

Development of Dual Fluorescent Microsphere Immunological Assay for Detection of Pseudorabies Virus gE and gB IgG Antibodies

chihai ji

SCAU <https://orcid.org/0000-0002-3225-6110>

Jingyu Wang

South China Agricultural University

Yuchen Zeng

SCAU

Haoming Pan

South China Agricultural University

Yingfang Wei

South China Agricultural University

Guan Liang

South China Agricultural University

Jun Ma

South China Agricultural University

Lang Gong

South China Agricultural University

Wei Zhang

South China Agricultural University

Guihong Zhang

South China Agricultural University

Heng Wang (✉ wangheng2009@scau.edu.cn)

<https://orcid.org/0000-0001-8471-6337>

Methodology article

Keywords: pseudorabies virus, fluorescent-microsphere immunoassays, gE protein, gB protein, specificity, sensitivity

Posted Date: May 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-24925/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Pseudorabies, also known as Aujeszky's disease, is an acute viral infection caused by pseudorabies virus (PRV). Swine are one of the natural hosts of pseudorabies, therefore, the disease brings huge economic losses to the swine industry. Establishment of a differential diagnosis technique that can distinguish between wild-type infected and vaccinated pigs, and monitor vaccine-induced IgG is crucial for eventual eradication of pseudorabies.

Results The aim of this study was to develop a rapid dual detection method for PRV gE and gB protein IgG antibodies with high specificity and sensitivity. PRV gE codons at amino acid residues (aa) 52–238 and gB codons at aa 539–741 were expressed to obtain recombinant PRV gE and gB proteins by pMAL-c5x vector. After purification with Qiagen Ni–NTA agarose affinity, the two proteins were analyzed by SDS-PAGE and immunoblotting assay. Two single fluorescent-microsphere immunoassays (FMIA) were established by coupling two recombinant proteins (gE and gB) with two magnetic microbeads and an effective dual FMIA was developed by integrating the two single assays. Optimal serum dilution for each assay, correlation with other common swine virus-positive sera and comparison with ELISA for two PRV antigens were tested for validation. Compared with ELISA, the specificity and sensitivity were 99.26% and 92.3% for gE IgG antibody detection and 95.74% and 96.3% for gB IgG antibody detection by dual-FMIA.

Conclusion We provide a new method for monitoring PRV protective antibody in vaccinated pigs and differentiating wild-type-PRV-infected from vaccinated pigs

Background

Pseudorabies is an important infectious disease in the pig industry, and it was first described by Aladár Aujeszky in the 1900s and identified in 1930s. It causes reproductive failure in sows, high mortality of newborn piglets, and respiratory symptoms in fattening and growing pigs [1]. Since 2011, an outbreak of severe pseudorabies in Northern China caused huge economic losses and spread rapidly in large numbers of vaccinated pig farms throughout the country [2, 3]. Increased virulence was identified during the epidemic, and commonly used vaccines provided little protection against the pathogen [4, 5].

Pseudorabies virus (PRV) is a member of the genus *Alphaherpesvirinae* that belongs to the *Herpesviridae* [6]. PRV is an enveloped, double-stranded DNA virus with a genome of approximately 150 kb that encodes about 70 proteins [7]. The viral envelope glycoprotein plays an important role in immunity and virus-cell interaction against PRV infection [8, 9], and has therefore always been one of the hotspots of PRV research. At present, 11 PRV glycoproteins have been identified; among which, gB, gC and gD are the main protective antigens and they stimulate production of neutralizing antibodies and virus-specific cellular immune responses in pigs [10]. Mouse and pig serum induced by PRV gB protein can neutralize virus infection *in vitro*, and gB protein can protect both mice and pigs from PRV infection after immunization [11]. The gE is an important antigen for virulence and viral (neuronal) spread. It can induce homotypic T cell aggregation and transmit the virus to highly susceptible cells by activating extracellular

signal-regulated kinase 1/2 signaling pathways [12, 13]. A major epitope in the gE amino acids (aa) 52–238 domain and eight epitopes in the gB aa 539–741 domain were identified [14, 15]. Generally, vaccination is a valuable method to control PRV. The advantage of using PRV gE protein as a marker to differentiate wild-type PRV infected from vaccine-immunized animals is because gE gene is expressed in all wild-type strains, and gE-deficient vaccine is safer than other gene-deficient vaccines [16–18]. Some European countries and North America have eradicated PRV from domestic pigs by using gE-deleted vaccine [19, 20]. The use of gene deletion vaccine combined with differential diagnosis can effectively differentiate vaccinated from wild-type infected animals, and is an important measure to prevent and control pseudorabies. Therefore, we used gE and gB proteins as diagnostic antigens in the present study.

Microsphere technology with two different sizes of microspheres were used to analyze two antibodies by flow cytometry in the 1970s [21]. Fluorescent-microsphere immunoassay (FMIA) is a new clinical laboratory diagnostic method with high throughput, sensitivity and specificity for simultaneous detection of several analytes in complex samples [22]. Additionally, FMIA can detect multiple antigens in a single pathogen. Luminex technology, which uses colored magnetic polystyrene beads to capture antigens, is well-known and widely applied for detection of analytes [23]. Based on its multiplexing capability, this method has been used for differentiating infected from vaccinated animals for viral pathogens such as foot and mouth disease virus [24], West Nile virus [25], avian influenza virus [26] and Rift Valley fever virus [27]. ELISA is a traditional diagnostic method for detecting viral antigen or antibody [28]. Currently, ELISA is used to detect gE and gB IgG antibodies of PRV for monitoring the level of viral infection and protective antibody in clinical diagnosis [29, 30]. One disadvantage of ELISA is that it usually detects only a single target molecule and it incurs a high cost when more than one molecule is detected. The development of an efficient diagnostic technique for monitoring protective antibody in vaccinated pigs and differentiating PRV wild-type-infected from vaccinated animals provides an important basis for prevention and control of PRV infection. Compared with ELISA, FMIA has the following advantages. (1) High throughput, which can detect 500 indexes of a sample or a reaction system simultaneously. (2) High sensitivity, which can detect tiny target substances, i.e., the minimum detection concentration is 0.01 pg/mL. (3) High accuracy, which needs 100 microspheres to detect each sample to determine the fluorescence value. (4) Cost and time saving compared with traditional ELISA when more than three indexes need to be detected simultaneously. However, one limitation is that the high initial investment cost, because the Luminex flex Map 3D detection platform is more expensive than the optical density reader. Fluorescent microspheres are protein carriers with good performance, which can couple antigen or antibody and detect the corresponding antibody or antigen specifically. Therefore, they are often used in immunoassays and development of diagnostic kits.

We generated gE-12 and gB-28 microsphere complexes by coupling recombinant PRV gE and gB proteins to Number 12 and 28 magnetic microbeads. The double fluorescence microsphere immunological detection method for gE and gB protein IgG antibodies was developed. We compared the results of our assay with those of ELISA. Our research provides a novel method for the simultaneous detection of PRV gE and gB protein IgG antibodies in clinical practice, and discrimination between PRV-infected and vaccinated animals.

Results

Expression and verification of recombinant PRV gE and gB protein

To develop a dual-detection method, we first amplified gE aa 52–238 domain and gB aa 539–741 domain and constructed two recombinant plasmids pMAL-c5x PRV gE and pMAL-c5x PRV gB. Two recombinant plasmids were identified by sequencing and the correct size by PCR. Two expression plasmids were transfected into *E. coli* BL21 (DE3). Extracts of pMAL-c5x PRV gE and pMAL-c5x PRV gB transformed cells were analyzed by SDS-PAGE and stained by Coomassie Brilliant Blue after induction with IPTG. The pMAL-c5x vector was used as a negative control and the results showed obvious bands of molecular mass 63.3 and 66.3 kDa that corresponded with PRV gE and gB recombinant proteins [including vector pMAL-c5x (added 6 × His tag) with 43.3 kDa] (Fig. 1A and B). PRV gE and gB recombinant proteins were abundant in the supernatant and consistent with the expected size of recombinant tagged protein, which demonstrated that the recombinant proteins were well expressed.

To verify that the recombinant proteins were the targets, supernatants were analyzed by immunoblotting with the primary antibodies (anti-MBP mAb and anti-His mAb) and secondary antibody (mouse IgG (H + L) goat anti-mouse antibody) after ultrasonic decomposition. The specific bands were obtained consistent with the size of the target proteins (Fig. 2), indicating that MBP-tagged and His-tagged proteins were successfully expressed in the recombinant proteins.

Purification And Verification Of Antigenicity Of Recombinant Proteins

To develop an accurate FMIA method for detection, we were able to obtain purified recombinant proteins. The supernatants of pMAL-c5x PRV gE and pMAL-c5x PRV gB in breaking cells were purified using Ni-Sepharose and eluents were analyzed by SDS-PAGE and stained by Coomassie Brilliant Blue. gE protein eluted with 300 mM imidazole solution had a higher concentration (Fig. 3A) and gB was a protein eluted with 200 mM imidazole solution with higher concentration (Fig. 3B). Two purified proteins were analyzed by immunoblotting with PRV-positive serum, which showed that two fusion proteins reacted specifically against the corresponding serum (Fig. 4A and C). No specific band appeared in the negative sera (Fig. 4B and D). This demonstrated that two purified recombinant proteins could be used to develop an FMIA method based on good reactivity and antigenicity.

Evaluation Of Specificity, Coupling Efficiency And Serum Dilution

After obtaining the purified recombinant proteins, products were analyzed by coupling efficiency. gE-12 and gB-28 microsphere complexes that were obtained from recombinant PRV gE and gB proteins coupled with Number 12 and 28 magnetic microbeads were detected (Fig. 5A). Compared with negative serum, strong median fluorescent intensity (MFI) signals were detected in positive serum of antigen-targeted gB and gE. This indicated that the coupling evaluation of PRV gE/gB and magnetic microbeads was so high that the FMIA could detect the products, and that two PRV antigens (gB and gE) targeted on the FMIA were used to detect antibodies in pig serum.

To select the optimal serum dilution, PRV gE/gB positive and negative sera were diluted 10-, 20-, 40-, 80-, 160-, and 320-fold with PBS. After fluorescence analysis, the MFI signals were over 15,000 with the 10-fold dilution ratio in PRV gE/gB positive serum, while the MFI signals in PRV gE/gB negative serum were below 3,000 (Fig. 5B). The significant differences ($P < 0.05$) indicated that optimal serum dilution ratio was 10-fold (Fig. 5C).

High specificity of single FMIA is required for developing a dual FMIA test, so we detected PRV gE and PRV gB positive sera using single FMIA, and other sera positive for other common pig pathogens such as classical swine fever virus (CSFV), porcine circovirus (PCV) and porcine reproductive and respiratory syndrome virus (PRRSV) or PRV-negative serum served as controls (Fig. 6A). The results showed that there was no cross-reaction with these sera. For PRV gE IgG antibody detection, there were high MFI signals in the corresponding gE-positive sera, which was similar to PRV gB IgG antibody detection (Fig. 6B). The results confirmed that this single FMIA method had strong specificity.

Construction Of Prv Ge And Gb Fmia

After obtaining the coupling microbeads, gE-12 and gB-28 microsphere complexes were added to the same detection reaction to establish a gE and gB IgG dual detection method. To evaluate the accuracy of dual FMIA, we compared the MFI values of gE/gB singleplex with multiplex assays by analysis of pig sera. Our results showed that the values of MFI obtained from the single assay were similar to some of those of the dual assay (Fig. 7A and B). Thus, there was little influence between gE-12 and gB-28 microsphere complex in the detection of pig sera.

Comparison Of Elisa And Fmia

To evaluate the accuracy of dual FMIA, we compared the results with the gE and gB ELISA tests. We used dual FIMA to test 284 gE and 409 gB clinical sera detected by IDEXX ELISA. According to receiver operating characteristic (ROC) curve analysis, we determined that the criterion value of optimal FMIA was 5991.5 for gE antibody detection. The sensitivity was 92.3% [95% confidence interval (CI) 63.9–98.7%], and the specificity was 99.26% (95% CI 97.4–99.9%) (Fig. 8A). The area wrapped by the curve and x axis was 0.981 ($P < 0.001$) (Fig. 8B). Similarly, we chose the criterion value of optimal FMIA was 2862 for gB antibody detection. For 409 gB-positive clinical sera, the sensitivity was 96.3% (95% CI 81–99.4%) and

the specificity was 95.74% (95% CI 89.5–98.8%) (Fig. 8C). The area wrapped by the curve and x axis was 0.989 ($P < 0.001$) (Fig. 8D). The results demonstrated that the FMIA detection method for gE/gB IgG antibody and IDEXX ELISA had similar correlation.

Discussion

Pseudorabies has been prevalent in many pig farms in China since the 1970s, causing serious economic losses to the industry. To control and eradicate this disease, vaccination and serological diagnosis of antigens have always been considered effective methods. gE-deleted vaccine, constructed by homologous recombination or CRISPR/Cas9, provides safe and consistent protection in piglets and sows [31, 32]. Virus surveillance is considered to be a complicated, time-consuming and expensive process. To date, some commercial ELISA kits have been used to detect anti-PRV gB or gE IgG in pigs [33, 34]. A recent study comparing different commercial and in-house ELISAs and a commercial immunoblot test, all based on different HEV proteins, found overall good agreement between tests on experimental samples collected in the late stage of HEV infection [35].

In recent years, FMIA has been used to diagnose human and animal pathogens. Moreover, the method has been used for differentiating and detecting yeast [36] or antibodies against pneumococcal polysaccharides and *Erysipelothrix rhusiopathiae* [37]. For rapid detection of PRV, fluorescent immunochromatographic strip and indirect sandwich ELISA have been used [38, 39], while there is no FMIA method for detection of PRV IgG. In our research, expression of recombinant antigen was the basis for development of an immunoassay with high specificity and sensitivity. gE-deleted vaccines are used in many countries, and as one of the most conserved herpesvirus glycoproteins, gB can cause a host response. PRV gB always serves as the target protein for development of immunological detection assays. We developed a dual fluorescent microsphere immunoassay for detection of PRV gB and gE IgG antibodies.

We selected gE and gB as the antigens to develop our immunological assay for PRV detection. PRV gE fragment (aa 52–238) and gB fragment (aa 539–741), which contained major epitopes, were expressed in *E. coli* by pMAL-c5X vector and recombinant proteins were obtained for subsequent identification. After purification and verification, two recombinant proteins were coupled with Number 12 and 28 magnetic microbeads respectively, and gE-12 and gB-28 microsphere complexes were formed. Coupling microsphere complexes were used as substrates to verify the feasibility of FMIA for detecting gE/gB antibodies in positive/negative sera, respectively. A dual FMIA for detection of PRV gE and gB IgG antibody was developed by integrating the single FMIAs. Subsequently, we measured the specificity of the dual FMIA for detection of 1000 pig serum samples positive for PRRSV, PCV, and CFSV. The results demonstrated that dual FMIA had strong specificity and the coupled antigens did not cross-react with other pig-virus-positive sera. Dual FMIA was compared with a commercial gE/gB ELISA kit (IDEXX Laboratories) to determine the accuracy of the detection. For detection of gE IgG, ROC curve analysis showed that the area wrapped by the curve and x axis was 0.981, indicating that dual FMIA had good accuracy for detection of gE with high sensitivity (92.3%) and specificity (99.26%). Also, the area was

0.989 for gB IgG detection, with high sensitivity (96.3%) and specificity (95.74%), which indicated the accuracy of dual FMIA for detection of gB. The results of dual FMIA detection showed good concordance and compatibility with those of gE/gB ELISA. Also, our dual FMIA method has the advantage that it can test several targets simultaneously; thus, it may be used for detecting several targets against PRV in one sample.

FMIA is a novel diagnostic and monitoring immunoassay method that requires fewer samples and less time and is more economic than traditional ELISA [40]. This method overcomes the disadvantage that ELISA needs to analyze antigens separately. Compared with the PRV IgG commercial ELISA kit, the sensitivity of our dual FMIA was higher. FMIA can detect multiple antibodies in pig sera and provides an important method for the surveillance of pig diseases. By optimizing the reaction conditions, the multi-detection ability of FMIA provides a novel high-throughput and high-sensitivity detection platform for diagnosing pathogens, monitoring disease and epidemiological investigation.

Methods

Virus strain, serum samples and antibodies

PRV Guangdong strain was preserved in our laboratory and the complete genome sequence is available in NCBI GenBank (KU056477.1/KT948041.1). Negative sera and antiserum against PRV gE/gB, CSFV, PCV and PRRSV were preserved in the Diagnostic Laboratory, College of Veterinary Medicine, South China Agricultural University and stored at -80°C. IRDye 800CW goat anti-Mouse IgG (H + L) was purchased from LI-COR Biosciences. Rabbit F (ab) anti-pig IgG H&L (Biotin) and goat anti-mouse IgG H&L (Biotin) were obtained from Abcam. Micro Plex®-C Microspheres (Number 12 and 28 magnetic microbeads) were purchased from Luminex. Microplates 96 orifice was purchased from Greiner.

Expression Of Recombinant Prv Ge And Gb Protein

Primers for amplifying gB and gE encoding regions were designed using Oligo 7.0 (Table 1). After amplification by nested PCR, gB and gE genes from PRV were cloned in the pMAL-c5x vector to obtain pMAL-c5x PRV gE and pMAL-c5x PRV gB plasmids. The plasmids were transformed into *E. coli* DH5 α , and expression was induced with 0.3 mM IPTG in an orbital incubator at 16°C for 8 h.

The induced bacterial solution was centrifuged at 8,000 $\cdot g$ for 10 min, the supernatant was discarded, and bacteria were resuspended in PBS (pH 7.0). After ultrasonic disruption, the products were incubated with ice until the solution became clear. Ultrasonic treatment works for 3 seconds and intermits for 5 seconds, with a working cycle of 8 seconds in total. The bacterial solution was centrifuged at 12,000 $\cdot g$ for 20 min at 4°C. The supernatant was collected and the precipitate containing the recombinant protein was resuspended in PBS for analysis of protein solubility.

Proteins were purified with Qiagen Ni-NTA agarose affinity isolation for native His-tagged proteins. Purified proteins were analyzed by SDS-PAGE. After staining with 0.025% Coomassie Blue, the protein bands were visualized. For immunoblotting, proteins were transferred to polyvinylidene fluoride membranes. After blocking with 5% skimmed milk powder at 37°C for 2 h, membranes were incubated with PRV gE/gB positive serum at 4°C for 8 h and with goat anti-mouse IgG at room temperature for 1 h. The bands visualized with 3,3',5,5'-tetramethylbenzidine substrate were analyzed by western blotting (Amersham Biosciences).

Table 1
Primers used for PCR

Primers	Primer sequence 5'→ 3'
gE-F1	GACCATGCGGCCCTTTCTGCT
gE-R1	CCATTTCGTCACCTTCCGGTTTCTCC
gE-F2	GCGGAATTCATGGAGGCCGACGACGATGAC
gE-R2	GGGAAGCTTTCAATGATGATGATGATGATGGGGCGAGAAGAGCTGCGA
gB-F1	GACAAGCCCGAGGTGTAC
gB-R1	TGGAAGAAGTTGGCGATG
gB-F2	GCGGAATTCATGGACCACATCCAGGCGCAC
gB-R2	GGGAAGCTTTCAATGATGATGATGATGATGGTAGAACTTGAGCGCGTG

Coupling Of Recombinant Proteins To Fluorescent-encoded Microspheres

Coupling was applied with a two-step amide reaction (Luminex xMAP). Number 12 and 28 magnetic microbeads were coupled with PRV gE and gB antigens by adding NaH₂PO₄ buffer, Sulfo-NHS (N-Hydroxysulfosuccinimide sodium salt-Sulfo-NHS) and EDC (1-thyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride) solutions. Phosphorylation of fluorescent-encoded microspheres was achieved with covalent amide bond formation. The 12-PRV gE and 28-PRV gB microsphere complexes were resuspended in PBS-TBN (PBS, 0.1% BSA, 0.02% Tween-20, 0.05% Na₃N₃, pH = 7.4) solution and stored at 4°C in the dark.

Selection Of Optimal Serum Dilution

PRV gE/gB-positive and gE/gB-negative sera were diluted with PBS-1% BSA 10-, 20-, 40-, 80-, 160- and 320-fold. We added 50 µL (250 µg/mL) microspheres and 50 µL (50 beads/µL) of the sample to be tested (monoclonal antibody or serum) to each well in a 96-well plate, and incubated at room temperature for

1 h. Then, 100 μ L biotin-labeled rabbit anti-porcine IgG antibody (250 μ g/mL) was added to each well, followed by addition of 100 μ L streptavidin-algae Red albumin (SA-PE, 1:1,000) and incubation for 30 min at room temperature (500 *g* /min). Finally, the products were resuspended with 125 μ L sheath liquid in per well and MFI was read in the Luminex Flex 3D liquid phase detection system. The optimum serum dilution was determined based on the MFI values of each group.

Establishment And Evaluation Of Dual Fmia

According to the Luminex xMAP system, gE-12 and gB-28 magnetic microbeads were diluted to 50 beads/ μ L using PBS-TBN, transferred to 96-well microplates, and each well add 50 μ L gE-12 and 50 μ L gB-28. After washing with PBS three times, 50- μ L experimental samples were added to each well after incubation for 30 min at room temperature. The coupling method was as described above (see coupling of recombinant proteins to fluorescent-encoded microspheres). Ninety-six pig pathogen serum samples were detected using each single and dual FMIA, and the FMI values were obtained to evaluate the comparability of the two assays. The correlation coefficients were analyzed by linear regression analysis.

Comparison With Elisa

PRV gE and gB antibodies in pig sera were tested by FMIA and compared with IDEXX ELISA kit test results. The MFI value was analyzed by ROC curve using MedCalc software to determine the optimal criterion value, specificity and sensitivity. The chi-square test was used to evaluate the coincidence rate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 and SPSS 19.0.

Conclusion

We constructed a dual FMIA method by expressing PRV gE and gB recombinant proteins and coupling with fluorescent microspheres. Compared with a commercial ELISA kit, our dual FMIA method showed higher specificity and sensitivity. Thus, it provides a new method for monitoring PRV protective antibody in vaccinated pigs and differentiating wild-type-PRV-infected from vaccinated pigs simultaneously.

Declarations

Funding

This work was supported by the National Key Research and Development Program of China (2016YFD0500707), the Science and Technology Project in Guangdong province of China(2015B020203006), the Industry Technology System of Modern Agriculture Construction Fund (CARS-35) and the Natural Science Foundation of Guangdong province (2018A030313712).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

Conceived and designed the experiments: HW and GHZ. Performed the experiments: CHJ, YFW and JM. Sample collection: JYW, YCZ, HMP, GL. Analyzed the data: LG. Contributed to the writing: CHJ and WZ. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

Author details

¹ Guangdong Provincial Key Laboratory of Prevention and Control for Severe Clinical Animal Diseases, College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province, 510642, People's Republic of China

² Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, Guangzhou, Guangdong Province, 510642, People's Republic of China

³ Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, Guangdong Province, 510642, People's Republic of China

⁴ National Engineering Research Center for Breeding Swine Industry, Guangzhou, Guangdong Province, 510642, People's Republic of China

References

1. Bouma A. Determination of the effectiveness of Pseudorabies marker vaccines in experiments and field trials. *BIOLOGICALS*. 2005;33(4):241–5.

2. Yu X, Zhou Z, Hu D, Zhang Q, Han T, Li X, Gu X, Yuan L, Zhang S, Wang B, et al. Pathogenic pseudorabies virus, China, 2012. *EMERG INFECT DIS*. 2014;20(1):102–4.
3. Wang Y, Qiao S, Li X, Xie W, Guo J, Li Q, Liu X, Hou J, Xu Y, Wang L, et al. Molecular epidemiology of outbreak-associated pseudorabies virus (PRV) strains in central China. *VIRUS GENES*. 2015;50(3):401–9.
4. Wu R, Bai C, Sun J, Chang S, Zhang X. Emergence of virulent pseudorabies virus infection in northern China. *J VET SCI*. 2013;14(3):363–5.
5. Luo Y, Li N, Cong X, Wang CH, Du M, Li L, Zhao B, Yuan J, Liu DD, Li S, et al. Pathogenicity and genomic characterization of a pseudorabies virus variant isolated from Bartha-K61-vaccinated swine population in China. *VET MICROBIOL*. 2014;174(1–2):107–15.
6. Pomeranz LE, Reynolds AE, Hengartner CJ. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev*. 2005;69(3):462–500.
7. Klupp BG, Hengartner CJ, Mettenleiter TC, Enquist LW. Complete, annotated sequence of the pseudorabies virus genome. *J VIROL*. 2004;78(1):424–40.
8. Kratchmarov R, Kramer T, Greco TM, Taylor MP, Ch'Ng TH, Cristea IM, Enquist LW. Glycoproteins gE and gI are required for efficient KIF1A-dependent anterograde axonal transport of alphaherpesvirus particles in neurons. *J VIROL*. 2013;87(17):9431–40.
9. Szpara ML, Tafuri YR, Parsons L, Shamim SR, Verstrepen KJ, Legendre M, Enquist LW. A wide extent of inter-strain diversity in virulent and vaccine strains of alphaherpesviruses. *PLOS PATHOG*. 2011;7(10):e1002282.
10. Xiao S, Chen H, Fang L, Liu C, Zhang H, Jiang Y, Hong W. Comparison of immune responses and protective efficacy of suicidal DNA vaccine and conventional DNA vaccine encoding glycoprotein C of pseudorabies virus in mice. *VACCINE*. 2004;22(3–4):345–51.
11. Grabowska AK, Lipinska AD, Rohde J, Szewczyk B, Bienkowska-Szewczyk K, Rziha HJ. New baculovirus recombinants expressing Pseudorabies virus (PRV) glycoproteins protect mice against lethal challenge infection. *VACCINE*. 2009;27(27):3584–91.
12. Babic N, Klupp B, Brack A, Mettenleiter TC, Ugolini G, Flamand A. Deletion of glycoprotein gE reduces the propagation of pseudorabies virus in the nervous system of mice after intranasal inoculation. *VIROLOGY*. 1996;219(1):279–84.
13. Setas Pontes M, Devriendt B, Favoreel HW. Pseudorabies virus triggers glycoprotein gE-mediated ERK1/2 activation and ERK1/2-dependent migratory behavior in T cells. *J VIROL*. 2015;89(4):2149–56.
14. Zaripov MM, Morenkov OS, Fodor N, Braun A, Schmatchenko VV, Fodor I, Brown A. Distribution of B-cell epitopes on the pseudorabies virus glycoprotein B. *J GEN VIROL*. 1999;80(Pt 3):537–41.
15. Yong T, Huan-Chun C, Shao-Bo X, Ya Q, Qi H, Yu R. Development of a latex agglutination test using the major epitope domain of glycoprotein E of pseudorabies virus expressed in *E. Coli* to differentiate between immune responses in pigs naturally infected or vaccinated with pseudorabies virus. *VETERINARY RESEARCH COMMUNICATIONS*. 2005;29(6):487–97.

16. An TQ, Peng JM, Tian ZJ, Zhao HY, Li N, Liu YM, Chen JZ, Leng CL, Sun Y, Chang D, et al. Pseudorabies virus variant in Bartha-K61-vaccinated pigs, China, 2012. *EMERG INFECT DIS*. 2013;19(11):1749–55.
17. Gu Z, Dong J, Wang J, Hou C, Sun H, Yang W, Bai J, Jiang P. A novel inactivated gE/gI deleted pseudorabies virus (PRV) vaccine completely protects pigs from an emerged variant PRV challenge. *VIRUS RES*. 2015;195:57–63.
18. Zhang C, Guo L, Jia X, Wang T, Wang J, Sun Z, Wang L, Li X, Tan F, Tian K. Construction of a triple gene-deleted Chinese Pseudorabies virus variant and its efficacy study as a vaccine candidate on suckling piglets. *VACCINE*. 2015;33(21):2432–7.
19. Pikalo J, Zani L, Huhr J, Beer M, Blome S. Pathogenesis of African swine fever in domestic pigs and European wild boar - Lessons learned from recent animal trials. *VIRUS RES*. 2019;271:197614.
20. Muller T, Hahn EC, Tottewitz F, Kramer M, Klupp BG, Mettenleiter TC, Freuling C. Pseudorabies virus in wild swine: a global perspective. *ARCH VIROL*. 2011;156(10):1691–705.
21. Wood WI, Gitschier J, Lasky LA, Lawn RM. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. *Proc Natl Acad Sci U S A*. 1985;82(6):1585–8.
22. Van Gageldonk PG, van Schaijk FG, van der Klis FR, Berbers GA. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to *Bordetella pertussis*, diphtheria and tetanus. *J IMMUNOL METHODS*. 2008;335(1–2):79–89.
23. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. *J IMMUNOL METHODS*. 2009;340(1):55–64.
24. Clavijo A, Hole K, Li M, Collignon B: **Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle.** *VACCINE* 2006, **24**(10):1693–1704.
25. Balasuriya UB, Shi PY, Wong SJ, Demarest VL, Gardner IA, Hullinger PJ, Ferraro GL, Boone JD, De Cino CL, Glaser AL, et al. Detection of antibodies to West Nile virus in equine sera using microsphere immunoassay. *J VET DIAGN INVEST*. 2006;18(4):392–5.
26. Watson DS, Reddy SM, Brahmakshatriya V, Lupiani B. A multiplexed immunoassay for detection of antibodies against avian influenza virus. *J IMMUNOL METHODS*. 2009;340(2):123–31.
27. Lindahl JF, Ragan IK, Rowland RR, Wainaina M, Mbotha D, Wilson W. A multiplex fluorescence microsphere immunoassay for increased understanding of Rift Valley fever immune responses in ruminants in Kenya. *J VIROL METHODS*. 2019;269:70–6.
28. Korppi M, Leinonen M, Ruuskanen O. Pneumococcal serology in children's respiratory infections. *Eur J Clin Microbiol Infect Dis*. 2008;27(3):167–75.
29. Wu CY, Wu CW, Liao CM, Chien MS, Huang C. Enhancing expression of the pseudorabies virus glycoprotein E in yeast and its application in an indirect sandwich ELISA. *J Appl Microbiol*. 2017;123(3):594–601.

30. Li X, Sun Y, Yang S, Wang Y, Yang J, Liu Y, Jin Q, Li X, Guo C, Zhang G. Development of an immunochromatographic strip for antibody detection of pseudorabies virus in swine. *J VET DIAGN INVEST.* 2015;27(6):739–42.
31. Yin Y, Xu Z, Liu X, Li P, Yang F, Zhao J, Fan Y, Sun X, Zhu L. A live gI/gE-deleted pseudorabies virus (PRV) protects weaned piglets against lethal variant PRV challenge. *VIRUS GENES.* 2017;53(4):565–72.
32. Liang X, Sun L, Yu T, Pan Y, Wang D, Hu X, Fu Z, He Q, Cao G. A CRISPR/Cas9 and Cre/Lox system-based express vaccine development strategy against re-emerging Pseudorabies virus. *Sci Rep.* 2016;6:19176.
33. Li X, Sun Y, Yang S, Wang Y, Yang J, Liu Y, Jin Q, Li X, Guo C, Zhang G. Development of an immunochromatographic strip for antibody detection of pseudorabies virus in swine. *J VET DIAGN INVEST.* 2015;27(6):739–42.
34. Wu CY, Wu CW, Liao CM, Chien MS, Huang C. Enhancing expression of the pseudorabies virus glycoprotein E in yeast and its application in an indirect sandwich ELISA. *J Appl Microbiol.* 2017;123(3):594–601.
35. Owolodun OA, Gimenez-Lirola LG, Gerber PF, Sanford BJ, Feagins AR, Meng XJ, Halbur PG, Opriessnig T. Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay. *J Virol Methods.* 2013;193(2):278–83.
36. Balada-Llasat JM, LaRue H, Kamboj K, Rigali L, Smith D, Thomas K, Pancholi P. Detection of yeasts in blood cultures by the Luminex xTAG fungal assay. *J CLIN MICROBIOL.* 2012;50(2):492–4.
37. Gimenez-Lirola LG, Xiao CT, Halbur PG, Opriessnig T. Development of a novel fluorescent microbead-based immunoassay and comparison with three enzyme-linked immunoassays for detection of anti-Erysipelothrix spp. IgG antibodies in pigs with known and unknown exposure. *J Microbiol Methods.* 2012;91(1):73–9.
38. Shen H, Chen H, Cheng Z, Ma L, Huang L, Xiao M, Xiao W, Xie K, Tang Y. A novel fluorescent immunochromatographic strip combined with pocket fluorescence observation instrument for rapid detection of PRV. *ANAL BIOANAL CHEM.* 2018;410(29):7655–61.
39. Wu CY, Wu CW, Liao CM, Chien MS, Huang C. Enhancing expression of the pseudorabies virus glycoprotein E in yeast and its application in an indirect sandwich ELISA. *J Appl Microbiol.* 2017;123(3):594–601.
40. Han X, Wang H, Chen H, Mei L, Wu S, Jia G, Cheng T, Zhu S, Lin X. Development and primary application of a fluorescent liquid bead array for the simultaneous identification of multiple genetically modified maize. *BIOSENS BIOELECTRON.* 2013;49:360–6.

Figures

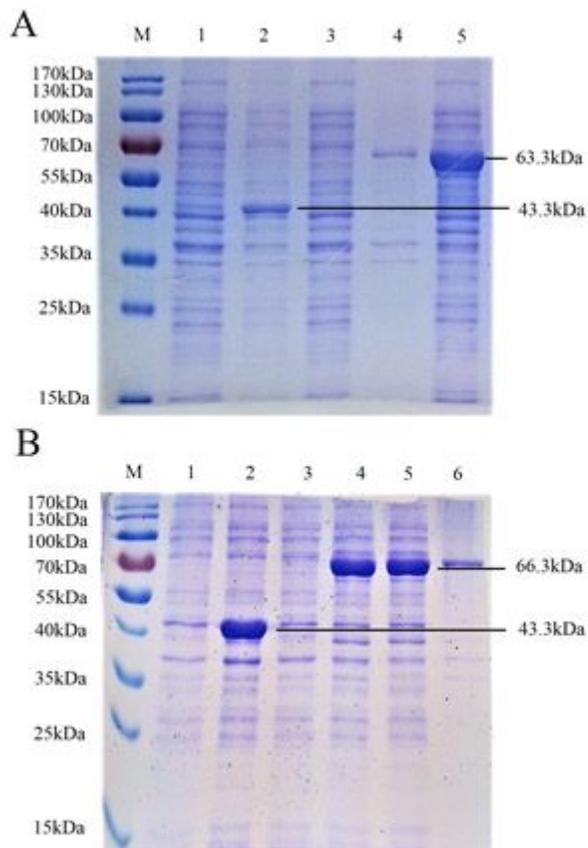


Figure 1

Identification of PRV gE and gB proteins from the pMAL-c5x plasmid by SDS-PAGE. (A) M, molecular weight marker; lane 1, untreated *E. coli* pMAL-c5x; lane 2, *E. coli* pMAL-c5x after induction by IPTG; lane 3, untreated *E. coli* pMAL-c5x PRV gE; lane 4: precipitate of *E. coli* pMAL-c5x PRV gE after induction by IPTG and ultrasonic disruption; lane 5: supernatant of *E. coli* pMAL-c5x PRV gE after induction by IPTG and ultrasonic disruption. (B) M, molecular weight marker; lane 1, untreated *E. coli* pMAL-c5x; lane 2, *E. coli* pMAL-c5x after induction by IPTG; lane 3, untreated *E. coli* pMAL-c5x PRV gB; lane 4: precipitate of *E. coli* pMAL-c5x PRV gB after induction by IPTG and ultrasonic disruption; lane 5: supernatant of *E. coli* pMAL-c5x PRV gB after induction by IPTG and ultrasonic disruption; lane 6: precipitate of *E. coli* pMAL-c5x PRV gB after induction by IPTG and ultrasonic disruption.

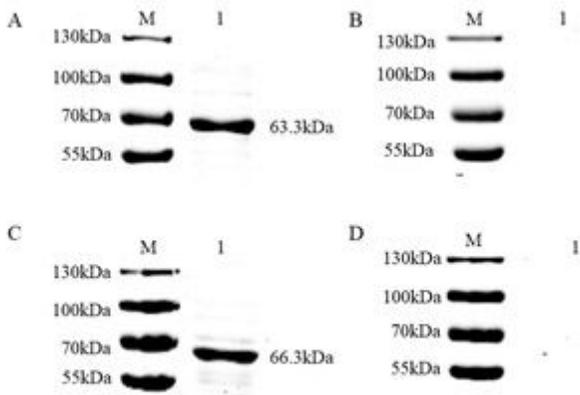


Figure 2

Identification of PRV gE and gB proteins from the pMAL-c5x plasmid by immunoblotting. (A) Detection of PRV gE by His tagging; M, molecular weight marker; lane 1, supernatant of *E. coli* pMAL-c5x PRV gE after induction by IPTG and ultrasonic disruption. (B) Detection of PRV gE by MBP tagging; M, molecular weight marker; lane 1, supernatant of *E. coli* pMAL-c5x PRV gE after induction by IPTG and ultrasonic disruption. (C) Detection of PRV gB by His tagging; M, molecular weight marker; lane 1, supernatant of *E. coli* pMAL-c5x PRV gB after induction by IPTG and ultrasonic disruption. (D) Detection of PRV gB by MBP tagging; M, molecular weight marker; lane 1, supernatant of *E. coli* pMAL-c5x PRV gB after induction by IPTG and ultrasonic disruption.

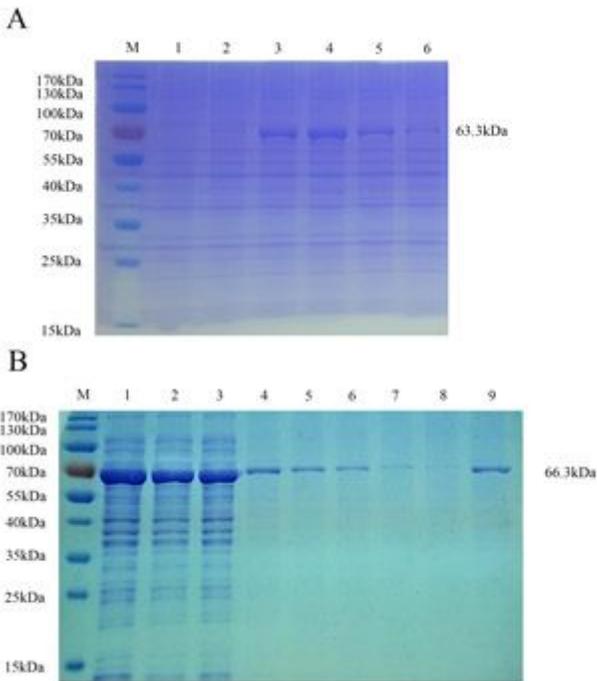


Figure 3

Identification of PRV gE and gB recombinant proteins after purification by SDS-PAGE. (A) Purification of PRV gE recombinant protein washed by different concentrations of imidazole solution and analyzed by SDS-PAGE. M, molecular weight marker; lane 1, PRV gE recombinant eluted with 80 mM imidazole solution; lane 2, PRV gE recombinant eluted with 100 mM imidazole solution; lane 3, PRV gE recombinant eluted with 200 mM imidazole solution; lane 4, PRV gE recombinant eluted with 300 mM imidazole solution; lane 5, PRV gE recombinant eluted with 400 mM imidazole solution; lane 6, PRV gE recombinant eluted with 500 mM imidazole solution. (B) Purification of PRV gB recombinant protein washed by different concentrations of imidazole solution and analyzed by SDS-PAGE. M, molecular weight marker; lane 1, supernatant of PRV gB recombinant after induction by IPTG and ultrasonic disruption; lane 2, PRV gB recombinant eluted with 100 mM imidazole solution; lanes 3 and 4, PRV gB recombinant eluted with 20 mM imidazole solution; lanes 5 and 6, PRV gB recombinant eluted with 40 mM imidazole solution; lanes 7 and 8, PRV gB recombinant eluted with 60 mM imidazole solution; lane 9, PRV gB recombinant eluted with 200 mM imidazole solution.

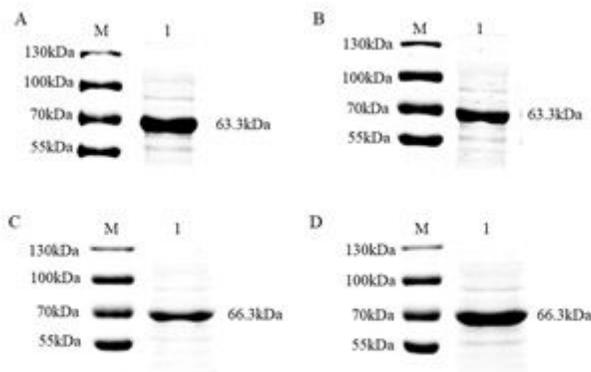


Figure 4

Antigenicity verification of PRV gE and gB recombinant proteins. PRV gE recombinant protein were detected with immunoblotting using PRV gE-positive serum (A) and pig negative serum (B) as the antibodies, respectively. PRV gB recombinant protein were detected with immunoblotting using PRV gB-positive serum (C) and pig negative serum (D) as the antibodies, respectively.

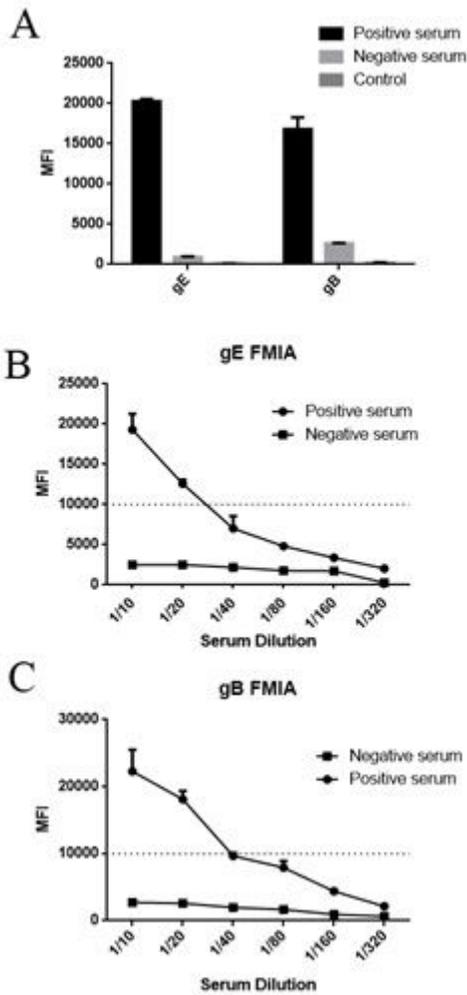


Figure 5

MFI value for PRV gE/gB positive and negative sera. (A) MFI value of FMIA was determined to test PRV gE and gB positive/negative sera (B and C). Samples were diluted 10-, 20-, 40-, 80-, 160- and 320-fold using PBS, and the MFI value of FMIA was determined.

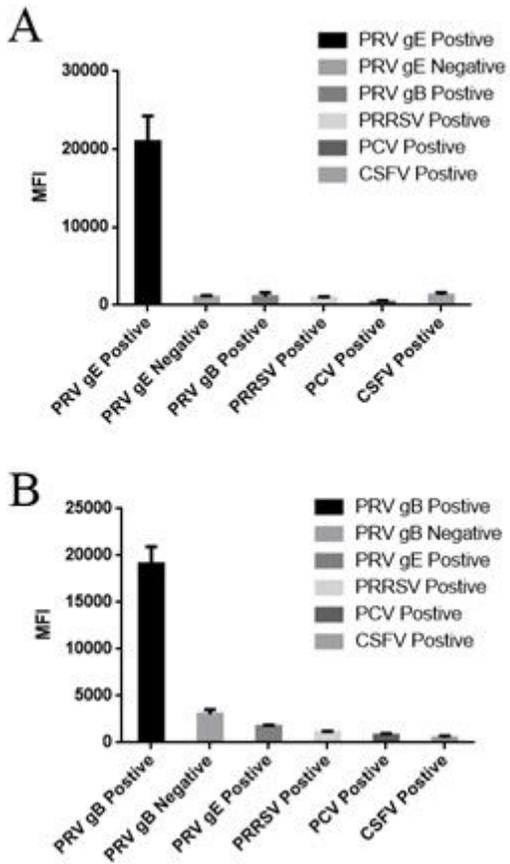


Figure 6

Antigenic cross-reactivity detection by dual FMIA. (A) The MFI value using PRV gE IgG method to detect gE-positive serum was >20,000, while other common pig pathogens or negative serum had low MFI values. (B) The MFI value using PRV gB IgG method to detect gE-positive serum was >18,000, while other common pig pathogens or negative serum had low MFI values.

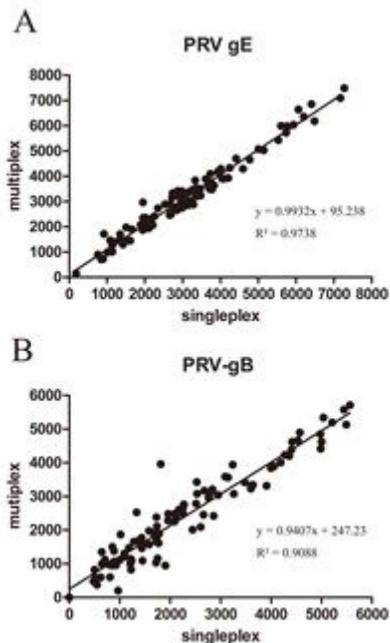


Figure 7

Comparison of the MFI values detected from singleplex and multiplex assays of PRV gE IgG detection (A) and PRV gB IgG detection (B).

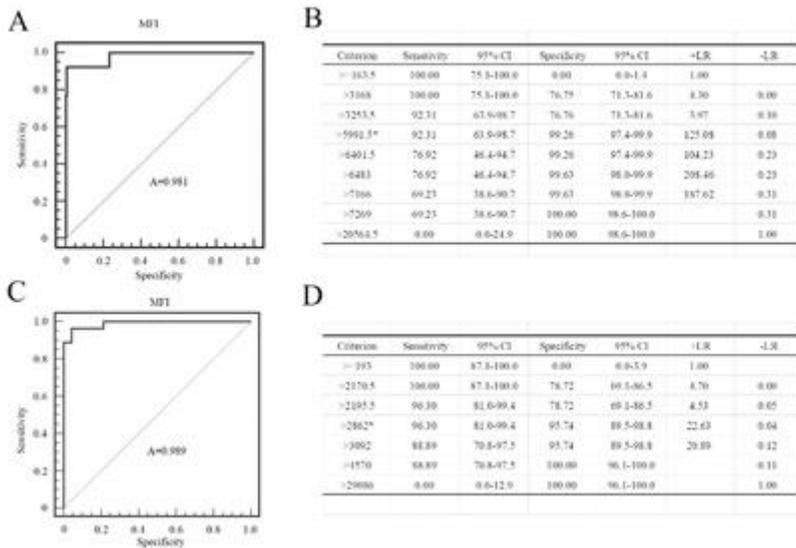


Figure 8

Statistical analysis of PRV gE and gB FMIA results. Reactivities of PRV gE-positive sera with ROC curve analysis (A) and the criterion value (MFI value above 5991.5 is positive and below is negative) of the assay (5991.5) is marked by * (B). Reactivities of PRV gB-positive sera with ROC curve analysis (C) and the criterion value of the assay (2862) is marked by* (D). We used SigmaPlot 10.0 for analysis of the sensitivity and specificity of the assay.