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*Original article*

# Development of a blocking immunoperoxidase monolayer assay for differentiation between pseudorabies virus-infected and vaccinated animalss

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## Abstract

Pseudorabies (PR) outbreaks have devastated many swine farms in several parts of China since late 2011. The outbreak-associated pseudorabies virus (PRV) variant strains exhibited some typical amino acid changes in glycoprotein E (gE), a diagnostic antigen used for discriminating between PRV-infected and vaccinated animals (DIVA). To counteract the potential impact of epitope variations on current serological diagnostics of PRV, we produced monoclonal antibodies (mAbs) against gE protein of one representative PRV variant strain and developed a blocking immunoperoxidase monolayer assay (b-IPMA) for DIVA. The b-IPMA was based on the inhibition of binding between PRV-infected cells and mAb by PRV-specific antibodies present in clinical swine sera and was validated by comparison with a commercial PRV gpI Antibody Test Kit (IDEXX Laboratories, USA). The diagnostic sensitivity, diagnostic specificity and agreement were determined to be 99.25%, 98.18% and 99.02% respectively upon testing 509 serum samples. b-IPMA detected only PRV-specific antibodies and showed no cross-reactivity with antibodies elicited by gE-deleted vaccine or other common swine pathogens. Thus, b-IPMA has the potential to be used for high-throughput screening of PRV-infected animals in veterinary clinics.

**Key words:** pseudorabies virus variant strains, anti-pseudorabies virus monoclonal antibody, blocking immunoperoxidase monolayer assay, differentiation between pseudorabies virus-infected and vaccinated animals

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## Introduction

Pseudorabies virus (PRV), also known as Aujeszky's disease virus or suid herpesvirus 1, is an alpha-herpes virus with a double-stranded linear DNA genome that can infect a broad range of wild and domestic animals (Mettenleiter 2000). Pigs are the natural host for PRV and the reservoir of the virus in nature (Marcaccini et al. 2008). PRV-infected pigs exhibit neurological signs, abortions, severe respiratory diseases, retarded growth and listlessness and are a constant danger for spreading of the virus into susceptible populations because of life-long latent infection (Maes et al. 1997).

Glycoprotein E (gE) is the major virulence determinant of PRV, but is not essential for virus replication (Jacobs and Kimman 1993). In this context, gE-deleted vaccines (for example Bartha-K61 vaccine) have been developed and used in the elimination program of pseudorabies (PR). Indirect enzyme-linked immunosorbent assays (ELISAs) and blocking ELISAs have been mainly applied as the corresponding serological tests to detect antibodies against gE protein, which have allowed the differentiation between PRV-infected and vaccinated animals (DIVA).

In China, more than 80% of pigs were vaccinated with gE-deleted Bartha-K61 vaccine (An et al. 2013) and PR outbreaks had been well controlled before 2011. However, a massive PR outbreak in Bartha-K61-vaccinated swine farms occurred in late 2011 and caused huge economic losses. Phylogenetic analyses showed that these outbreak-associated PRV strains belonged to an independent branch of the phylogenetic tree, and pathogenicity characterization indicated that outbreak-associated PRV variant strains were more pathogenic than previous isolates (Yu et al. 2014, Wang et al. 2015). Alignment of amino acid sequences of the gE ectodomain revealed that, compared with Becker-USA strain, PRV variant strains contained 11 amino acid substitutions and one insertion of aspartic acid (Asp, D) or glycine (Gly, G) at position 48 (Wang et al. 2015). These substitutions and insertions were located within the immunodominant region of gE protein (52-238 aa), which challenged current diagnostics of PRV antibodies using blocking ELISAs. Thus, novel diagnostic methods are needed for the detection of antibodies against these outbreak-associated PRV variant strains. Although indirect ELISA is an easy and straightforward method to detect serum antibodies, it is influenced greatly by the purity of the protein antigen. Even a trace contamination of non-relevant protein from *Escherichia coli* (*E. coli*) would affect the end result because the pigs might have raised antibodies against *E. coli* under natural growing conditions. In contrast, blocking ELISAs, which measure the inhibition of the binding of murine monoclonal antibodies (mAbs) against one or two epitopes on gE protein by serum antibodies, are more specific and can eliminate the dependency on protein purity (Jacobs and Kimman 1994, Kimman et al. 1996). Hence, the blocking format is more preferable than the indirect format for developing immunoassays to detect serum antibodies in veterinary clinics.

Here, we labelled monoclonal antibodies (mAbs) against two epitopes on gE protein of the outbreak-associated PRV variant strain with horseradish peroxidase (HRP) and developed a blocking immunoperoxidase monolayer assay (b-IPMA) for the detection of serum antibodies against gE protein and then the DIVA. The b-IPMA was validated with a commercial PRV gpI Antibody Test Kit and Western blot in terms of diagnostic specificity, diagnostic sensitivity and agreement. The results showed that b-IPMA is gE-specific, easy to perform and capable of being used as a complementary serological test together with gE-deleted vaccines for DIVA.

## Materials and Methods

### Cells and virus

Baby hamster kidney cells (BHK-21) were cultured in DMEM media containing 10% fetal bovine serum (FBS). PRV variant strain TY-Henan-2014 was isolated from an outbreak-affected swine herd in 2014 and stored in our laboratory. A gE gene deleted vaccine strain of PRV Bartha-K61 was propagated in the BHK-21 cells and kept at -80°C. mAbs 10C3F3 and 3E6E5 against two immunodominant epitopes were produced by immunizing BALB/c mice with *E. coli*-expressed gE protein and screening with IPMA and ELISA. Clinical serum samples were collected from swine farms in different regions of central China's Henan province from 2005 to 2014 and tested using a PRV gpI Antibody Test Kit (PRVgpI-Ab, IDEXX Laboratories, Westbrook, ME). Reference sera (positive and negative) against PRV, classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus (JEV), foot and mouth disease virus (FMDV), porcine parvovirus (PPV) and *Streptococcus suis* were purchased from the China Institute of Veterinary Drug Control.

### mAb-HRP conjugation

Mouse immunoglobulin (IgG) was extracted from ascites using ammonium sulfate precipitation and purified by protein G affinity chromatography. Covalent

conjugation of horseradish peroxidase (HRP) to the purified IgG was done according to a previously described method (Tsang et al. 1995).

### Blocking immunoperoxidase monolayer assay (b-IPMA)

The b-IPMA was performed as follows: BHK-21 cells were seeded onto 96-well cell culture plates and allowed to grow into a confluent monolayer within 24 h. After washing twice with DMEM, the cells were infected with 200 $TCID_{50}$ of PRV variant strain TY-Henan-2014 or Bartha-K61 vaccine strain and grown in DMEM containing 2% FBS for 24 h. The cells were then washed with phosphate buffered saline (PBS) three times and fixed with cold methanol containing 2% $H_2O_2$ for 15 min at room temperature. 5% skimmed milk was added to block available binding sites on the plate at 37°C for 1 h. The prepared plates were stored at -20°C before use. For testing clinical swine sera, 50 µl of sera diluted 1:2 in PBS was added and allowed to incubate with the cells at 37°C for 30 min. After washing six times with PBS containing 0.05% Tween-20 (PBST), 50 µl of mAb-HRP conjugate was added and incubated with the plate at 37°C for 30 min. Finally, AEC substrate buffer (3-amino-9--ethylcarbazole) was added for color development for 5 min before the addition of double distilled water (DDW) to wash the plates and stop the color reaction. The wells were then observed under light microscopy or with an internal CCD camera within a smartphone. Each assay was independently read with the naked eye by two people. If gE-specific antibodies were present in the sera, it would completely block the binding of mAb-HRP conjugate to PRV-infected BHK-21 cells on the plates and then no colored cells would be observed. The absence of gE-specific antibodies in the sera had no influence on the binding of 10C3F3-HRP and 3E6E5-HRP with the plates, and thus the color development would be clearly observed. To ensure a complete blocking effect from PRV-positive swine sera, the amount of PRV inoculum, infection time and the working conditions for serum samples and mAb-HRP conjugates were optimized. The samples would be re-tested in b-IPMA if different results were obtained.

### Specificity and sensitivity of b-IPMA

The specificity of b-IPMA was determined by testing reference sera against common swine pathogens including PRV, CSFV, PCV2, PRRSV, JEV, FMDV, PPV and *Streptococcus suis*. The sensitivity of b-IPMA was evaluated by diluting and observing the blocking effects of 12 positive sera representing strong (n = 4), medium (n = 4) and weak (n = 4) reactions in PRV gpI Antibody Test Kit.

### Validation of b-IPMA

The performance of b-IPMA was validated with a commercial PRV gpI Antibody Test Kit (IDEXX Laboratories, USA) on testing 509 serum samples in parallel. Considering the PRV gpI Antibody Test Kit as a gold standard, the diagnostic sensitivity (DSN), diagnostic specificity (DSP) and agreement were calculated according to the formula: DSN = TP/(TP + FN) × 100; DSP = TN/(TN + FP) × 100, and Agreement = (TP + TN)/total number of serum samples tested × 100, where TP, FN, TN and FP indicated true-positive, false-negative, true-negative and false-positive, respectively. Western blot was used to confirm discrepant results between the two assays.

### Western blot

*E. coli*-expressed gE protein of PRV was subjected to 12% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skimmed milk, the transferred membrane was incubated with 1:100 diluted swine sera for 1 h at 37°C. HRP-conjugated goat anti-pig IgG was used as secondary antibodies, and an ECL substrate kit was used to give the signal.

## Results

### Preparation of recombinant gE protein

To express the gE protein of the PRV variant strain, plasmids pET28a-gE(51-409aa), pET32a-gE(51-409aa), pET28a-gE(35-255aa), pET32a-gE(35-255aa) and pET28a-gE(51-255aa) were constructed and tested for expression in *E. coli* BL21(DE3) cells and *E. coli* Rosetta(DE3) cells, respectively. Only recombinant plasmid pET28a-gE(51-255aa) gave the expression of gE protein (27.1 kDa) in *E. coli* Rosetta(DE3) cells, indicating that the presence of rare codons and hydrophobic regions might affect the expression of gE protein. Western blot analysis showed that recombinant gE protein possessed high-immunogenicity (Fig. 1). Only positive swine sera, 3E6E5 and 10C3F3 gave positive reactions, while negative swine sera and the control mAb 1G7D8, specific for the envelope protein of JEV, possessed no reaction with gE protein.

### Characterization of anti-PRV mAb by ELISA

mAbs 10C3F3 and 3E6E5 were produced against two different immunodominant epitopes on gE protein. The affinity constant (Ka) of anti-PRV mAbs 10C3F3

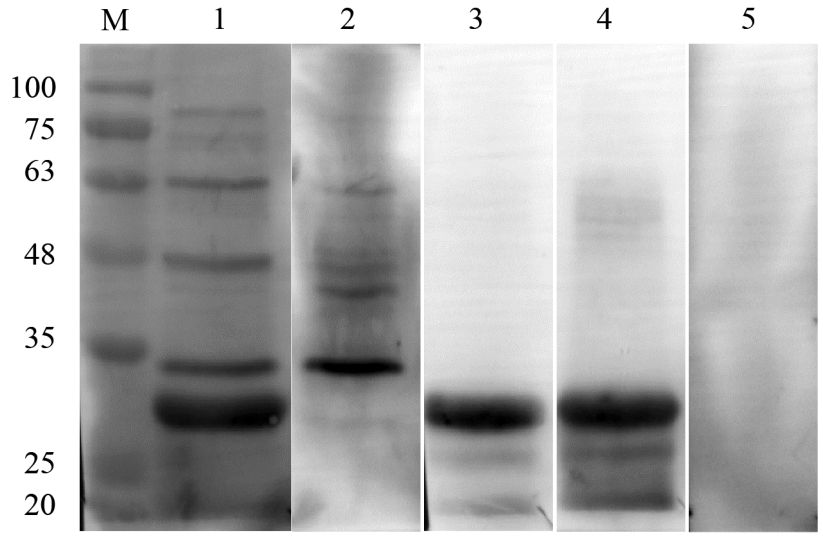


Fig. 1. Immunogenicity of gE protein analyzed by Western blot. M. pre-stained protein marker. Recombinant gE protein (27.1 kDa) was respectively reacted with PRV-positive swine sera (1), PRV-negative swine sera (2), 3E6E5 (3), 10C3F3 (4), and 1G7D8 (5).

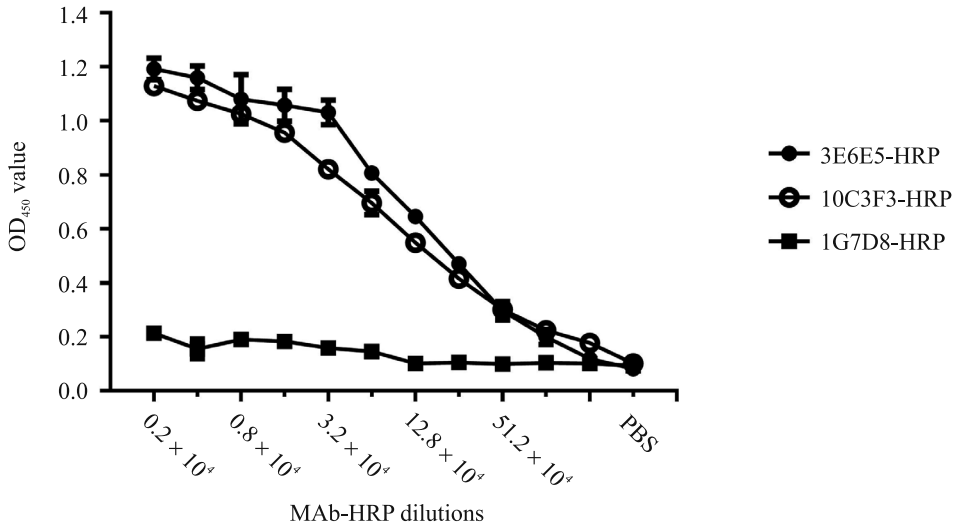


Fig. 2. Titers of mAb-HRP conjugate. The titers of 10C3F3-HRP and 3E6E5-HRP were measured using gE protein-based indirect ELISA. 100 ng of *E. coli*-expressed gE protein was coated onto each well. 1G7D8-HRP, specific for the envelope protein of JEV, was used as control.

(IgG1/κ) and 3E6E5 (IgG2a/κ) were $2.5\times10^{11}$ L/mol and $1.2\times10^{10}$ L/mol, respectively. Both the titers of 10C3F3-HRP and 3E6E5-HRP were determined to be $1{:}5.12\times10^{5}$ in gE protein-based indirect ELISA (Fig. 2). The titers of 10C3F3-HRP and 3E6E5-HRP in IPMA both reached 1:8,000-16,000. The control mAb-HRP, 1G7D8-HRP, showed no reactions with gE protein in ELISA or PRV-infected cells in IPMA.

### Characterization of anti-PRV mAb by IPMA

Both 10C3F3-HRP and 3E6E5-HRP were PRV variant strain specific and possessed no reaction with the gE-deleted Bartha vaccine strain in IPMA (Fig. 3). In b-IPMA, 10C3F3-HRP and 3E6E5-HRP were diluted to a titer of 1:10,000. The specific reactions between mAb-HRP conjugate and PRV-infected cells could only be completely inhibited by PRV-positive swine sera, while Bartha-K61 vaccinated swine sera and negative swine sera exhibited no blocking effect (Fig. 3). To be noted, there was no difference in observation and evaluation of the final results using light microscopy or smartphone-based CCD camera.

### Specificity and sensitivity of b-IPMA

The specificity of b-IPMA was determined by testing reference sera against common swine pathogens. Only PRV-positive swine sera completely blocked the reaction between mAb-HRP conjugate and infected

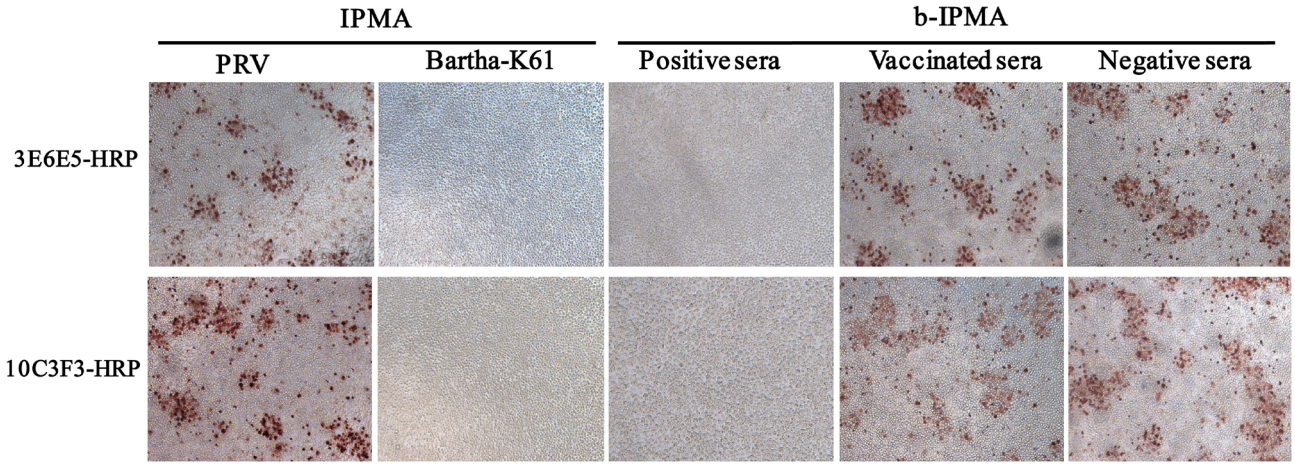


Fig. 3. Characterization of anti-PRV mAb by IPMA. Specificity of 10C3F3-HRP and 3E6E5-HRP was determined using indirect IPMA (left) and the blocking effect of clinical swine sera was evaluated by b-IPMA (right). Serum controls include PRV-positive swine sera, Bartha-K61 vaccinated swine sera and PRV-negative swine sera. Clear red-brown spots could be observed only in reactions between mAb-HRP conjugate and PRV-infected BHK-21 cells in IPMA, but these reactions would be blocked by positive swine sera, not by vaccinated sera or negative swine sera in b-IPMA.

Table 1. Comparison between b-IPMA and PRV gpI Antibody Test Kit.

| gPI-ELISA | b-IPMA | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Positive | 396 | 3 | 399 |
| Negative | 2 | 108 | 110 |
| Total | 398 | 111 | 509 |

cells in b-IPMA, while positive sera against CSFV, PCV2, PRRSV, JEV, FMDV, PPV and *Streptococcus suis* all failed to block the binding of mAb-HRP conjugate with infected cells. This indicated that b-IPMA was specific for detecting antibodies against PRV and had no cross-reactivity with antibodies against other swine pathogens. The sensitivity of b-IPMA was evaluated by testing PRV-positive sera displaying strong, medium and weak reactions in a commercial PRV gpI Antibody Test Kit. In b-IPMA, all four strong positive sera possessed a nearly complete blocking effect even at a dilution of 1:16. Medium positive sera could give complete blocking when diluted at 1:8, while the weak positive samples showed a complete blocking effect at 1:4. Hence, a 1:2 dilution of serum sample would give enough PRV-specific antibodies to occupy all available binding sites for mAb-HRP conjugate.

### Comparison between b-IPMA and PRV gpI Antibody Test Kit

In parallel, 509 field serum samples were tested by b-IPMA and a PRV gpI Antibody Test Kit (Table 1). The DSN, DSP and agreement were calculated as 99.25%, 98.18% and 99.02%, respectively. Confirmation of discrepant results by Western blot showed that the 2 false positive samples in b-IPMA were positive, while the 3 false negative samples were negative. This indicated that the developed b-IPMA had better performance for high throughput screening of PRV-specific antibodies.

## Discussion

Pseudorabies had been well controlled with the use of gE-deleted modified live vaccines and a DIVA strategy in China until 2011, when massive PR outbreaks occurred in Bartha-K61 vaccinated swine herds (An et al. 2013). Outbreak-associated PRV isolates were antigenically different from previous strains and exhibited enhanced pathogenicity that compromised the protection provided by the traditional Bartha-K61 vaccine (Luo et al. 2014, Yu et al. 2014, Wang et al. 2017). Novel gene-deleted PRV vaccines based on current circulating strains were then produced, and evaluated in swine populations, and they showed complete protection against viral challenges (Gu et al. 2015, Hu et al. 2015, Zhang et al. 2015, Wang et al. 2016). These novel vaccines all possessed the deletion of the gE gene and would allow the DIVA strategy to be used with serological assays.

Previously, five epitopes were mapped within amino acids 52-238 on gE protein (Jacobs et al., 1990). Gene fragments containing these epitopes have been expressed in *E. coli*, yeast and the baculovirus-insect cell system and used for the development of differential ELISAs based on indirect, blocking or sandwich formats (Ro et al. 1995, Kimman et al. 1996, Morenkov et al. 1996, Ao et al. 2003, Serena et al. 2011). In this study, we developed a blocking IPMA using mAbs produced against the gE protein of one representative PRV variant strain, TY-Henan-2014, for DIVA. Initially, four plasmids pET28a-gE(51-409aa), pET32a-gE(51-409aa), pET28a-gE(35-255aa) and pET32a-gE(35-255aa) were constructed and tested for expression in *E. coli* BL21(DE3) cells and *E. coli* Rosetta(DE3) cells. However, none of these plasmids gave expression in these two host cells. Finally, recombinant plasmid pET28a-gE(51-255aa) was tested in trial and it managed to give expression of water-soluble gE protein in *E. coli* Rosetta(DE3) after optimization of temperature and concentration of isopropyl β-D-thiogalactoside. This indicated that the presence of rare codons and hydrophobic regions might affect the expression of gE protein. mAbs 10C3F3 and 3E6E5 were produced by immunizing BALB/c mice with purified gE protein and screening with IPMA and ELISA. 10C3F3 and 3E6E5 were confirmed to be against two different immunodominant epitopes and then conjugated with horseradish peroxidase (HRP) respectively. For development of b-IPMA, 10C3F3-HRP and 3E6E5-HRP were first titrated in IPMA and then mixed in their highest titers for use in b-IPMA. The use of two mAb-HRP conjugates against different epitopes on gE protein would help assure the specificity and long-term usefulness of the assay.

To give a proper signal for judgment of results and allow a complete blocking effect from PRV-positive swine sera, we optimized the amount of PRV inoculum, infection time and the working conditions for serum samples and mAb-HRP conjugates. In the end, 200 $TCID_{50}$ of PRV was used to infect the cells for 24 h. After fixation with pre-cooled (-20°C) methanol containing 2% $H_2O_2$ at RT for 15 min and sealing with 5% skimmed milk at 37°C for 1 h, serum samples diluted 1:2 in PBS were added and allowed to incubate with the plate at 37°C for 30 min. Upon washing with PBST, 10C3F3-HRP and 3E6E5-HRP diluted 1:10,000 in PBS was then allowed to incubate with the plate at 37°C for another 30 min. AEC substrate buffer was added for color development for 5 min before termination with distilled water. The results were read using light microscopy or with a smartphone-based CCD camera. The use of a smartphone camera gave consistent results with light microscopy and would facilitate high throughput read-out of results.

Specificity evaluation showed that b-IPMA only detected PRV-specific antibodies and showed no cross-reactivity with antibodies elicited by gE-deleted vaccine, CSFV, PCV2, FMDV, PPV, PRRSV, JEV and *Streptococcus suis*. A 1:2 dilution of serum sample would provide enough PRV-specific antibodies to occupy all available binding sites on PRV-infected cells for mAb-HRP conjugate.

By validation with a PRV gpI Antibody Test Kit for detecting 509 field serum samples, the DSN, DSP and agreement were determined to be 99.25%, 98.18% and 99.02%, respectively. b-IPMA gave 2 false positive and 3 false negative results compared with the PRV gpI Antibody Test Kit. Western blot analysis showed consistent results with b-IPMA for these discrepant samples, which indicated that the developed b-IPMA possessed better performance that can be used for high throughput screening of PRV-specific antibodies.

In conclusion, we developed a b-IPMA based on two mAb-HRP conjugates for the differentiation between PRV-infected and vaccinated animals (DIVA). The b-IPMA used a blocking format in which the binding between mAb-HRP conjugate and PRV-infected cells would be blocked by clinical PRV-positive serum samples. It is gE-specific, easy to perform and can fulfill the need to be used as a complementary serological test together with gE-deleted vaccines. Hence, this study offers a useful immunoassay for the high-throughput screening of anti-PRV antibodies against outbreak-associated PRV strains currently circulating in China.

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