Yaru Ning
Southwest University
Jiuqing Xin
Chinese Academy of Agricultural Sciences Harbin Veterinary Research Institute

Methodology article

Keywords: Mycoplasma hyopneumoniae, indirect ELISA, convalescent sera, hyperimmune sera, IgG

Posted Date: March 30th, 2020

DOI: https://doi.org/10.21203/rs.2.22701/v2

Abstract

Background: Mycoplasma hyopneumoniae is the primary pathogen of enzootic pneumonia (EP). Vaccination with 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 colonization. Therefore, after immunization with inactivated bacterin, M. hyopneumoniae colonized on the respiratory tract and lungs stimulates the humoral immune responses and produce 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 is laborious for nasal swab collection, and the amount of each swab sample obtained from the nasal cavity is less compared to the serum sample. Establishment of a discriminative ELISA detecting humoral IgG from convalescent sera but not hyperimmune sera facilitates to evaluate the natural infection of M. hyopneumoniae after inactivated bacterin vaccination.

Result: We expressed and purified a recombinant protein named Mhp366-N which contains an epitope recognized 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.

Conclusions: 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.

Background

Enzootic pneumonia (EP), caused by *Mycoplasma hyopneumoniae*, is one of the most common and significant economically infectious diseases in pig husbandry worldwide [1]. As a chronic respiratory disease, it is characterized by high morbidity, low mortality with dry and non-productive cough, causing a reduced average daily weight gain and poor feed conversion efficiency [2, 3]. *M. hyopneumoniae* infection increases susceptibility of pigs to other organisms and causes porcine respiratory disease complex (PRDC) [1, 4].

Control of this disease can be achieved by applying several techniques, such as the optimization of management practices and housing conditions, and the application of antimicrobials and vaccination [1, 5]. Nowadays, two types of vaccines are used clinically. One is inactivated, adjuvanted whole-cell

bacterins applied worldwide, while the other one is attenuated live vaccine which has been licensed and used clinically in China [6]. Vaccination with an inactivated bacterin is the most popular and practical measure to control EP. However, these commercial vaccines can only offer partial protection, having a limited effect on the transmission of microorganism and cannot prevent colonization [7]. Therefore, after immunization with inactivated bacterin, *M. hyopneumoniae* colonized on or subsequently adhered to the respiratory tract and lungs stimulates the humoral immune responses and produce IgG and IgA antibodies [8].

ELISA is a widely used serological method to detect *M. hyopneumoniae* antibodies. The indirect ELISA kit (IDEXX Laboratories, Westbrook, Maine, USA) 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 [9, 10]. Nevertheless, this ELISA method is laborious for nasal swab collection, 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-immunized pigs [11]. In addition, Mhp366 from *in vitro* grown *M. hyopneumoniae* strains was not detected by using a polyclonal serum raised against Mhp366 [11]. Based on these characteristics of Mhp366, we have developed an indirect ELISA for detecting humoral immunodominant proteins of *M. hyopneumoniae* which can discriminate between inactivated bacterin-induced hyperimmune sera and convalescent sera [12]. 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.

Results

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 (Fig. 1A). The results were also confirmed by Western blot analysis, since the target protein could react strongly with anti-His-tag antibody (Fig. 1B). Purification of the soluble recombinant protein was achieved by Ni chelating affinity chromatography as C-terminal 6×His-tagged fusion (Fig. 1C).

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 summarized in Table 1. Eight weeks after immunization, 14 serum samples from farm A were positive to *M. hyopneumoniae* and no *P36* gene was detected by nested PCR for all 20 piglets. For the farm B, 9 positive samples were detected by nested PCR method, while, the prevalence of *M. hyopneumoniae* was 60% (12/20) by IgG detection. The numbers of positive and negative diagnostic results confirmed by both molecular biology and anti-*M hyopneumoniae* IgG antibody were 7 and 6, respectively. Finally, we randomly picked up 12 *M. hyopneumoniae* hyperimmune sera from farm A and 5 convalescent sera from farm B for the following assay.

Optimization of ELISA procedure

Firstly, we investigated the effect of the antigen concentration. As shown in Fig. 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 Fig. 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. Fig. 2C demonstrates that complete blocking saturation was obtained at 30 min. With further 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 1:4000. As seen in Fig. 2D, the P/N ratio enhanced with the increase of the serum dilution and reached a maximum at 1:1000, and then subsequently decreased upon further increase of the serum dilution to 1:4000. Moreover, the incubation time of the serum with immobilized antigen could also affect the sensitivity of the final assay. In this regard, we have investigated the incubation time from 0.5 h to 2 h and we found that the highest P/N ratio was obtained at 0.5 h (Fig. 2E).

Finally, we investigated the effect of HRP-conjugate rabbit anti-pig IgG (H+L) secondary antibody by 2-fold 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 Fig. 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 h to 2 h increased the assay sensitivity (Fig. 2G). The optimal incubation time of the secondary antibody was 2 h. After the above-mentioned conditions were checked, the colorimetric

reaction time was optimized. As shown in Fig. 2H, the highest P/N value was obtained when the enzyme reacted with substrate for only 10 min.

Calculation of the cut-off value

Average OD_{450} value of 12 hyperimmune sera was 0.178, and the SD was 0.047. Therefore, the cut-off value of our indirect ELISA was calculated as 0.319 (mean ELISA value + 3SD=0.319). For better interpretation, any pig serum that had an OD_{450} value of 0.319 or higher than 0.319 was classified as convalescent serum. Serum with an OD_{450} value lower than 0.319 was classified as hyperimmune serum.

Reproducibility, specificity and sensitivity

Reproducibility was measured by determining intra- and inter-assay variation. The intra-assay CV of 2 hyperimmune serum and 2 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 7 porcine respiratory disease pathogens' antisera, including 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. As shown in Fig. 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 5 convalescent sera, the OD_{450} values decreased gradually. Five convalescent sera were still positive at 1:500, 1:1000 and 1:2000 dilutions, 4 sera showed positive result at 1:4000 dilution, 2 sera were positive at 1:8000 dilution, and 5 sera gave negative result at 1:16000 or more dilutions (Fig. 3B). As a result, the convalescent serum could be diluted up to 2000 times in this assay.

Comparisons among different ELISA methods

Serum samples collected from farm C and D were detected by commercial ELISA kit and discriminative IgG-ELISA for convalescent and hyperimmune sera (Table 2). At farm C, 15 samples were positive and 85 were negative by using a commercial ELISA kit. One sample which was determined as positive by commercial ELISA kit was judged as seropositive by the sIgA ELISA kit. However, seroconversion was not

observed with discriminative IgG-ELISA testing. On the other hand, nested PCR result showed that 12 laryngeal samples were positive for *M. hyopneumoniae* DNA and others were negative. For farm D, 4 and 2 serum samples obtained from piglets of 7 and 14 days old, respectively, were positive for IgG detection by commercial ELISA kit. Nevertheless, no antibody was detected with both sIgA ELISA and discriminative IgG-ELISA, although 1 laryngeal sample was 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.

Discussion

Detection of IgG antibody 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 *M. flocculare* [13]. 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 [10]. Commercial inactivated vaccines are the most popular strategy to control EP and are applied in more than 70% of the pig herds [1]. Therefore, the stimulation of anti-*M. hyopneumoniae* IgG could be the result of natural infection or vaccination. The commercial ELISA kits cannot distinguish 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 a sIgA ELISA method based on P97 protein to overcome the aforementioned issues [9]. 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 [14-16]. 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-immunized pigs. Although Mhp366 has a length of 555 amino acid residues with a calculated molecular weight of 64.4 kDa, its epitope recognized by the convalescent sera covers the amino acid positions 68-88 (^{68}QKENSQKNDVVNSQNKTEKTE^{88}). Therefore, we amplified 637 bp fragment of *mhp366* gene which covered the differential diagnostic region from the stating site.

Reproducibility was measured by determining intra- and inter-assay variation. 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 a good reproducibility. In addition to that, our ELISA test was able to discriminate between *M. hyopneumoniae* and other 7 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 1:1000, 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 [17] or even 98 days of age [18] after application of vaccine. Based on our finding, after 7 weeks immunization 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 immunization or infection [19].

Inactivated vaccines reduce the number of pathogens in the respiratory tract [20]. However, some studies indicate that vaccination does not significantly reduce the transmission of this respiratory pathogen in vaccinated herds compared to unvaccinated ones [20-22]. In 100 bacterin-vaccinated pigs of 10-11 weeks old, *M. hyopneumoniae* genetic material from 15 pigs were amplified.

The pathogens localized 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 post infection [23], whereas, seroconversion due to natural *M. hyopneumoniae* infection occurred in pigs within 8-24 weeks of old [24, 25]. 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 recognized 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, chromogenic time, are different from ones established in this study.

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.

Methods

Cloning of *mhp366-N* gene fragment

Plasmid pGEX-6P-2-mhp366 was extracted from recombinant bacteria GST-Mhp366 [26] using HiPure Plasmid Micro Kit (Magen, China). Nucleotide fragment *mhp366-N* which contains the corresponding peptide segment recognized 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) by 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.

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 h 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, China).

Animal source

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.

Serum samples used in this study were collected from 4 farms. Pigs from farm A were *M. hyopneumoniae*-free and no EP-like clinical syndromes occurred or lung lesions were observed. Pathogen and serology detection were carried out in recent 2 years. *M. hyopneumoniae* organism and nucleotide are free by culture and nested PCR. Also, the sera are negative by immunological diagnosis with commercial ELISA kit (IDEXX laboratories, Westbrook, Maine, USA). While, pigs from farm 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 farm C and D. All pigs were weaned on 21st day.

Sample collection and preparation

Twenty pigs from farm A were immunized with a commercial *M. hyopneumoniae* inactivated vaccine (MYPRAVAC SUIS, Hipra Lab) on the 7th day and 21st day after their bearing. MYPRAVAC SUIS is a whole-cell, inactivated bacterin based on J strain, with mineral oil and aluminum hydroxide as adjuvants. Fifty six days after the last immunization, serum samples were collected from the front cavity veins of immunized 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 [16]. Pigs from farm C and D were also vaccinated with MYPRAVAC SUIS on the 7th and 21st day. Twenty pigs of 21 weeks old and 100 pigs of 10-11 weeks old were chosen from farm B and C, respectively. Five piglets of 7 days old before immunization and other 5 piglets of 14 days old shot on 7th day from farm D were picked up randomly. Laryngeal swabs were collected from corresponding pigs at farm B [16], while, nasal and laryngeal swabs were collected from corresponding pigs at farm C and D, as previously described [9, 16].

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 releasing into 1 mL sterile PBS at 4℃ overnight. *M. hyopneumoniae* was determined by nested PCR from laryngeal swabs as described previously [9]. 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, sIgA was detected from the supernatant according the procedure of sIgA ELISA kit [9].

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

Optimization of ELISA procedure and working condition

The 96-well microtiter plates (Corning incorporated, 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℃ after 37℃ for 1 h. 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 h, 1 h or 2 h. After five washes with PBST, 100 μL serum samples diluted from 1:50 to 1:8000 were added and incubated at 37℃ for 0.5 h, 1 h or 2 h. 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 1:10000 to 1:80000) at 37℃ for different times (0.5 h, 1 h and 2 h). 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 0.2078 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 (ThermoFisher Scientific, Ratastie 2, FI-01620 Vantaa, Finland). All samples were run in triplicate, and each experiment was performed at least twice. Each working condition was optimized and determined with the highest P/N ratio between convalescent serum samples (P) and hyperimmune serum samples (N).

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 3 standard deviations (SD), as described previously [27, 28].

Evaluation of reproducibility

Reproducibility of intra- and inter- assay variation between runs was performed as described by Feng et al. [9] with minor modification. In brief, 2 hyperimmune and 2 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 3 plates from different batch were chosen for inter-assay (between runs) reproducibility. Mean values, SD and coefficient of variations (CV) were calculated.

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 2 convalescent sera were used as negative and positive controls, respectively.

Five convalescent sera were diluted with blocking buffer as follows: 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000 and 1:64000. 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.

Application and comparison of ELISA discriminating hyperimmune sera and convalescent sera with commercial kits

Samples from farm 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 conduct in duplicate. *M. hyopneumoniae* DNA was tested by nested PCR from laryngeal swabs as described previously (Feng et al., 2010).

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, German) for 2 h at 100 V using a trans-blotting apparatus (Bio-Rad, USA). The membrane was blocked overnight at 4℃ in 5% skimmed milk-TBST and was detected by His-tag (4C2) monoclonal antibody (Bioworld Technology, China) with a 1:8000 dilution at RT for 1 h. The primary antibody binding was incubated with a 1:20000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Proteintech, China) at RT for 1 h and visualized with an enhanced chemiluminescence kit (CWBio, China).

List Of Abbreviations

CV: coefficient of variations; ELISA: Enzyme-linked immunosorbent assay; GST: glutathione *S*-transferase; HRP: horseradish peroxidase; IPTG: isopropyl-β-□-thiogalactoside; EP: enzootic pneumonia; OD: optical density; PRDC: porcine respiratory disease complex; SD: standard deviations.

Declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable.

Availability of data and materials

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by State Key Laboratory of Veterinary Biotechnology Foundation (SKLVBF201905), Fundamental Research Funds for the Central Universities (XDJK2020B012), and Chongqing Technology Innovation and Application Development Project (cstc2019jscx-msxmX0402). The funding bodies had no role in the design of the research, the collection, analysis, and interpretation of data, and the writing of the manuscript.

Authors' contributions

HD and JX conceived and designed the study and analyzed the data. HD, YW, ZX, YT, ZW and YN performed the experiments, interpreted the results. HD, CT and JX wrote the manuscript. All authors reviewed the results and approved the final version of manuscript.

Acknowledgements

Not applicable.

Author details

[1] Laboratory of Veterinary Lemology, College of Animal Science and Technology, Southwest University, Chongqing 400715, China. [2] Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing 400714, China. [3] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150069, China.

References

1. Maes D, Sibila M, Kuhnert P, Segalés J, Haesebrouck F, Pieters M. Update on *Mycoplasma hyopneumoniae* infections in pigs: Knowledge gaps for improved disease control. Transbound Emerg Dis. 2018; Suppl 1: 110-124.
2. Sibila M, Bernal R, Torrents D, Riera P, Llopart D, Calsamiglia M, Segalés J. Effect of sow vaccination against *Mycoplasma hyopneumoniae* on sow and piglet colonization and seroconversion, and pig lung lesions at slaughter. Vet Microbiol. 2008; 127(1-2): 165-170.
3. Cvjetković V, Sipos S, Szabó I, Sipos W. Clinical efficacy of two vaccination strategies against *Mycoplasma hyopneumoniae* in a pig herd suffering from respiratory disease. Porcine Health Manag. 2018; 4: 19.
4. Chae C. Porcine respiratory disease complex: Interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hyopneumoniae*. Vet J. 2016; 212: 1-6.
5. Simionatto S, Marchioro SB, Maes D, Dellagostin OA. *Mycoplasma hyopneumoniae*: From disease to vaccine development. Vet Microbiol. 2013; 165(3-4): 234-242.
6. Feng ZX, Wei YN, Li GL, Lu XM, Wan XF, Pharr GT, Wang ZW, Kong M, Gan Y, Bai FF, Liu MJ, Xiong QY, Wu XS, Shao GQ. Development and validation of an attenuated *Mycoplasma hyopneumoniae* aerosol vaccine. Vet Microbiol. 2013; 167(3-4): 417-424.
7. Matthijs AMF, Auray G, Jakob V, García-Nicolás O, Braun RO, Keller I, Bruggman R, Devriendt B, Boyen F, Guzman CA, Michiels A, Haesebrouck F, Collin N, Barnier-Quer C, Maes D, Summerfield A. Systems immunology characterization of novel vaccine formulations for *Mycoplasma hyopneumoniae* Front Immunol. 2019; 10: 1087.
8. Tao Y, Shu J, Chen J, Wu Y, He Y. A concise review of vaccines against *Mycoplasma hyopneumoniae*. Res Vet Sci. 2019; 123: 144-152.
9. Feng ZX, Shao GQ, Liu MJ, Wang HY, Gan Y, Wu XS. Development and validation of a SIgA-ELISA for the detection of *Mycoplasma hyopneumoniae* Vet Microbiol. 2010; 143(2-4): 410-416.
10. Bai Y, Gan Y, Hua LZ, Nathues H, Yang H, Wei YN, Wu M, Shao GQ, Feng ZX. Application of a sIgA-ELISA method for differentiation of *Mycoplasma hyopneumoniae* infected from vaccinated pigs. Vet Microbiol. 2018; 223: 86-92.
11. Meens J, Bolotin V, Frank R, Böhmer J, Gerlach GF. Characterization of a highly immunogenic *Mycoplasma hyopneumoniae* lipoprotein Mhp366 identified by peptide-spot array. Vet Microbiol. 2010; 142(3-4): 293-302.
12. Ding H, Zhou Y, Wang H. Development of an indirect ELISA for detecting humoral immunodominant proteins of *Mycoplasma hyopneumoniae* which can discriminate between inactivated bacterin-induced hyperimmune sera and convalescent sera. BMC Vet Res. 2019; 15(1): 327.
13. Maes D, Verdonck M, Deluyker H, de Kruif A. Enzootic pneumonia in pigs. Vet Q. 1996; 18(3): 104-109.
14. Fablet C, Marois C, Kobisch M, Madec F, Rose N. Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. Vet

Microbiol. 2010; 143(2-4): 238-245.

15. Vangroenweghe F, Karriker L, Main R, Christianson E, Marsteller T, Hammen K, Bates J, Thomas P, Ellingson J, Harmon K, Abate S, Crawford K. Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in preweaned piglets utilizing an antemortem tracheobronchial mucus collection technique and a real-time polymerase chain reaction assay. J Vet Diagn Invest. 2015; 27(5): 606-610.
16. Pieters M, Daniels J, Rovira A. Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet Microbiol. 2017; 203: 103-109.
17. Sibila M, Nofrarías M, López-Soria S, Segalés J, Valero O, Espinal A, Calsamiglia M. Chronological study of *Mycoplasma hyopneumoniae* infection, seroconversion and associated lung lesions in vaccinated and non-vaccinated pigs. Vet Microbiol. 2007; 122(1-2): 97-107.
18. Martelli P, Terreni M, Guazzetti S, Cavirani S. Antibody response to *Mycoplasma hyopneumoniae* infection in vaccinated pigs with or without maternal antibodies induced by sow vaccination. J Vet Med B Infect Dis Vet Public Health. 2006; 53(5): 229-233.
19. Erlandson KR, Evans RB, Thacker BJ, Wegner MW, Thacker EL. Evaluation of three serum antibody enzyme-linked immunosorbent assays for *Mycoplasma hyopneumoniae*. J Swine Health Production. 2005; 13: 198-203.
20. Meyns T, Dewulf J, de Kruif A, Calus D, Haesebrouck F, Maes D. Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. Vaccine. 2006; 24(49-50): 7081-7086.
21. Villarreal I, Maes D, Vranckx K, Calus D, Pasmans F, Haesebrouck F. Effect of vaccination of pigs against experimental infection with high and low virulence *Mycoplasma hyopneumoniae* Vaccine. 2011; 29(9): 1731-1735.
22. Villarreal I, Meyns T, Dewulf J, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D. The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. Vet J. 2011; 188(1): 48-52.
23. 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 GQ. Use of serological and mucosal immune responses to *Mycoplasma hyopneumoniae* antigens P97R1, P46 and P36 in the diagnosis of infection. Vet J. 2014; 202(1): 128-133.
24. Djordjevic SP, Eamens GJ, Romalis LF, Saunders MM. An improved enzyme linked immunosorbent assay (ELISA) for the detection of porcine serum antibodies against *Mycoplasma hyopneumoniae*. Vet Microbiol. 1994; 39(3-4): 261-273.
25. Leon EA, Madec F, Taylor NM, Kobisch M. Seroepidemiology of *Mycoplasma hyopneumoniae* in pigs from farrow-to-finish farms. Vet Microbiol. 2001; 78(4): 331-341.
26. Zhou Y, You F, Zhong J, Wang H, Ding H. Development of an ELISA for identification of immunodominant protein antigens of *Mycoplasma hyopneumoniae*. Sheng Wu Gong Cheng Xue. Bao. 2018; 34(1): 44-53.

27. Poolperm P, Varinrak T, Kataoka Y, Tragoolpua K, Sawada T, Sthitmatee N. Development and standardization of an in-house indirect ELISA for detection of duck antibody to fowl cholera. J Microbiol Methods. 2017; 142: 10-14.
28. Tankaew P, Singh-La T, Titaram C, Punyapornwittaya V, Vongchan P, Sawada T, Sthitmatee N. Evaluation of an In-house indirect ELISA for detection of antibody against haemorrhagic septicemia in Asian elephants. J Microbiol Methods. 2017; 134: 30-34.

Tables

Farm	No. of pigs	PCR result of LS		Commercial ELISA results of sera	
		+	-	+	-
A	20	0	20	14	6
B	20	9	11	12	8
LS: laryngeal swabs.					

Table 1
Prevalence of M. hyopneumoniae infection and M. hyopneumoniae positive sera in selected pigs from 2 farms

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	+	15	1	0	12
	-	85	99	100	88
Sucking pigs of age 7 days from farm D	+	4	0	0	1
	-	1	5	5	4
Sucking pigs of age 14 days from farm D	+	2	0	0	1
	-	3	5	5	4
Total	+	21	1	0	14
	-	89	109	110	96

+: positive, -: negative.

Figures

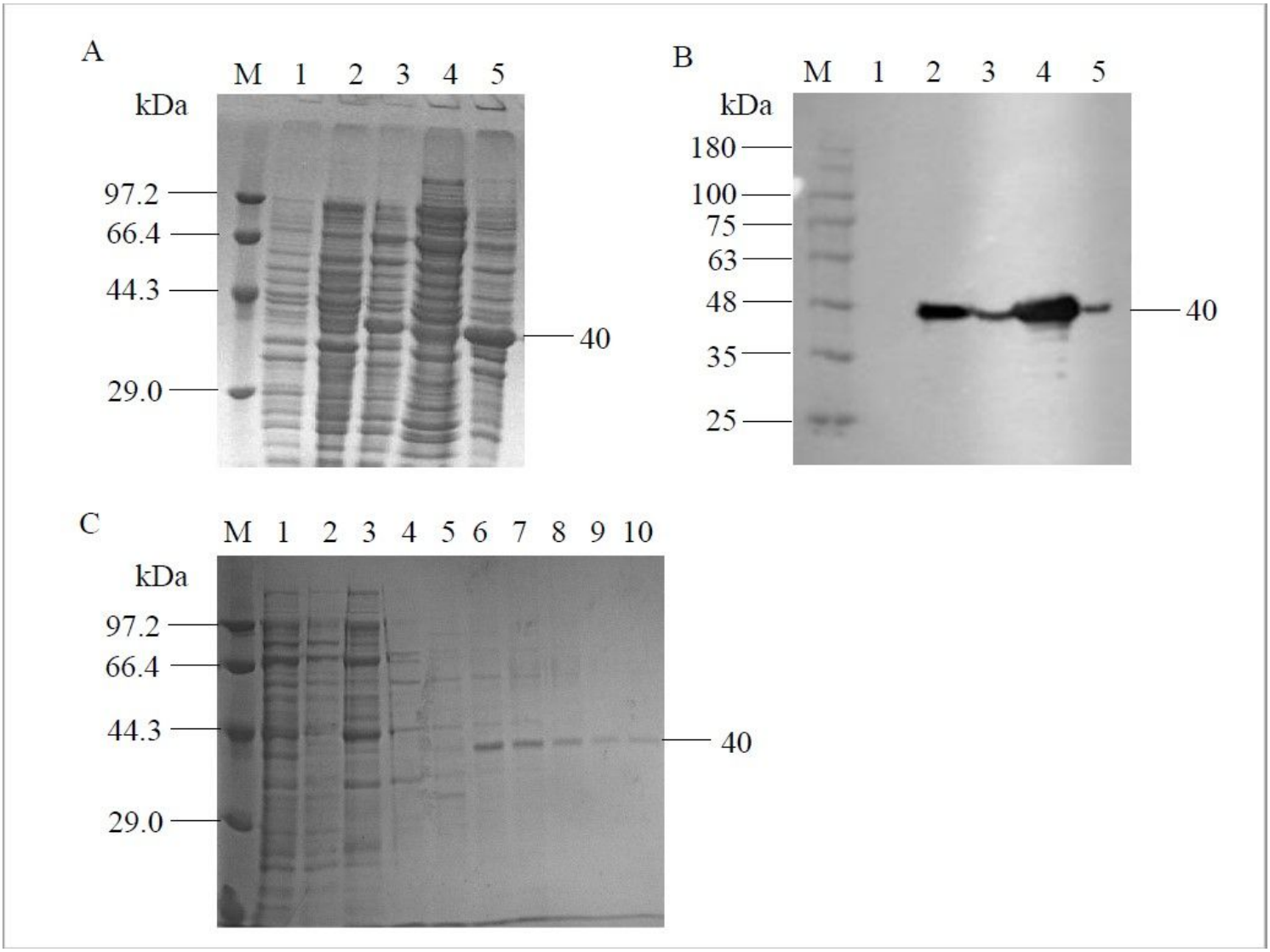

Figure 1

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 soluble form (Lane 4) and inclusion body (Lane 5) with the induction of IPTG (Lane 3), although it still expressed 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) and run through (Lane 2). After other proteins were washed with a linear imidazole gradient of 0.1 M (Lane 3), 0.2 M (Lane 4) and 0.5 M (Lane 5), purified protein was collected (Lane 6-10).

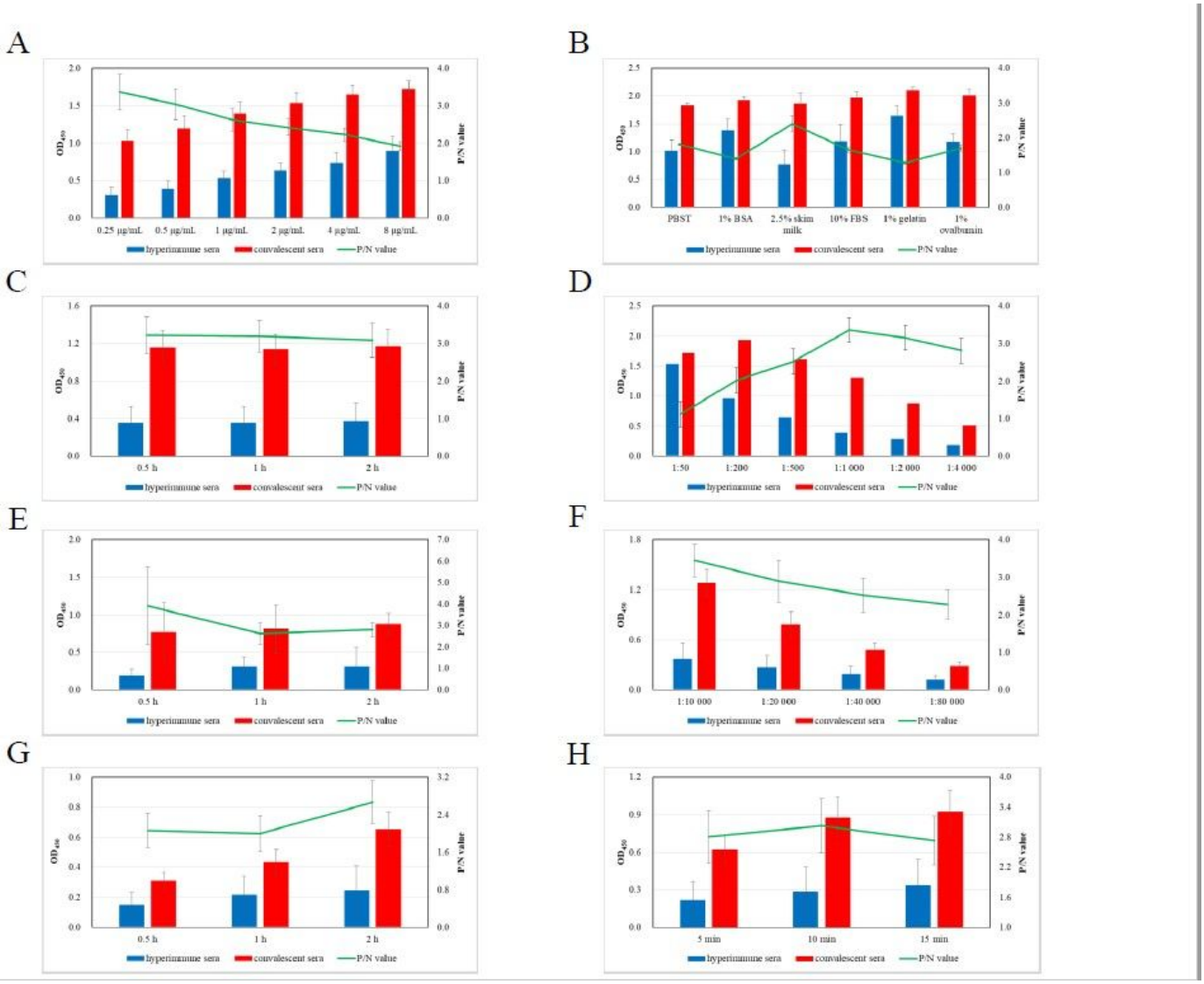

Figure 2

Optimization 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 h (C). The optimal dilution of serum and secondary antibody were 1:1000 (D) and 1:10000 (F) diluted in blocking buffer. The optimal incubation times of serum and secondary antibody were 0.5 h (E) and 2 h (G), respectively. The optimal colorimetric reaction time was observed after exposing to substrate solution for 10 min (H).

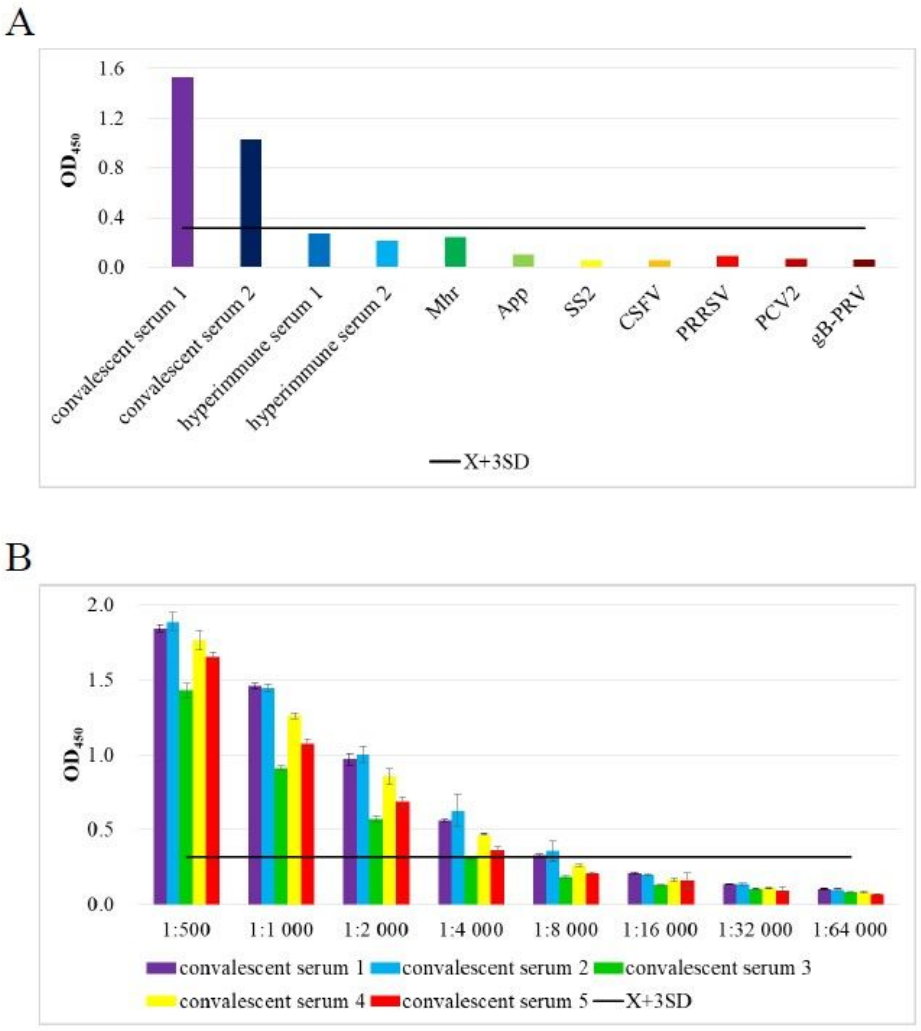

Figure 3

Specificity and sensitivity detection of ELISA. (A) The results were positive when used 2 convalescent sera. However, negative results were gotten 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, 2 hyperimmune sera as primary antibodies. (B) Five convalescent sera were still positive at 1:500, 1:1000 and 1:2000 dilutions, 4 sera were positive at 1:4000 dilution, 2 sera were positive at 1:8000 dilution, and 5 sera were negative at 1:16000 or more dilutions. The convalescent serum could be diluted up to 2000 times in this assay.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklist.pdf