Virologica Sinica 37 (2022) 922–933

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Research Article

A simple nanobody-based competitive ELISA to detect antibodies against African swine fever virus

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ARTICLE INFO

Keywords:
African swine fever virus (ASFV)
Nanobody
ASFV-p30
Nanobody-HRP fusion Protein
Competitive ELISA

ABSTRACT

African swine fever virus (ASFV) infection is a big threat to the global pig industry. Because there is no effective vaccine, rapid, low-cost, and simple diagnosis methods are necessary to detect the ASFV infection in pig herds. Nanobodies, with advantages of small molecular weight and easy genetic engineering, have been universally used as reagents for developing diagnostic kits. In this study, the recombinant ASFV-p30 was expressed and served as an antigen to immunize the Bactrian camel. Then, seven nanobodies against ASFV-p30 were screened using phage display technique. Subsequently, the seven nanobodies fused horseradish peroxidase (nanobody-HRP) were secretory expressed and one fusion protein ASFV-p30-Nb75-HRP was selected with the highest sensitivity in blocking ELISA. Using the ASFV-p30-Nb75-HRP fusion protein as a probe, a competitive ELISA (cELISA) was developed for detecting anti-ASFV antibodies in pig sera. The cut-off value of cELISA was determined to be 22.7% by testing 360 negative pig sera. The detection limit of the cELISA for positive pig sera was 1:320, and there was no cross-reaction with anti-other swine virus antibodies. The comparative assay showed that the agreement of the cELISA with a commercial ELISA kit was 100%. More importantly, the developed cELISA showed low cost and easy production as a commercial kit candidate. Collectively, a simple nanobody-based cELISA for detecting antibodies against ASFV is developed and it provides a new method for monitoring ASFV infection in the pig herds.

1. Introduction

African swine fever (ASF) is a contagious viral disease of domestic and wild pigs caused by the ASF virus (ASFV) (Ley et al., 1984). The World Organization for Animal Health (OIE) has listed ASF as a notifiable animal disease in 2004 (Rowlands et al., 2008). ASFV acute infections in domestic pigs can cause 100% mortality rate (Blome et al., 2013; Sánchez-Vizcaíno et al., 2015). Since 2018, outbreaks of ASF have been reported in Asia, Europe, and South America, including China, Mongolia, Vietnam, Cambodia, North Korea, Myanmar, Laos, Belgium, Haiti, Dominican Republic (Zhou et al., 2018; Rai et al., 2020; Friker and Schüpbach, 2021). It has caused severe economic losses to the pig industry in these countries (Tran et al., 2021).

ASFV, the only member of the genus *Asfivirus* within the *Asfarviridae* family (Dixon et al., 2013), is a double-stranded DNA virus with a genome size of approximately 170–194 kb and contains 151–174 open reading frames (ORFs) encoding 150–200 viral proteins (de Villiers et al., 2010). However, to date, the functions of only approximately 50 proteins of ASFV were characterized (Jia et al., 2017). Among them, ASFV phosphoprotein p30 (ASFV-p30) is one viral structural protein with a size of about 30 kDa (Indrabalan et al., 2021). Some previous studies documented that the protein is translated in the early stage of viral infection and can induce a strong immune response in pigs (Afonso et al., 1992; Gómez-Puertas et al., 1998). So the protein was universally used as an antigen to detect anti-ASFV antibodies in pig serum samples (Barderas et al., 2000; Reis et al., 2007; Petrovan et al., 2019).

Currently, there are still no commercial vaccines and specific drugs for combatting ASFV infection in pigs (Gaudreault and Richt, 2019). Rapid diagnosis of domestic pigs positive for ASFV is the main technical means for preventing and controlling the disease (Zhang et al., 2021; Zhou et al., 2022). Many testing methods for detecting viral particles, nucleic acid, and antibodies have been developed (Wang et al., 2017; Wang D. et al., 2020b). Among these assays, real-time quantitative PCR (qPCR) for detecting ASFV DNA was widely used (Lin et al., 2020; Wang

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https://doi.org/10.1016/j.virs.2022.09.004
Received 4 May 2022; Accepted 17 July 2022
Available online 8 September 2022

A. et al., 2020a; Wang Y. et al., 2020c). Besides, serological diagnosis of ASF is very important as a complement to qPCR testing, especially for some recovered pigs in which viral DNA in the sera and faeces of pigs is very low and cannot be detected with qPCR (Kazakova et al., 2017; Teklue et al., 2020; Sun et al., 2021). For example, many enzyme-linked immunosorbent assays (ELISAs), including indirect and blocking ELISAs, were developed for detecting anti-ASFV antibodies in pig sera (Tabares et al., 1981). However, the available indirect and blocking ELISAs were developed mostly based on the polyclonal and monoclonal antibodies (Yu et al., 2021; Yuan et al., 2021). These assays also show some drawbacks (Oura et al., 2013). For example, they need a great pure antigen and horseradish peroxidase (HRP) labeled monoclonal antibodies (mAb) or second antibodies, resulting in the complex production technology for further development of the commercial diagnostic kits.

Nanobody is a single domain antibody and is derived from the *Camelidae* heavy chain-only antibodies (VHH) (Vincke and Muyldermans, 2012), with some advantages including small molecular weight (~15 kDa) and easy genetic engineering and expression (Hamers-Casterman et al., 1993; Vanlandschoot et al., 2011). Therefore, the nanobody-horseradish peroxidase (HRP) fusion has been used as a probe for developing competitive ELISA (cELISA) for the diagnosis of viral infection (Du et al., 2019; Ji et al., 2020; Lu et al., 2020). Compared with the ELISA assays based on the traditional antibodies, the ones using nanobody-HRP fusion protein showed advantages, such as short testing time, simple operation, and accessibility to commercial production (Sheng et al., 2019). However, all available commercial ELISA kits for detecting anti-ASFV antibodies were developed based on the polyclonal and monoclonal antibodies.

In the present study, we screened nanobodies against ASFV-p30 and produced nanobody-HRP fusion proteins. Then we developed a cELISA based on the fusion protein for detecting antibodies against ASFV in the pig sera. The cELISA showed good specificity, high sensitivity, and high agreement with the commercial ELISA kit. Additionally, the developed assay in the study showed simple operation and reduced reaction time comparing with the commercial kit and is a good assay to detect the ASFV infection in pig herds.

2. Materials and methods

2.1. Cells

HEK293T cells from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured with Dulbecco's Modified Eagle's Medium (Life Technologies Corp, USA) supplemented with 10% fetal bovine serum (FBS, Gibco USA) and penicillin/streptomycin at 37 °C in 5% CO_2 incubator. Porcine alveolar macrophages (PAMs) were collected based on the previous descriptions with some modifications (Ai et al., 2021). Briefly, the lung was collected from a 30-day-old pig and washed with phosphate buffer solution (PBS, 0.1 mol/L, pH 7.2). The lavage solution was recovered and collected after gently tapping the lungs. The collected fluid was centrifuged at 300 ×*g* for 10 min, and then the PAM cells were resuspended by 10% FBS RPMI 1640 medium (Biological Industries, Israel). Finally, the cells were cultured at 37 °C in a 5% CO_2 incubator.

2.2. Serum samples

Three hundred and sixty pig sera were collected from three pig farms in China in 2017. Because there were no outbreaks of ASFV infection in the pigs in China before 2018, these sera were considered negative pig sera for anti-ASFV antibodies and used to determine the cut-off value of cELISA. In addition, 420 negative pig sera from 2015 to 2017 in China were also collected to determine the specificity of cELISA. A total of 115 positive pig sera for anti-ASFV antibodies were collected from 5 farms occurring ASFV infection in October 2021 to determine the sensitivity of cELISA. These sera were collected from the farms located in Shaanxi Province and kindly provided by Shaanxi Center for Animal Disease Control and Prevention. Totally 895 pig sera samples were confirmed negative or positive for anti-ASFV antibodies by a commercial ELISA kit (Svanovir ASFV-Ab; Boehringer Ingelheim Svanova, Uppsala, Sweden). The kit is suggested by the OIE for the serological assays of ASFV infection and is an indirect ELISA using the recombinant ASFV-p30 as a coating antigen.

Thirty-six pig sera were kindly provided by Qingqing Song, the chief of The Spirit Jinyu Biological Pharmaceutical Co. These serum samples were collected from six challenged pigs with an ASFV isolate (GenBank accession No. MW656282) at 0, 7, 14, 21, 28, and 35 day-post-inoculation (dpi), and were used for detecting the sensitivity of cELISA. The positive pig sera for antibodies against clinical swine fever virus (CSFV, n = 22), porcine reproductive and respiratory syndrome virus (PRRSV, n = 23), porcine circovirus type 2 (PCV2, n = 46), porcine pseudorabies virus (PRV, n = 28), porcine epizootic diarrhea virus (PEDV, n = 16) and swine influenza virus (SIV, n = 19) were collected from clinical pigs. These positive pig sera were confirmed using the commercial ELISA kits (IDEXX, Westbrook, ME, USA). 460 clinical pig sera from 8 farms from January to October 2021 were used to evaluate the agreement between the developed cELISA and the commercial ELISA kit (Svanovir ASFV-Ab; Boehringer Ingelheim Svanova, Uppsala, Sweden).

2.3. Expression and purification of the recombinant ASFV-p30

ASFV-p30 coding sequences (GenBank accession No. MK128995.1) were synthesized by the GENEWIZ Company and ligated into the pUC-56 vector. The recombinant plasmids were named pUC-56-p30. The genes were amplified using the primers ASFV-p30-F and ASFV-p30-R (Supplementary Table S1) using the pUC-56-p30 plasmids as templates by PCR. Then, the PCR products were recovered from 1% agarose gel and cloned into the pET-21b (+) vector with His tag (Novagen, USA) digested by *Bam*H I and *Xho* I enzymes (TaKaRa, Japan). After validated by Sanger sequencing, the recombinant plasmids were transformed into *Escherichia coli* (*E. coli*) strain BL21 (DE3) cells (TransGen Biotech, Beijing, China) and ASFV-p30 protein was expressed based on the previous descriptions with some modifications (Duan et al., 2020). Briefly, the positive bacteria were inoculated into 500 mL Luria broth (LB) liquid medium [0.01% (W/V) Tryptone, 0.005% (W/V) Yeast Extract, 0.01% (W/V) NaCl] containing 100 μg/mL of ampicillin (NOVON Scientific, USA). When the OD_{600nm} of bacteria liquid reached approximately 0.6–0.8, the isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After being cultured for 6 h, the bacterial cells were collected by centrifugation with 8000×*g* for 10 min at 4 °C. Then, the pellets were resuspended with PBS and sonicated at 30 min (25 W, 20 kHz frequency, 3 s on and 3 s off) of ultrasound. After being centrifuged again, the precipitations were suspended in Lysis Buffer (8 mol/L Urea, 100 mmol/L NaH_2PO_4, 100 mmol/L Tris·HCl, pH = 8.0). According to the instructions manual, the dissolved proteins were purified with 4 mL Ni NTA Beads 6FF (SMART, Changzhou, China).

SDS-PAGE and Western blot assays were used to analyze the expression, purification, and antigenicity of the recombinant ASFV-p30. For the Western blot, the positive pig serum sample for anti-ASFV antibodies was the primary antibody and the second antibody is HRP labeled goat anti-pig IgG (Abcam, Cambridge, MA, USA). Then, the results were shown by the chemiluminescence using an automatic luminescence instrument (BIO-RAD).

2.4. Bactrian camel immunization and library construction

As previously described, a healthy 4-year-old male Bactrian camel was immunized with the purified ASFV-p30 by subcutaneous route (Vincke et al., 2012). The purified proteins (1.2 mL, 1 mg/mL) for the first immunization were emulsified with the same volume of Freund's complete adjuvant. The same amount of proteins was emulsified for the

other four immunizations with Freund's incomplete adjuvant. Total five times immunizations were performed at 2-week intervals. After the last immunization, the serum samples were collected from the immunized camels to detect anti-ASFV-p30 antibodies by indirect ELISA using the recombinant ASFV-p30 proteins as the coating antigen. Peripheral blood lymphocytes were also isolated from 250 mL of blood with Leucosep® tubes (Greiner Bio-One, Germany) at 3000×*g* for 30 min. Then, total RNA was extracted from the lymphocytes using a Neasy® Plus Mini RNA extraction kit (QIAGEN, Germany). SuperScript® III Reverse Transcriptase (Invitrogen, USA) was used to synthesize the first strand of cDNA using 50 μg of RNA as a template with Oligo $(dT)_{18}$ and random primers (Invitrogen, USA). Nested PCR was used to amplify the *VHH* gene by using the primers CALL001 and CALL002 for the first round and VHH-FOR and VHH-REV for the second round (Supplementary Table S1). Then, the second PCR products (~400 bp) were digested by *Pst* I and *Not* I and ligated into the phagemid pMECS vector digested with the same two enzymes (Vincke et al., 2012). The recombinant phagemids were electro-transformed into the competent *E. coli* TG1 cells. After that, the cells were resuspended with 500 μL of SOC mediums [2% (W/V) Tryptone, 0.5% (W/V) Yeast Extract, 0.05% (W/V) NaCl, 2.5 mmol/L KCl, 10 mmol/L $MgCl_2$, 20 mmol/L Glucose] and incubated at 37 °C for 1 h. Cells were cultured on LB agar plates containing 2% glucose and 100 μg/mL ampicillin at 37 °C for 8 h. Finally, the colonies were scraped from the plates and tested with primer pairs MP57 and GIII (Supplementary Table S1) to evaluate the library's storage capacity. 48 clones were randomly sequenced to identify the diversity of the library, and the nanobodies were grouped according to the hypervariable complementary-determining region 3 (CDR3) sequence.

2.5. Screening of specific nanobodies against ASFV-p30

To screen the anti-ASFV-p30 nanobodies, four rounds of phage rescue and biopanning were performed as described previously (Liu et al., 2015). Briefly, the phages were rescued via M13K07 phagemid. For biopanning, the plates were coated with the purified ASFV-p30 (5 μg/well, four wells each time). After four rounds of biopanning, the enrichment of specific phage particles was evaluated with polyclonal phage ELISA. The 96 single colonies were randomly selected from the plate and added to 96-well plates. Then, 200 μL of LB/AMP-GLU medium [100 mL LB medium containing 1 mol/L glucose, 0.01 (W/V) ampicillin] was added to each well and cultured at 37 °C for 8 h. The 10 μL of each clone was added to 1 mL TB medium [1.2% (W/V) Tryptone, 2.4% (W/V) Yeast Extract, 0.4% (W/V) Glycerol, 17 mmol/L KH_2PO_4, 72 mmol/L $K_2HPO_4{\cdot}3H_2O$] in a 24-well plate and expressions of nanobodies were induced by IPTG. After the 24-well plate containing bacteria was centrifuged at 2000×*g* for 15 min, the supernatants were discarded, and the pellets were frozen and thawed three times. Then, after centrifugation, the supernatants containing their periplasmic extracts of nanobodies were tested by the indirect ELISA. Finally, all positive clones that contained *VHH* genes were sequenced and classified according to their CDR3 sequence.

2.6. Expressions of nanobody-HRP fusion proteins against ASFV-P30 protein

The nanobody-HRP fusion proteins against ASFV-p30 were expressed in HEK293T cells based on a previous study (Sheng et al., 2019). Briefly, the positive pMECS plasmids containing *VHH* genes and pCMV-N1-HRP vector were digested by *Pst* I and *Not* I enzymes. Then, each *VHH* gene from the pMECS plasmids was separately ligated into the vector pCMV-N1-HRP by a DNA Ligation Kit (New England Biolabs, England). After Sanger sequencing, the positive recombinant plasmids were extracted and transfected into HEK293T cells with polyetherimide agents (PEI, Polysciences Inc. Warrington, USA). At 48 h-post-transfection (hpt), the transfected cells were fixed with 4% paraformaldehyde at 4 °C for 30 min. After the fixed cells were blocked with 5% BSA, they were detected with anti-His mAbs at 37 °C for 1 h, and then were incubated at 37 °C for 1 h using FITC-goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, USA). Finally, the cells were observed under fluorescence microscopy (Leica AF6000, Germany).

In addition, the supernatants were collected and tested for secretory expression of the fusion in the HEK293 cells with ELISAs. To determine that the nanobody-HRP fusions still have the HRP bioactivity for reacting with the tetramethylbenzidine (TMB), the ELISA plate was directly coated with the supernatant containing the fusions. For determing the fusions still specifically reacted with the antigen, the purified ASFV-p30 was used as coating antigen and the supernatants containing nanobody-HRP fusions as the primary antibodies. The procedures of the ELISAs were described as the below.

2.7. Indirect and direct ELISA

To determine the antibody titers against the ASFV-p30 protein in the immunized Bactrian camel, the periplasmic extracts containing nanobodies and the supernatants containing nanobody-HRP fusion proteins were reacted with ASFV-p30 protein using the indirect and direct ELISAs, respectively. Briefly, the 96-well plates were coated with the supernatants (100 and 200 μL) from the tranfected HEK293T cells or purified recombinant ASFV-p30 (400 ng/well) using PBS as a coating buffer at 4 °C for 12 h. After being washed with PBS'T (0.05% Tween-20 in PBS), the plates with the supernatants as coating antigen were directly added with TMB and incubated at room temperature for 15 min. Finally, the OD_{450nm} values of the plates were read using an automatic microplate reader (Bio-Rad, USA) after the reaction was stopped by 3 mol/L H_2SO_4. The plates with ASFV-p30 protein as coating antigen were added with sera from camel, periplasmic extracts, or supernatants respectively and incubated at 37 °C for 1 h. For the indirect ELISA, after being washed again, the plates were added with the rabbit anti-camel polyclonal antibodies following HRP-labeled goat-rabbit IgG or HRP-labeled HA monoclonal antibodies and incubated at 37 °C for 1 h. After being washed, the plates were added with TMB and incubated at room temperature for 15 min. Finally, the OD_{450nm} values of the plates were read using an automatic microplate reader (Bio-Rad, USA) after the reaction was stopped by 3 mol/L H_2SO_4. For the direct ELISA, after the supernatants were added and the plates were washed, TMB was directly supplemented, then the reactions were stopped.

2.8. Indirect immunofluorescence assay (IFA)

ASFV infection in PAM cells was performed in the enhanced biosafety level 3 (P3) biosafety laboratory in The Spirit Jinyu Biological Pharmaceutical Co., Ltd and approved by the Ministry of Agriculture and Rural Affairs. Briefly, the PAM cells were plated into 96-well-plates at the density of 1×10^5 cells/well. After being plated for 4 h, an ASFV isolate (GeneBank accession No. MW656282) was inoculated into the wells for culturing with an MOI of 0.1. At 48 h post infection, the cells were fixed with 4% paraformaldehyde at 4 °C for 30 min. Then, the fixed cells were blocked with 5% BSA in PBS. After being washed five times with PBS, the cells were incubated with supernatants containing nanobody-HRP fusion proteins at 37 °C for 1 h. Then, after being washed again, they were incubated with anti-His mAbs and followed with FITC-goat anti-mouse IgG antibodies at 37 °C for 1 h. Then, the stained cells were observed under fluorescence microscopy (Leica AF6000, Germany).

2.9. Establishment of a competitive ELISA to detect anti-ASFV antibodies in pig sera

To select the best nanobody-HRP fusion protein for developing the cELISA, the blocking ELISA was performed with positive and negative pig sera for anti-ASFV antibodies based on the previous descriptions (Sheng et al., 2019). Briefly, the 96-well ELISA plates were coated with purified ASFV-p30 (400 ng/well) at 4 °C overnight. After the plates were washed

and blocked with 2.5% skimmed-milk in PBS'T, they were incubated with positive and negative pig sera for anti-ASFV antibodies at 37 °C for 1 h. Then, after being washed again, the plates were added with different supernatant containing nanobody-HRP fusion proteins and incubated at 37 °C for 1 h. Finally, TMB was added for color reaction, and 3 mol/L H_2SO_4 was added to stop the reaction. After the OD_{450nm} values were read, the ratio values between positive pig sera and one negative serum (P/N) value were calculated. Then, the nanobody-HRP fusion protein was selected when the P/N was the smallest in the blocking ELISA. In addition, to determine the affinity of the selected nanobody-HRP fusion protein binding to ASFV-p30, the direct ELISA was performed using different amounts of the purified ASFV-p30 protein as coating antigens. The procedures of the direct ELISA were similar to the above descriptions.

The checkerboard titration with direct ELISA was used to determine the optimal amount of coating antigen and concentration of nanobody-HRP fusion protein. Briefly, different dilutions of ASFV-p30 protein (10, 20, 40, 80, 160, 320, and 640 ng/well) were used to coat the ELISA plate at 4 °C overnight. After being washed with PBS'T, the plate was added with the selected nanobody-HRP fusion protein using different dilutions of 1:10, 1:100, 1:1,000, and 1:10,000. Then, the color reaction was added with TMB and stopped with 3 mol/L H_2SO_4. The optimized amount of coated ASFV-p30 and dilution of nanobody-HRP fusion protein were selected when the OD_{450nm} value was approximately 1.0 in the direct ELISA.

The optimal dilution of testing pig sera in the cELISA was determined. First, the positive and negative pig sera were diluted at 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. Briefly, the ELISA plates were coated with the optimized amount of purified ASFV-p30 and incubated at 4 °C overnight. Then, the plates were washed with PBS'T and blocked with 2.5% skimmed milk in PBS'T (blocking buffer). After being washed three times, the mixtures between positive and negative pig sera dilutions and optimized nanobody-HRP fusion protein dilution were added to the wells and incubated at 37 °C for 1 h. After being washed, the plates were added to TMB for colorimetric reaction. After the reactions were stopped, the OD_{450nm} values were read. The optimal dilution of testing pig sera was selected when the P/N was the smallest.

The incubation time between the mixture of testing pig sera and nanobody-HRP fusion protein with coated ASFV-p30 and the time of colorimetric reaction with TMB were also optimized using the checkerboard titration. Briefly, the plates were coated using the optimized amount of ASFV-p30. After being blocked and washed, the wells were added into the mixture between the positive or negative sera and nanobody-HRP fusion protein and incubated at 37 °C for 15, 30, 45, and 60 min. After being washed, the plate was added to TMB and incubated at room temperature for 10 and 15 min. Then, the reactions were stopped, and the OD_{450nm} value was read. Then, the optimal times for incubation and color reaction were selected when the P/N was the smallest.

After the above conditions of cELISA were determined, the procedures were performed as follows. First, the ELISA plates were coated using the optimized amount of ASFV-p30 (100 μL/well) in PBS buffer at 4 °C overnight. Second, after being blocked with blocking buffer (200 μL/well) at room temperature for 1 h and washed three times with PBS'T, the plates were added into the mixture (100 μL/well) of optimized dilutions of testing pig sera and nanobody-HRP fusion protein and incubated for optimal times at 37 °C. Third, after being washed again, the plates were added to TMB (100 μL/well) and incubated at room temperature for the optimized time. Finally, after the reactions were stopped with 3 mol/L H_2SO_4 (50 μL/well), the OD_{450nm} values were read with an automatic microplate reader.

2.10. Validation of the developed competitive ELISA

To determine the cut-off value of cELISA, a total of 360 negative pig sera for anti-ASFV antibodies were tested with the assay. The formula of [1 − (OD_{450nm} value of testing pig serum sample/OD_{450nm} value of negative sample)] × 100% was used to calculate the percent competitive inhibition (PI). Then, the cut-off value of cELISA was set at the mean PI of 360 negative pig sera plus 3 standard deviations (SD) to ensure 99% confidence for negative pig sera within this range.

Specificity of the cELISA was evaluated using 420 negative pig sera obtained from 2015 to 2017 in China. In addition, a total of 132 positive pig sera for antibodies against CSFV (n = 22), PRRSV (n = 23), PCV2 (n = 46), PRV (n = 28), PEDV (n = 16) and SIV (n = 19) were also tested with the cELISA to evaluate the specificity of the assay.

To determine the sensitivity of cELISA, 115 positive pig sera for anti-ASFV antibodies were tested with the cELISA. In addition, doubling dilutions (from 1:10 to 1:1280) of each 6 positive and negative pig sera for anti-ASFV antibodies were tested using cELISA to determine the lowest detection dilution.

The reproducibility of cELISA was determined by testing each five positive and negative pig sera (separately selected from the above 115 positive and 420 negative ones) to analyze the intra-assay and inter-assay variabilities. Then, the coefficient of variation (CV) was calculated using the PI values of different sera to evaluate the inter-assay variation (between plates) and the intra-assay variation (within a plate). Each serum sample was tested using three different plates to determine the inter-assay CV and three replicates within each plate to calculate the intra-assay CV.

The assay's stability was also evaluated for the following development of commercial kits for ASFV antibody detection. Briefly, the 96-well plates were coated with the purified recombinant ASFV-p30 protein using PBS as a coating buffer at 4 °C for 12 h. After being washed with PBS'T and blocked with blocking buffer, the plates were dried in the fume hood and vacuumed. Then, the dried plates and optimized dilution of nanobody-HRP fusion protein were stored at 4 °C. The direct ELISA and cELISA were performed respectively with the procedure mentioned above at 0, 28, 56, 84, and 112 days to evaluate the stability of the cELISA.

2.11. Comparisons of the competitive ELISA with a commercial ELISA kit

The 460 clinical pig sera from the eight pig farms and the 36 sequential pig sera from six challenged pigs were tested with the developed cELISA and a commercial ELISA kit (Svanovir ASFV-Ab; Boehringer Ingelheim Svanova, Uppsala, Sweden). Then, the coincidence rates between the cELISA and commercial ELISA kit were calculated using Microsoft Excel's CORREL function.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Kappa index values were calculated to estimate the coincidence between the cELISA and the commercial ELISA kit. These calculations were performed using SPSS software (Version 20, http://www.spss.com.cn). All the serum samples were tested for three times with these assays.

3. Results

3.1. Expression and purification of the recombinant ASFV-p30

Using the recombinant plasmids pUC-56-p30 as templates, the genes encoding ASFV-p30 were successfully amplified. Then, the recombinant plasmids containing ASFV-p30 were transformed into the *E. coli* BL21 (DE3). SDS-PAGE analysis showed that the recombinant ASFV-p30 with His-tag and some amino acids from the vector were successfully expressed with the expected size of 35 kDa and the highly purified target was obtained after purification with Ni-resin (Fig. 1A). Additionally, Western blot analysis showed that the recombinant ASFV-p30 reacted with the positive pig sera for anti-ASFV antibodies, indicating the protein has a good antigenicity (Fig. 1B).

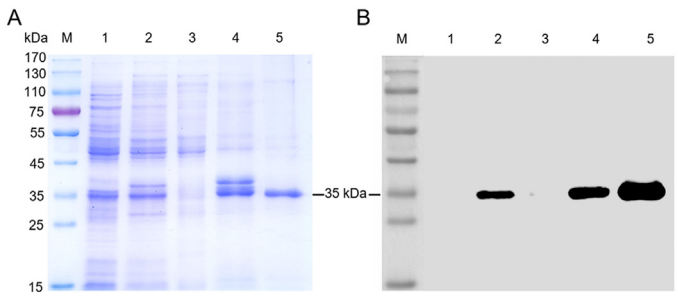

Fig. 1. Expression and purification of the recombinant ASFV-p30. **A** SDS-PAGE analysis of the recombinant ASFV-p30 expressed with *E coli*. **B** Western blot assay of the recombinant ASFV-p30. Positive pig sera for anti-ASFV antibodies was used as primary antibodies. M: molecular weight markers; lane 1: pET21b vector control; lane 2: Induction with 0.1 mmol/L IPTG; lane 3: soluble protein in the supernatant after sonication; lane 4: inclusion body in precipitation after sonication; lane 5: purified ASFV-p30.

3.2. Construction of a phage display VHH library against ASFV-p30

After the last immunization, the titers of anti-ASFV-p30 antibodies in the immunized camel sera reached $1:10^7$ with indirect ELISA detection (Fig. 2A). The lymphocytes were also isolated from the immunized camel and used to extract total cellular RNA. Then, the target band of approximately 400 bp in size was successfully amplified with reverse transcription nested PCR. Through digestion, ligation, and transformation, a phage display VHH library was successfully constructed, which contains approximately 4.5×10^8 individual transformants colonies. Then, 48 clones were randomly selected and were used as templates to identify the positive ones by PCR. Totally 97.9% positive rates were determined (Fig. 2B). The positive PCR products were sequenced, and the results showed great diversity in the library (Supplementary Fig. S1).

3.3. Screening of specific nanobodies against ASFV-p30

Through four rounds of panning with indirect ELISA using ASFV-p30 as coating antigen, the phages against the protein were significantly enriched (Table 1). Then, the periplasmic extracts from the 96 clones in the plates of four round of biopanning were produced and used to evaluate the reaction with ASFV-p30 by indirect ELISA. The results showed that 94 clones could specifically bind to ASFV-p30 (Fig. 2C). Sequences comparisons of the amino acids of the 94 clones showed that 7 different sequences were obtained (ASFV-p30-Nb2, -Nb23, -Nb25, -Nb27, -Nb37, -Nb75 and -Nb89). The conserved residues at the positions of 36, 43, 44, and 46 were all hydrophilic amino acids in the 7 different sequences (Fig. 2D), indicating that they all were nanobodies (Conrath et al., 2005). In addition, the titers of the 7 nanobodies in the periplasmic extracts were 10^4 for ASFV-p30-Nb23, -Nb37, -Nb75, 10^3 for ASFV-p30-Nb2, -Nb27, -Nb89 and 10^2 for ASFV-p30-Nb25 (Fig. 2E).

3.4. Production of nanobody-HRP fusion proteins against ASFV-p30 in HEK293T cells

After the ASFV-p30 recombinant plasmids were transfected into HEK293T cells for 48 h, the supernatant was collected. The IFA assay showed that the seven nanobody-HRP fusion proteins were successfully expressed in the HEK293T cells (Fig. 3A). The direct ELISA with the supernatants as coating antigen showed that the seven nanobody-HRP fusion proteins were secreted into the medium and still have HRP activity when reacted with the substrate of TMB (Fig. 3B). Then, the 7 nanobody-HRP fusion proteins were named accordingly as ASFV-p30-Nb2-HRP, -Nb23-HRP, -Nb25-HRP, -Nb27-HRP, -Nb37-HRP, -Nb75-HRP and -Nb89-HRP. In addition, the seven fusions did not react with PRRSV-N protein, which was expressed using the same expressing vector and the bacterial system as recombinant ASFV-p30, indicating the binding specificity of the fusion proteins (Fig. 3C). The results of IFA also revealed that the seven nanobody-HRP fusion proteins could bind to the p30 protein when ASFV infected PAM cells (Fig. 3D).

3.5. Development of competitive ELISA for detecting anti-ASFV antibodies

To compare the sensitivity of the seven nanobody-HRP fusion proteins, blocking ELISA was performed and the results showed that the P/N value of ASFV-p30-Nb75-HRP antibody was the smallest (Fig. 4A), suggesting that the fusion protein was the best probe for the development of cELISA. Using the different amounts of ASFV-p30 protein as the coating antigen, the results revealed that the affinity constants for ASFV-p30-Nb75-HRP were determined to be 0.630 ± 0.049 μg/μL (Fig. 4B).

Besides, the results of the checkerboard titration assay showed that the optimal amount of ASFV-p30 as coating antigen was 80 ng/well, and the optimized dilution of supernatant containing ASFV-p30-Nb75-HRP fusions was 1:1,000 because the OD_{450nm} value of direct ELISA was approximately 1.0 using these conditions (Table 2).

The optimal dilution of testing pig sera was 1:10 because the P/N was the smallest for the cELISA testing positive and negative pig sera (Table 3). Finally, the results of the checkerboard assay for determining the optimal times of incubation and colorimetric reaction showed that the P/N value was smallest when the incubating time of the mixture with ASFV-p30 protein was 45 min and the colorimetric reaction time was 15 min (Table 4).

3.6. Cut-off value of the competitive ELISA

All 360 negative pig sera for anti-ASFV antibodies were tested with the cELISA. The mean PI value was 7.48%, and the SD value was 5.08%. Then, the cut-off value of cELISA was calculated to be 22.7% (mean PI + $3 \times$ SD), indicating that if the PI value of testing pig serum sample is $\geq$ 22.7%, the sample is positive. Conversely, the sample is negative.

3.7. Specificity, sensitivity, reproducibility and stability of the competitive ELISA

All 420 negative pig sera from the pig farm in 2015–2017 were tested with the cELISA and the results showed that all the pig sera were negative. The PI values of 352 pig sera testing with the cELISA were below 5%, and only 11 pig sera were from 15% to 22.7% (Fig. 5A). These results indicated that the cELISA has good specificity for testing clinical pig sera. Additionally, for antibodies of other swine viruses, the results showed that all PI values of these pig sera were below 22.7%, indicating that the cELISA cannot detect antibodies against other swine viruses in pig sera except ASFV (Fig. 5B).

The cELISA test of 115 positive pig sera for anti-ASFV antibodies showed that 113 were positive, and the PI value of these samples was from 43% to 87%. However, two samples were negative, the PI values were from 21.4% to 22.5%, and the PI values of the 2 ones were close to the cut-off value of cELISA (Fig. 6A). For the different dilutions of the 6 positive pig sera randomly selected from the above 113 positive samples, all sera at the dilution of 1:640 were negative using the cELISA, while those at 1:320 were positive (Fig. 6B). In addition, when the cELISA was used to test 36 sequential pig sera from the challenged pigs, the results showed that seropositivity was first observed at 7 dpi in four of the six pigs and the other two pigs seroconverted at 14 dpi and until 35 dpi, they were still positive (Fig. 6C), which were same with the commercial ELISA kit.

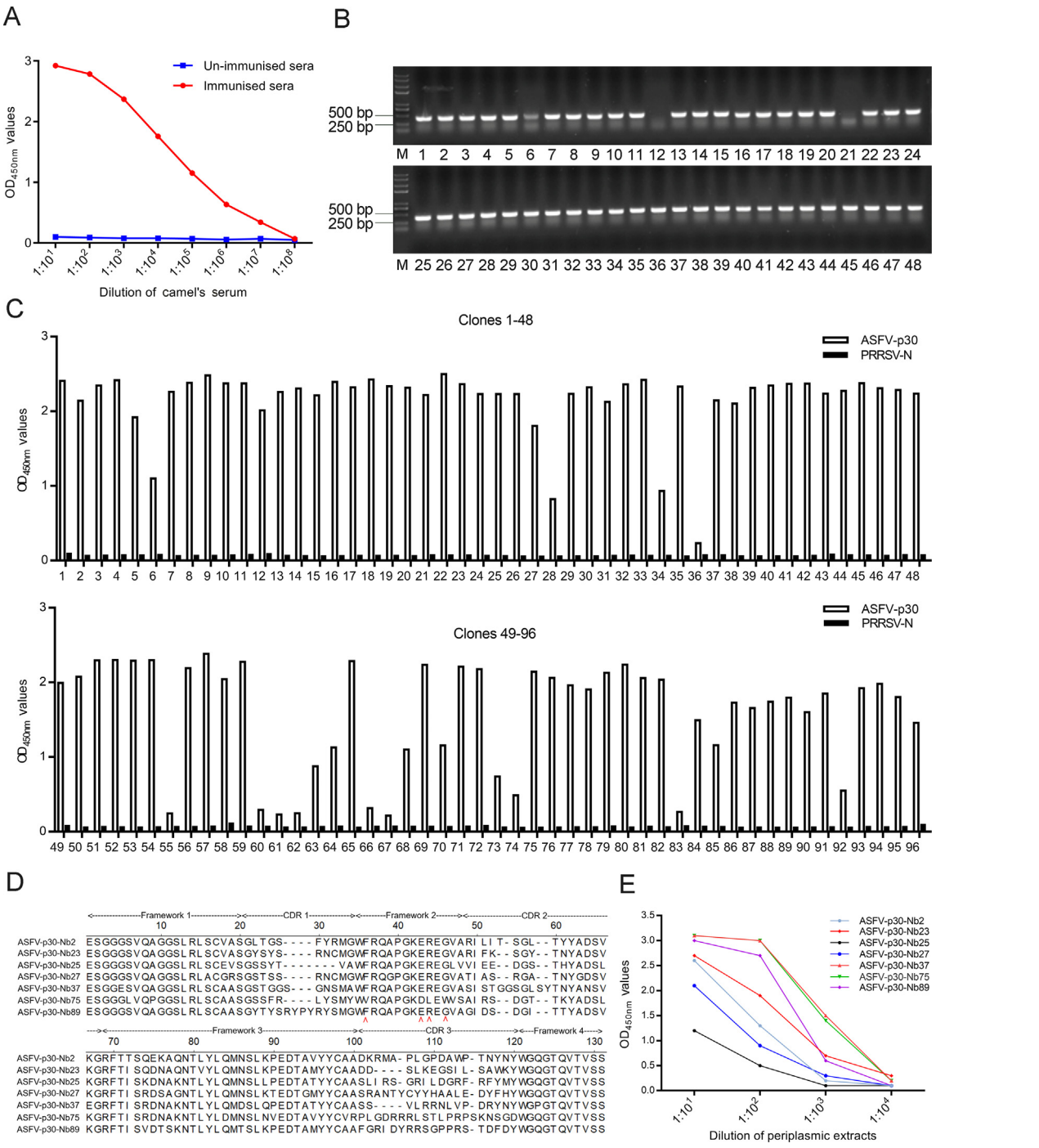

Fig. 2. Screening and identification of nanobodies against the ASFV-p30. **A** Titers of antibodies against ASFV-p30 in the sera from the immunized camel. **B** Total 48 clones were randomly picked to estimate the correct insertion rate by PCR. **C** Identification of the periplasmic extracts from the 96 clones specifically binding to the ASFV-p30 with indirect ELISA. 94 clones were identified as positive. **D** Alignment of amino acid sequences of seven screened nanobodies. **E** Titration of the seven screened nanobodies binding with the ASFV-p30 in the periplasmic extracts.

Table 1
Enrichment of phage particles against ASFV-p30 specific nanobodies during four rounds of panning.

Round of panning	Input phage (PFU/well)	P output phage (PFU/well)	N output phage (PFU/well)	Recovery (P/input)	P/N
1st round	5×10^{10}	3.9×10^{4}	9×10^{3}	7.8×10^{-7}	4.3
2nd round	5×10^{10}	2.0×10^{3}	0	4.0×10^{-8}	2.0×10^{3}
3rd round	5×10^{10}	9.0×10^{4}	2.0×10^{2}	1.8×10^{-6}	4.5×10^{2}
4th round	5×10^{10}	1.0×10^{6}	3.0×10^{1}	3.0×10^{-5}	3.3×10^{5}

ASFV, African swine fever virus; p30, phosphoprotein; PFU, plaque forming unit.

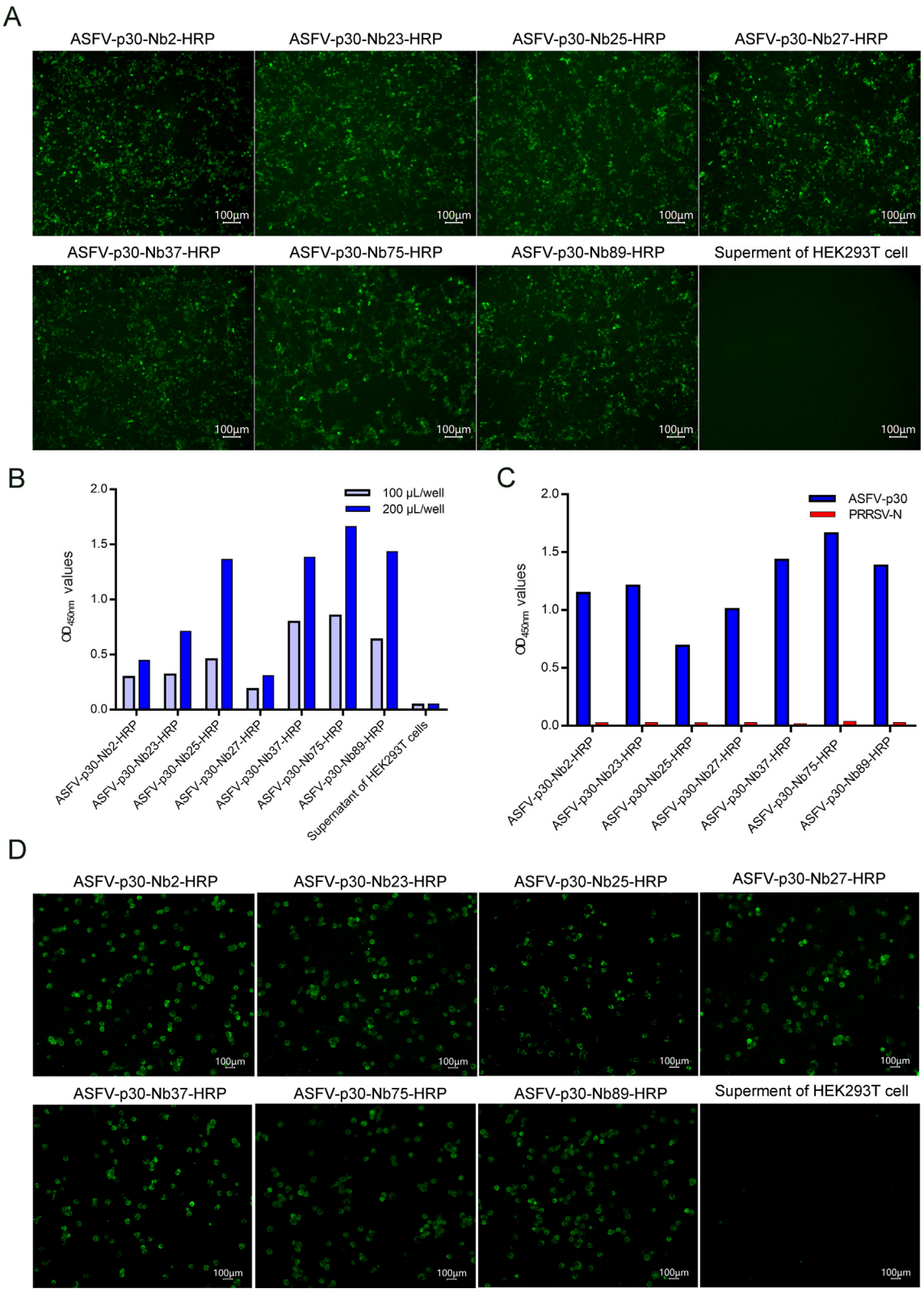

Fig. 3. Characterization of seven ASFV-p30-Nbs-HRP fusion proteins secretory expressed in the HEK293T cells. **A** Expression of seven ASFV-p30-Nbs-HRP fusion proteins in the HEK293T cells. Anti-His mAb was used as the primary antibody. **B** ASFV-p30-Nbs-HRP fusion proteins in the culture medium of HEK293T cells showed HRP activity reacting with the substrate of TMB. **C** Specific reactions between different concentrations of ASFV-p30-Nbs-HRP and ASFV-p30 using direct ELISA. **D** ASFV-p30-Nbs-HRP specifically binds to p30 in ASFV-infected PAM cells in IFA assay. The anti-His mAbs were used as the primary antibody reacting with His tag of the ASFV-p30-Nbs-HRP and the FITC-goat anti-mouse IgG antibodies was used as the second antibody.

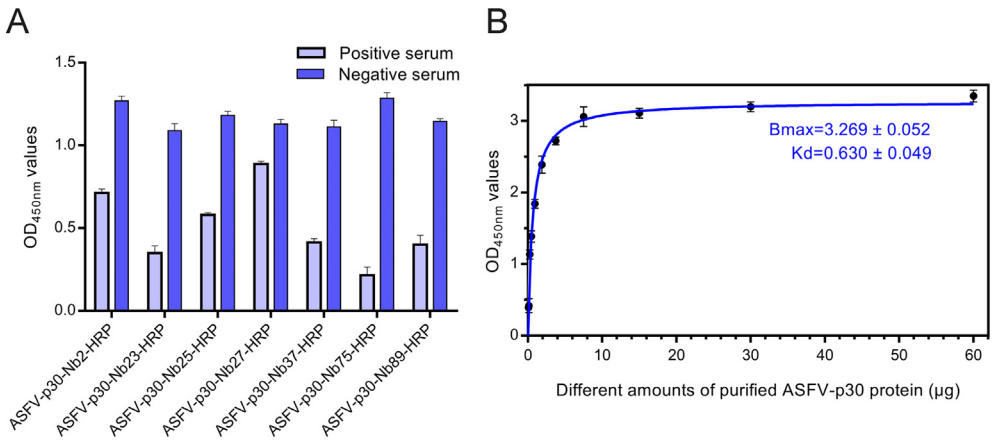

Fig. 4. Selection of the best nanobody-HRP fusion protein for cELISA. **A** Analysis of the seven screened nanobodies blocking the binding of ASFV-p30 and the ASFV pig serum. **B** Binding affinity analysis of ASFV-p30-Nb75-HRP to different amounts of ASFV-p30 proteins by direct ELISA.

Table 2
Optimization of ASFV-p30 protein amounts using the indirect ELISA.

Different amounts of ASFV-p30 proteins (ng)	Different dilutions of ASFV-p30-HRP fusions in the medium			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
10	0.7985	0.373	0.208	0.062
20	1.393	0.545	0.3895	0.0845
40	1.8845	1.226	0.7205	0.1235
80	2.424	1.537	1.088	0.186
160	2.466	2.316	1.387	0.226
320	2.5795	2.4305	1.457	0.252
640	2.6115	2.4475	1.872	0.3215

The optimal amount of ASFV-p30 protein and dilution of ASFV-p30-Nb75-HRP were selected when the OD_{450nm} value of the direct ELISA was approximately 1.0. ASFV, African swine fever virus; p30, phosphoprotein; HRP, horseradish peroxidase.

By testing each five positive and negative pig sera in the triplicate of the same plate, the intra-assay CV of the PI was from 1.22% to 6.33% (Table 5). When these samples were tested in the three different plates with the cELISA, the inter-assay CV of the PI was from 1.53% to 9.98% (Table 5). These results indicated that the developed cELISA exhibits good reproducibility.

For the stability of the cELISA, the results of direct ELISA using the stored plates and nanobody-HRP fusion proteins as reagents showed that the OD_{450nm} values on different days were approximately 1.0 (CV = 5.60%), suggesting that the coated plates with ASFV-p30 and ASFV-p30-Nb75-HRP fusions have a long validity period (Fig. 7A) when the plates and fusions were stored at 4 °C. Second, the results of the cELISA showed that the coated plates and ASFV-p30-Nb75-HRP fusions maintained a good competitive effect in 112 days (CV = 7.93%) (Fig. 7B). These results indicated that the developed cELISA exhibits good stability as a commercial kit candidate.

Table 3
Dilution optimization of tested pig sera for cELISA.

No. serum	Sera type	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
1	Positive	0.33	0.70	1.21	1.57	1.64	1.60	1.69	1.77
	Negative	2.33	2.20	2.09	1.99	1.94	1.63	1.67	1.70
	P/N	0.14	0.32	0.58	0.79	0.85	0.98	1.01	1.04
2	Positive	0.34	0.69	1.19	1.57	1.64	1.59	1.70	1.82
	Negative	2.21	2.01	2.06	1.96	1.84	1.64	1.62	1.63
	P/N	0.15	0.34	0.58	0.80	0.89	0.97	1.05	1.12

Two positive and negative sera were tested using cELISA. The best dilution was selected when the OD_{450nm} value of positive to negative (P/N) sera was the smallest. cELISA, competive ELISA.

Table 4
Incubation time optimization using a checkerboard assay with cELISA.

Time of color reaction (min)	Sera type	Incubation times of pig sera, ASFV-Nb75-HRP fusions and coated antigens (min)			
		15	30	45	60
10	Positive	0.72	0.52	0.27	0.22
	Negative	0.71	0.77	1.02	1.06
	P/N	1.01	0.68	0.26	0.21
15	Positive	0.74	0.48	0.22	0.21
	Negative	0.76	0.81	1.12	1.09
	P/N	0.97	0.59	0.20	0.19

The best competition time and colorimetric reaction time were also selected when the OD_{450nm} value of positive to negative (P/N) sera was smallest. cELISA, competive ELISA.

3.8. Agreements of competitive ELISA and commercial ELISA kit

Four hundred and sixty clinical pig sera were tested with cELISA and a commercial ELISA kit, and the results showed that the positive rates of the two assays were the same as 19.3% (89/460) (Table 6). Meanwhile, for 36 sequential sera from challenged pigs, the results of cELISA and the commercial ELISA kit also showed 28 positive anti-ASFV antibodies. The above results indicated that the two assays had an agreement rate of 100%. Statistical analysis showed that the developed cELISA had a high level of consistency with the commercial ELISA kit (Kappa = 1) (Table 6).

4. Discussion

Antibody plays a valuable role in developing immunoassays for diagnosing diseases (Dovgan et al., 2019; Stravinskiene et al., 2019). The most commercial ELISA kits were produced using the traditional antibodies, including polyclonal antibodies and mAbs (Cao et al., 2021). However, their production is technically demanding, expensive, and time consuming (Gallardo et al., 2009; Tesfagaber et al., 2021). As a promising new generation antibody for diagnostic applications, nanobody has recently arisen as a substitute for conventional antibodies for developing immunoassays. The traditional antibody consists of two identical heavy chains and light chains connected by disulfide bonds. Usually, the two variable regions of the heavy and light chains bind to the antigen (Posner et al., 2019). Compared with traditional antibodies, only one variable region of nanobody binds to the antigen. These suggested that the binding affinity of traditional antibody to antigens is higher than that of the nanobody (Hamers-Casterman et al., 1993). So the nanobodies could

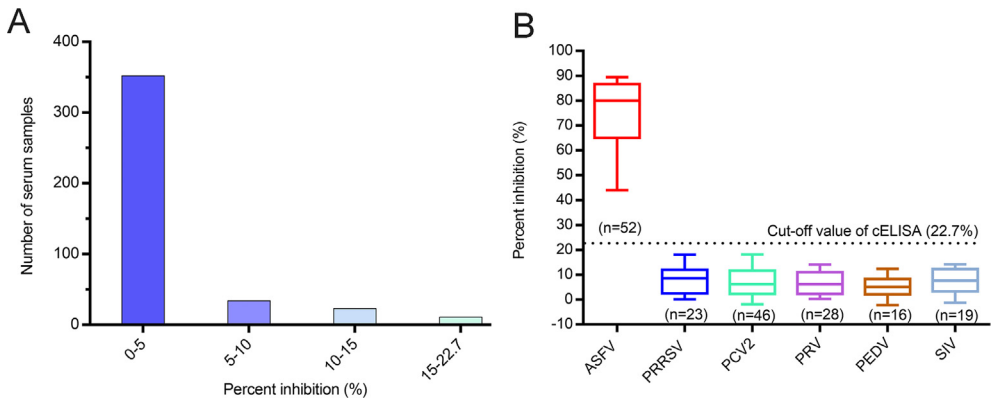

Fig. 5. Specificity of the developed cELISA for detecting anti-ASFV antibodies using the ASFV-p30-Nb75-HRP fusions as a probe. **A** Distribution of the PI values of the clinical negative pig sera for anti-ASFV antibodies in the cELISA. **B** Evaluation of the cELISA for detecting the antibodies against other pig disease viruses, including PRRSV, PCV2, PRV, PEDV, and SIV.

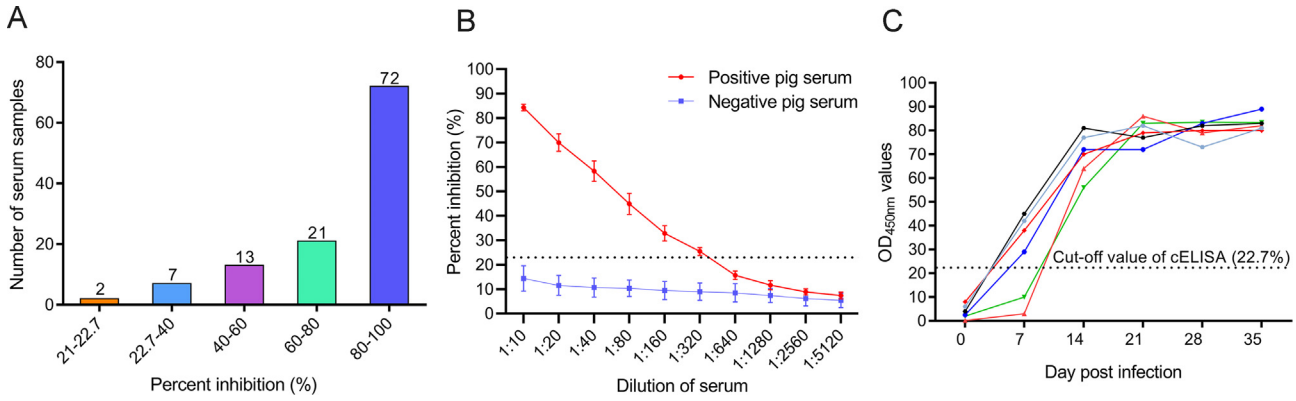

Fig. 6. Sensitivity of the developed cELISA with the ASFV-p30-Nb75-HRP fusion protein. **A** Distribution of the PI values of the clinical positive pig sera for anti-ASFV antibodies in the cELISA. **B** Determination of the largest dilution of positive pig sera for anti-ASFV antibodies. **C** Detection of antibodies against ASFV in the 36 sequential sera at different days post-inoculation from six pigs challenged with ASFV using the cELISA.

Table 5
Reproducibility of the cELISA determined by CV% value of intra and inter assay.

Type of precision	CV % value range of five serum samples	Median value
Intra assay precision (CV%)	1.22–6.33	3.78
Inter assay precision (CV%)	1.53–9.98	5.76

Intra assay precision: determined from three repetitions (well-to-well) of five serum samples in the same method. Inter assay precision: determined from three repetitions (plate-to-plate) at different time. cELISA, competitive ELISA; CV, coefficient of variation.

compete with the traditional antibodies in the sera and be universally used as the probe for developing cELISA to detect antibodies in the sera.

Additionally, since the nanobody molecule is smaller than the conventional antibody, recombinant engineering and protein expression *in vitro* are much simpler. Especially, the simple genetic structure of a nanobody allows easy to fuse labels with a nanobody (Barthelemy et al., 2008; Wang et al., 2016). For example, the nanobody-HRP fusion protein has been designed and universally used as the probe for developing cELISA to detect antibodies against different viruses because the fusions are inexpensive to produce and do not require enzyme-labeling or purification compared with the traditional antibodies (Sheng et al., 2019; Duan et al., 2021; Mu et al., 2021). Based on these advantages, in the present study, a nanobody-HRP fusion protein against anti-ASFV-p30 protein was also used as a probe to develop a cELISA for detecting anti-ASFV antibodies. Compared to traditional antibodies-based immunoassays, the assays showed simple operation, high sensitivity, and specificity. Importantly, the nanobody-HRP fusion protein was directly used for antibody detection without purification, which greatly simplifies the production process of subsequent commercial kits. In addition, the subsequent construction of cell lines with stable secretion and expression of fusion protein further simplifies the production process and reduces production costs. Therefore, the cELISA established in this study has a broad market application prospect.

ASFV infection is the biggest threat to the global pig industry. Since ASF was first reported in China in 2018, the disease has seriously caused the pig industry economic loss. Because there is no effective vaccine available to control ASFV outbreaks to date, highly sensitive and specific diagnostic assays are critical for the rapid detection of ASFV-infected pigs. Low-cost and high-throughput ELISA is an ideal complement to qPCR testing for ASF diagnosis. So far, many ELISAs have been developed based on the traditional antibodies for detecting antibodies against ASFV in pig sera (Lv et al., 2021; Yuan et al., 2021). The cost of nanobody-based cELISA is one-twentieth that of a standard antibodies-based commercial ELISA kit by calculating each reagent's price. The ELISA plates and coating reagents are the same as the traditional ELISA kit, but the developed cELISA using nanobodies does not need to use the enzyme-labeled secondary antibody. In addition, the nanobody-HRP fusion protein can be produced on a large scale. Therefore, there is a lower testing cost using the developed cELISA in the study to detect antibodies against ASFV in the pig sera than using the existing commercial ELISA kits. In addition, our study showed that the agreement between the developed cELISA and the commercial ELISA kit is 100%. The posibile reason is the limited amount of tested pig sera. In the future,

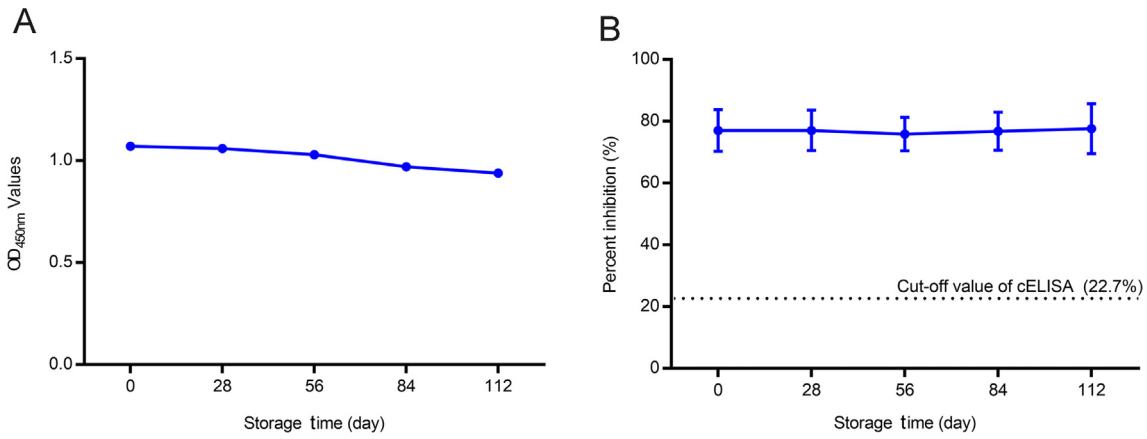

Fig. 7. Stability of the developed cELISA. **A** Binding analysis of the ASFV-p30-Nb75-HRP fusion protein to ASFV-p30 at different times using direct ELISA. **B** Analysis of the ASFV-p30-Nb75-HRP fusion protein blocked to react with ASFV-p30 by the positive pig sera for anti-ASFV antibodies using the cELISA.

Table 6
Comparisons of the developed cELISA with commercial ELISA kit by detecting the clinical and challenged pig serum samples.

Samples	cELISA	Number	Commercial ELISA Kit		Agreement (%)	Kappa value
			+	–		
Clinical sera	+	89	89	0	100	1
	–	371	0	371		
Challenged sera	+	28	28	0		
	–	8	0	8		

The Kappa value > 0.4 was regarded as no significant difference.

more pig sera are needed to evaluate the agreement between the developed cELISA and commercial ELISA kit. The developed cELISA in the study is more convenient in the practical operation because it does not need to use the enzyme-labeled secondary antibody. Therefore, the developed cELISA is a new method for detecting anti-ASFV antibodies in the pig sera to replace the existing commercial ELISA kits.

Among all of the ASFV proteins, p30 is one of the most immunogenic proteins and induces the highest level of viral antibody response during ASFV infection (Hernaez et al., 2008; Sánchez et al., 2013; Murgia et al., 2019). These indicate that p30 is a good antigen for developing diagnostic assays to detect ASFV infection. The p30 was also selected for screening the nanobodies and developing cELISA as the antigen in the study. Except p30, p72 and p54 are also universally used as antigens to detect anti-ASFV antibodies in the pig sera (Gallardo et al., 2009; Cubillos et al., 2013; Tesfagaber et al., 2021). However, the results of ELISA using the p72 as coated antigen in our other study showed that the seropositivity was first observed at 14 dpi from the challenged pigs with ASFV (data not shown). The results of the present study suggested that the cELISA with p30 may be more sensitive than the one with p72. In addition, for the p54, it was found that the expression of the protein with the bacterial system was difficult, and the amount was low. Then, according to the above results, compared with p72 and p54, the p30 protein is the better antigen for developing the ELISA to detect anti-ASFV antibodies in the pig sera.

5. Conclusions

In this study, a nanobody-HRP fusion proteins against ASFV-p30 were expressed and screened. Subsequently, using the screened ASFV-p30-HRP as a probe, a cELISA was successfully developed for detecting anti-ASFV antibodies in pig sera. This assay shows high sensitivity, good specificity, reproducibility, and stability. Importantly, it eliminates the use of commercial secondary antibodies, showing low cost, reduced detection time compared to the commercial ELISA kit. The developed cELISA provides a new method for supervising ASFV infection in pigs.

Data availability

The data supporting this study's findings are available on request from the corresponding authors.

Ethics statement

Animal experiments were performed based on the Guidance for Experimental Animal Welfare and Ethical Treatment by the Ministry of Science and Technology of China. The protocols of experimental animal procedures were carried out following the guidelines of the Northwest A&F University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Northwest A&F University.

Author contributions

Jiakai Zhao: investigation, data curation, writing-original draft. Jiahong Zhu: investigation, data curation, validation. Ying Wang: investigation, validation. Mengting Yang: data curation. Qiang Zhang: data curation. Chong Zhang: resources. Yuchen Nan: resources. En-Min Zhou: resources. Yani Sun: conceptualization, methodology, supervision. Qin Zhao: conceptualization, methodology, project administration, writing (review and editing).

Conflict of interest

The authors report no conflicts of interest in this work.

Acknowledgements

We thank Dr. Qingqing Song of The Spirit Jinyu Biological Pharmaceutical Co. Ltd. for the gift of the sequential serum samples from the challenged pigs. This work was supported by the Natural Science Foundation of China (grant no. 31941016 and 31972676), the Natural Science Foundation of Shaanxi Province of China (grant no. 2022JC-12), and the Key R&D Program of Shaanxi Province (grant no. S2022-YF-YBNY-0673).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.09.004.

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