

# Two novel protein chips for the detection of antibodies against porcine parvovirus

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## Research article

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# Abstract

**Background:** PPV is one of the most important pathogens causing porcine reproductive disorder. It has been shown in clinical cases to be a commonly mixed infection with other important swine diseases which can aggravate the severity of the disease and bring serious economic losses to the pig industry. Serological methods, such as hemagglutination inhibition assays (HAI), serum neutralization (SN), and the modified direct complement-fixation (MDCF) test were utilized earlier, whereas the enzyme-linked immunosorbent assay (ELISA) is the most frequently applied assay to detect PPV-specific antibodies

**Results:** We establish the visible protein chip and the cyanine dye 3 (Cy3)-labeled protein chip to detect the clinical serum from pigs. In this study, the recombinant protein VP2 of PPV was expressed in *E.coli*, purified with nickel magnetic beads, and then printed onto epoxy-coated glass slides for preparation of the protein chip. After a series of experiments, the conditions of antigen protein concentration, incubation time of primary antibody or secondary antibody, and optimal serum dilution fold were optimized, resulting in a successful visible protein chip and Cy3-labeled protein chip. The results showed that the positive serum, diluted up to 6000-fold, can be detected by the visible protein chip, and the positive serum, diluted up to 12,800-fold, can be detected by the Cy3-labeled protein chip, suggesting the high sensitivity of these protein chips. Moreover, the positive detection ratio, sensitivity, and specificity of these two kinds of protein chips were higher than those of commercial ELISA antibody detection kits.

**Conclusion:** Overall, these two protein chips can be used to rapidly diagnose clinical samples with high throughput. **Key words:** Protein chip; Porcine parvovirus (PPV); Antibody detection; Clinical serum

## Background

Porcine parvovirus (PPV) is a non-enveloped, single-stranded linear DNA virus, which belongs to the genus Parvovirus within the family Parvoviridae; the virus is round or hexagonal in shape[1, 2]. PPV is one of the most important pathogens causing porcine reproductive disorder. It has been shown in clinical cases to be a commonly mixed infection with Pseudorabies virus (PRV), classical swine fever virus (CSFV), Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Porcine Circovirus type 2 (PCV2), and Japanese Encephalitis virus (JEV), which can aggravate the severity of the disease and bring serious economic losses to the pig industry[3]. In 1965, PPV was first isolated in Germany as a contaminant of porcine primary cell culture that was used for CSFV propagation, and subsequently broke out in many countries and regions in the world. At present, seven genotypes of PPV have been found in the world, which have some differences in genomes and pathogenicity. PPV was first detected in China in 1980[4] and isolated and identified in 2017[5–10]. PPV1 is the most common type in pigs, which has a higher positive detection rate in Chinese pig herds and is harmful to the pig industry. VP2, the main nucleocapsid protein component of PPV, has hemagglutination activity and can induce neutralizing antibodies. Previous studies have shown that the major antigenic site regions of VP2 are amino acids 60–68, 81–88, 266–275, 351–357, and 398–404, where amino acids 378, 383, and 436 are critical sites to determine PPV virulence[11, 12].

Serological methods, such as hemagglutination inhibition assays (HAI), serum neutralization (SN), and the modified direct complement-fixation (MDCF) test were utilized earlier, whereas the enzyme-linked immunosorbent assay (ELISA) is the most frequently applied assay to detect PPV-specific antibodies[13]. With the maturity of gene chip technology, the protein chip has also been developed with the features of high throughput, large amount of detection information, rapidity and timeliness, small sample requirements, and high sensitivity[14]. The principle of the protein chip is to regularly immobilize various proteins (such as antigens or antibodies), polypeptide molecules, receptors, and ligands on a certain carrier (such as filters, gels, slides, nano-microspheres, and microplates). On top of this, by adding antibodies labeled with Cy3, Cy5, chemiluminescence, immunogold, etc., the reaction between the antigen and the antibody is amplified to analyze the components that can specifically interact with it[15]. The protein chip is a promising laboratory method in the diagnosis of animal diseases and in the monitoring of disease states, but its application is not extensive. A visible protein chip was established to detect avian influenza (AI), Newcastle disease (ND), infectious bronchitis (IB), and infectious bursal disease (IBD)[16]. The results showed that the sensitivity is significantly higher than traditional detection assays. In Xu et al[17], a novel protein chip was developed to detect bluetongue virus using a new solid material, iPDMS, as a solid phase carrier, which has a high degree of sensitivity and specificity. Compared with commercial IDEXX BTV ELISA kits, the total, negative, and positive coincidence rates were 95.12%, 99.28%, and 86.5%, respectively. Additionally, previous publication have described a novel detection platform that leverages horseradish peroxidase (HRP)-mediated silver precipitation within antibody immobilized porosity tuned polyethylene glycol diacrylate (PEGDA) hydrogel microparticles with the operational advantages of suspension arrays for sensitive quantification of biomarkers[18].

Herein we present a visible protein chip detection microarray and Cy3-labeled protein chip detection microarray for the detection of PPV antibodies in pig serum. In this study, we use epoxy-coated glass slides as diagnostic material. Additionally, chemical amplifications using Au nanoclusters or nanoparticles as catalytic centers have primarily been utilized for the silver enhancement/amplification reaction[19]. Cyanine dye 3(Cy3) was also applied to detect antigen–antibody reaction products specifically, which is a procedure that was applied relatively early to the field of protein chips; furthermore, Cy3, Cy5, and IR800 can also be used to label protein chips[20, 21]. Both of these methods were applied to evaluate the seroprevalence of PPV in serum samples collected from pig herds in China.

## Methods

**Virus and cells.** PK-15 cells were purchased from ATCC (CCL-33), and propagated in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum, 100 ug/mL streptomycin, and 100 IU/mL penicillin (GIBCO, Invitrogen). The attenuated strain, NADL-2, of PPV was obtained from the National Institute of Veterinary Drug Control.

**Gene synthesis and plasmid construction.** According to the advanced structure and GC content of the PPV VP2 gene sequence (JQ710896.1) and the codon usage frequency of the *Escherichia coli* host, the VP2 gene sequence (amino acids 156–438) was optimized using software of Rare Codon Caltor and

synthesized by Wuhan Jinkaili Bioengineering Co., Ltd. Hind III and BamHI restriction sites were introduced at the 5' and 3' ends of the VP2 gene, respectively. The pCold vector and the VP2 gene fragment were double digested and ligated with T4 Ligase. The ligation product was transformed into the E.coli DH5α competent state and cultured at 37 °C for 12 h. The selected positive clones were cultured in IR800 medium for 12 h. Plasmids were extracted and sequenced. The correct plasmid was identified as pCold-VP2.

**Expression and purification of PPV VP2 protein.** Rosetta2 (DE3) cells (Novagen, Shanghai, China) transformed with pCold-VP2 were cultured in LB medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, were grown at 37 °C until achieving the logarithmic phase (at OD600 of 0.6), and induced by Isopropyl-thio-galactoside (IPTG) at a final concentration of 0.1 mM for 16 h at 18 °C. The re-suspended cells were lysed by sonication on ice for 8 × 4 s with 3 s intervals. The lysate was centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant and the pellet resuspended in PBS were both analyzed by SDS–PAGE to observe the solubility of the target protein. The fusion proteins in the inclusion bodies were denatured using 8 µM urea at 4 °C overnight, and the supernatant was retained after centrifugation. His-tagged VP2 fusion proteins were bound to Ni-NTA resin (Qiagen, Hilden, Germany) and purified following the manufacturer's instructions. Finally, freshly purified proteins were treated with Detoxi-Gel™ Endotoxin Removing Gel (Thermo Fisher Scientific, Waltham, USA) to remove endotoxin derived from bacterial culture in the fusion proteins. Aliquots of purified protein were stored at –80 °C.

**Production of mouse anti-VP2 polyclonal antibody.** One group of Balb/c mice (Qinglongshan Animal Breeding Farm, Nanjing, China) were immunized at 100 µg VP2 protein with Freund's complete adjuvant (1:1, Sigma-Aldrich, USA) to form an emulsion. Another group of Balb/c mice (Qinglongshan Animal Breeding Farm, Nanjing, China) with the same dose of sterile saline was set as negative control. After the first immunization, the second and third immunizations were performed after Freund's incomplete adjuvant (1:1) emulsification every two weeks. After the third immunization, serum was collected from the orbital blood and stored in a refrigerator at -80 °C. The mice were sacrificed by cervical dislocation without anesthesia. After confirming the death, the mice were put into a freezer for centralized processing.

**Indirect immunofluorescence (IFA).** PK-15 cells were infected with PPV for 48 hours and fixed with 4% paraformaldehyde, permeabilized with 0.1% X-Triton, and blocked with 10% sheep serum. PK-15 cells were incubated with mouse anti-VP2 polyclonal antibody (1:500) for 2 h at 37 °C, washed with PBST 3 times, incubated with goat anti-mouse antibody (Alexa Fluor 488, abcam, USA) for 1 hour at 37 °C, and washed with PBST 3 times. The nucleus was dyed with DAPI for 20 minutes and observed with a confocal microscope.

**Antibody labeled.** The standard PPV positive antibody (National Institute of Veterinary Drug Control) was conjugated to Cyanine3 fluorescent dye using a Lightning-Link® HRP conjugation kit (Innova Biosciences, UK) according to the manufacturer's instructions. Briefly, 1 µL of LL-Rapid modifier reagent was added for each 10 µL of Standard PPV positive antibody and mixed gently. The mixture was

removed into a vial of Lightning-Link® Rapid mix and resuspended gently by withdrawing and re-dispensing the liquid once or twice. After incubating for 15 minutes at room temperature in the dark, 1 µL of LL-Rapid quencher reagent was added into every 10 µL of serum. The conjugate mixture could be used after 4 minutes.

**Protein chip preparation.** Before printing, 50% of the moisture of the spotter was prepared at 4 °C. VP2 protein of 0–0.4 mg/mL was printed on epoxy-coated glass slides with 2 × printing buffer (Capital Biochip Biotechnology Co. Ltd, Beijing, China); each dilution of VP2 protein was printed 3 times using the SmartArrayer™ microarray spotter (Capital Biochip Biotechnology Co. Ltd, Beijing, China). The spotted chip was placed in a 37 °C molecular hybridization instrument and hydrated for 10 h. After 5 min washing with PBST, 1% BSA was added to each sub-array for blocking. Then the prepared slides could be used immediately or could be stored at 4 °C after centrifuging and drying. The diluted standard PPV positive antibody serum was added to the chips and incubated for 1 h at 37 °C. After washing and drying, the protein chip was observed by scanning with a Luxscan-10K/A chip scanner (Capital Biochip Biotechnology Co. Ltd, Beijing, China).

**Establishment of visible protein chip.** To determine the optimal concentration of diagnostic antigen, PPV VP2 protein were diluted to 0.002 mg/mL, 0.02 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, and 0.4 mg/mL with 2 × printing buffer. The blocked chip was added with 100-fold diluted positive control serum and incubated at 37 °C for 1 h. After washing with PBST three times, 100-fold diluted rabbit anti-pig IgG was added on the chips and incubated at 37 °C for 1 h. After washing with PBST three times, the well-mixed silver staining solution A and B (Sigma-Aldrich, St. Louis, USA) were added into each well, and then the results were observed directly. Once the antigen concentration was determined, the optimized concentration of VP2 protein was printed on epoxy-coated glass slides for subsequent experiments. The positive control serum was diluted to 10-, 100-, 1000-, 2000-, 4000-, 5000-, and 6000-fold to determine the optimal serum dilution. The incubation time of primary and secondary antibodies was set to 30 min, 45 min, and 1 h, respectively, and a total of 9 groups of time gradient tests were performed to determine the optimal antigen–antibody reaction time.

**Establishment of Cy3-labeled protein chip.** To determine the optimal antigen concentration, PPV VP2 proteins were diluted with a series of concentrations (0.002, 0.02, 0.05, 0.1, and 0.2 mg/mL) with 2 × printing buffer. The blocked chip was added with 100-fold diluted positive porcine serum and incubated at 37 °C for 1 h. After washing with PBST, 100-fold diluted Cy3-labelled rabbit anti-porcine IgG was added and incubated at 37 °C for 1 h. After washing with PBST for 5 min, the chip was centrifuged and dried. The results were observed by scanning with a Luxscan-10K/A chip scanner. Once the antigen concentration was determined, the optimized concentration of VP2 protein was printed on epoxy-coated glass slides for subsequent experiments. PPV antibody positive serum was diluted 10~25,600-fold to determine the optimal serum dilution ratio. The incubation time of primary and secondary antibodies was set to 30 min, 45 min, and 1 h, respectively, and a total of 9 groups of time gradient tests were performed to determine the optimal antigen–antibody reaction time.

**Detection of clinic samples.** The established PPV visible protein chip was used to detect 9 negative and 42 positive swine serum samples that had been detected using an indirect ELISA assay according to the manufacturer's instructions (Wuhan Keqian Biology Co., Ltd, China). The established PPV Cy3-labeled protein chip detection microarray was used to detect 15 negative and 19 positive swine serum samples that had been detected by indirect ELISA. Then, 120 clinical swine serum samples [farmer permission] from the pig farms in Jiangsu province were tested using the two established protein chip technologies and compared with commercial PPV ELISA antibody detection kits.

## Results

**PPV VP2 protein preparation and its Specificity.** The PPV VP2 gene was cloned into the pColdI vector (Figure 1A). Upon induction by IPTG, PPV VP2 protein was expressed in the inclusion body (Figure 1B). After the expressed PPV VP2 protein was purified, a mouse anti-VP2 polyclonal antibody with good specificity was successfully prepared. The purified VP2 protein was proved to have good specificity (Figure 1C) and was used to prepare protein chips (Figure 1D).

**Protein chip preparation.** PPV VP2 protein was diluted to 0–0.4 mg/mL with 2 × printing buffer to determine the optimal protein concentration. Preparation of protein chips based on the dot sequence are shown in Figures 2A and 2B, respectively; the results showed 0.3 mg/mL as the optimal protein concentration of the visible protein chip detection microarray (Figure 2C,2E), and 0.1 mg/mL as the optimal protein concentration of the Cy3-labeled protein chip detection microarray (Figure 2D,2F).

**Optimization of the serum dilution.** PPV antibody positive serum was diluted 10–25,600-fold to determine the optimal serum dilution ratio. The visible protein chip detection microarray could dilute serum up to 6000-fold, and a 2000-fold dilution of serum was determined as a diagnostic concentration (Figure 3A). The Cy3-labeled protein chip detection microarray could dilute serum up to 25,600-fold, and it was determined that a 2000-fold dilution of serum was a diagnostic concentration (Figure 3B).

**Optimization of the incubation time of primary and secondary antibodies.** In order to determine the optimal antibody reaction time, the first and second antibody incubation gradients were set for 30 min, 45 min, and 1 h, respectively, and a total of 9 sets of time gradient tests were conducted at 37 °C in the hybridization apparatus. Other reaction conditions were unchanged. Silver staining or protein chip scanner observations were done. The results showed that the optimal incubation time for the visible protein chip detection microarray was 30–45 min for the primary antibody and 60 min for the secondary antibody (Figure 4A); the optimal incubation time for the Cy3-labeled protein chip detection microarray was 30–45 min for the primary antibody and 45–60 min for the secondary antibody (Figure 4B).

**Determining the positive–negative threshold.** When the visible protein chip appeared as dots, the sample could be judged as positive; otherwise, the sample was negative. When the SNR (the ratio of the median value of the signal intensity to the background median value) of the Cy3 fluorescent labeling protein chip was greater than or equal to 3.0, the sample could be judged as positive; otherwise, the sample was negative.

**Coincidence rate of the two protein chips.** The established visible protein chip detection microarray was used to detect 9 negative pig serum and 42 positive pig serum that had been detected using commercial PPV ELISA antibody test kits. The results showed that 51 samples were detected using a visible protein chip: 44 were positive and 7 were negative. One of the positive samples detected by the commercial PPV ELISA antibody test kits was detected as negative by the visible protein chip, and 3 negative samples detected by the commercial PPV ELISA antibody detection kits were detected as positive by the protein chip. The negative coincidence rate, the positive coincidence rate, and the total coincidence rate were 85.7%, 93.2%, and 92.2%, respectively.

The established Cy3-labeled protein chip detection microarray was used to detect 5 negative porcine serum and 19 positive porcine serum that were detected by the commercial PPV ELISA antibody test kits. The results showed that of the 34 detected serum, there were 22 positive samples and 12 negative samples detected by the Cy3-labeled protein chip detection microarray (Table 1). One of the positive samples detected by the commercial PPV ELISA antibody detection kits was detected as negative by the Cy3-labeled protein chip diagnostic microarray, and 4 negative samples detected by the commercial PPV ELISA antibody test kits were detected as positive by the Cy3-labeled protein chip. The negative coincidence rate, the positive coincidence rate, and the total coincidence rate was 91.7%, 81.8%, and 85.3%, respectively (Table 1 ).

**Application of the two protein chips.** One hundred and twenty clinical pig serum samples were tested using the two established protein chip technologies and compared with commercial PPV ELISA antibody detection kits. The results show that the positive rate of the two established protein chip technologies was higher than ELISA, and the positive rate of the Cy3-labeled protein chip was higher than the visible protein chip (Table 2).

## Discussion

Protein chips are widely used for high-throughput proteomic analysis, but the low sensitivity and narrow dynamic range have limited their capabilities in diagnostics and proteomics[14, 22]. Nitrocellulose (NC), polystyrene, poly methyl methacrylate (PMMA), nylon, poly vinylidene fluoride (PVDF), poly-L-lysine (PLL), Poly-electrolyte thin films, and polymer-coated initiator-integrated PDMS (iPDMS) can be used as diagnosis material. Choosing the right substrate for diagnostics is important; here, we use epoxy-coated glass slides as diagnostic material, which have the advantage of being able to efficiently bind to proteins. ELISA requires 200–1000 ng of antigen protein per sample, while the protein chip technology established in this experiment requires only 0.5–2 ng of antigen protein per sample; antigen and antibody can be efficiently bound, so when testing, they only need a micro-scale of serum to be detected. In this study, we set different concentrations of VP2 protein, and we determined 0.3 mg/mL as the optimal protein concentration of the visible protein chip detection micro-array and 0.1 mg/mL as the optimal protein concentration of the Cy3-labeled protein chip detection micro-array. In addition, one PPV antibody-positive pig serum sample was detected as positive when diluted 6000-fold using a visible protein chip, and by

12,800-fold dilution when using a Cy3-labeled protein chip. We ultimately set a 2000-fold dilution as the optimal dilution for the test sera.

As a reasonable incubation time significantly enables efficient binding of antigens and antibodies, the first and second antibody incubation gradients were set for 30 min, 45 min, and 1 h, respectively, to optimize the optimal primary and secondary antibody incubation times. The two kinds of protein chips established in this study are solid-phase protein chips, whereas previous studies also established a suspension protein array for the detection of animal virus antibodies[23]. The two protein chip technologies established have good sensitivity and specificity. In this study, the visible protein chip negative coincidence rate, positive coincidence rate, and total coincidence rate were 85.7%, 93.2%, and 92.2%, respectively. For the Cy3-labeled protein chip, the negative coincidence rate, the positive coincidence rate, and the total coincidence rate were 91.7%, 81.8%, and 85.3%, respectively. The specificity and sensitivity of the Cy3-labeled protein chip and the visible protein chip were higher than those of ELISA by about 4.2% and 1.7% respectively, while the Cy3-labeled protein chip was higher than that of the visualized protein chip by about 2.5%.

PPV is one of the major pathogens causing sow reproductive failure. It mainly causes reproductive difficulties in recently farrowed sows and skin lesions in newly born piglets. PPV is distributed all over the world and bring a lot of economic losses to the pig industry. At present, there are no effective antiviral drugs against PPV, and the effective control of PPV is still based on immunoprevention. In recent years, protein chips have been increasingly used in fields such as food inspection, proteomics, disease diagnosis, drug screening, agriculture, forestry, animal husbandry, and forensic identification. As a novel laboratory diagnostic technique, the two protein chips established in this study can be used to monitor the level of PPV antibodies in pigs with more sensitive and high-throughput and has important application prospects.

## Conclusion

These two protein chips are used to detect PPV antibodies, and they are highly compatible with commercial ELISA kits in terms of specificity and sensitivity. At the same time, these two protein chip methods also have the advantage of high throughput and have broad prospects in clinical use.

## Abbreviations

iPDMS: polydimethylsiloxane; LB: Luria Broth; PBS : phosphate buffer saline; DAPI: 4',6-diamidino-2-phenylindole; BSA : Bovine Serum Albumin

## Declarations

### Competing Interests

The authors declare that they have no competing interests.

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## **Availability of data and materials**

Raw data is available from the corresponding author on reasonable request.

## **Authors' contributions**

BZ designed and directed the study. YW, XDW, and JXH perform the experiment, collect results and analyze data. YW, JXH draft the first vision of the manuscript. XNC, XBH collect clinical serum samples. BZ critically revised the manuscripts. All the participants carefully reviewed this vision manuscript.

## **Ethics approval and consent to participate**

This study was carried out in strict accordance with the recommendations in the "Guidelines for the Care and Use of Laboratory Animals" by the Ministry of Health of China. The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (approval number: PZ2019012). Written informed consent to collect clinical serum samples from pig farms. This study minimized animal suffering during sample collection.

## **Consent for publication**

Not applicable Competing interest.

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## Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the supplemental files section.

## Figures



### Figure 1

PPV VP2 protein preparation and its Specificity. (A) Identification of prokaryotic expression vector digested by restriction enzyme identification. (B) Identification of PPV VP2 protein expression and purification by SDS–PAGE. (C) Detection of reactivity of anti-VP2 polyclonal antibodies by confocal microscopy. (D) Using the protein chip to verify the specificity of the PPV VP2 protein.



### Figure 2

Determination of the optimal spot concentration. (A,B) Dot sequence order of both protein chips. (C,D) Optimization of the antigen of both protein chips. (E,F) The antigen diagnostic concentration of the visible protein chip microarray is optimized. The amount of 0.3 mg/ml can be used as a diagnostic concentration after gray value analysis. The antigen diagnostic concentration of the Cy3-labeled protein chip diagnostic microarray is optimized. The amount of 0.1 mg/ml can be used as a diagnostic concentration.



### Figure 3

Optimization of the serum dilution. A 2000-fold dilution of serum was determined as a diagnostic concentration of the visible protein chip detection microarray (A) and the Cy3-labeled protein chip detection microarray (B).



### Figure 4

Optimization of the incubation time of primary and secondary antibodies. (A) The optimal incubation time for the visible protein chip detection microarray was 30–45 min for the primary antibody, and 60 min for the secondary antibody. (B) The optimal incubation time for the Cy3-labeled protein chip detection microarray was 30–45 min for the primary antibody, and 45–60 min for the secondary antibody.

## Supplementary Files

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