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Research Article

Nanobodies against African swine fever virus p72 and CD2v proteins as reagents for developing two cELISAs to detect viral antibodies

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ABSTRACT

African swine fever virus (ASFV) poses a significant threat to the global swine industry. Currently, there are no effective vaccines or treatments available to combat ASFV infection in pigs. The primary means of controlling the spread of the disease is through rapid detection and subsequent elimination of infected pig. Recently, a lower virulent ASFV isolate with a deleted *EP402R* gene (CD2v-deleted) has been reported in China, which further complicates the control of ASFV infection in pig farms. Furthermore, an *EP402R*-deleted ASFV variant has been developed as a potential live attenuated vaccine candidate strain. Therefore, it is crucial to develop detection methods that can distinguish wild-type and *EP402R*-deleted ASFV infections. In this study, two recombinant ASFV-p72 and -CD2v proteins were expressed using a prokaryotic system and used to immunize Bactrian camels. Subsequently, eight nanobodies against ASFV-p72 and ten nanobodies against ASFV-CD2v were screened. Following the production of these nanobodies with horse radish peroxidase (HRP) fusion proteins, the ASFV-p72-Nb2-HRP and ASFV-CD2v-Nb22-HRP fusions were selected for the development of two competitive ELISAs (cELISAs) to detect anti-ASFV antibodies. The two cELISAs exhibited high sensitivity, good specificity, repeatability, and stability. The coincidence rate between the two cELISAs and commercial ELISA kits was 98.6% and 97.6%, respectively. Collectively, the two cELISA for detecting antibodies against ASFV demonstrated ease of operation, a low cost, and a simple production process. The two cELISAs could determine whether pigs were infected with wild-type or CD2v-deleted ASFV, and could play an important role in monitoring ASFV infections in pig farms.

1. Introduction

African swine fever (ASF) is a devastating infectious disease in pigs and poses a significant threat to the global swine industry (Burmakina et al., 2016). It is listed as a notifiable disease by the World Organization for Animal Health (WOAH) (Dixon et al., 2013). The disease was first reported in Africa and then spread to Europe, Asia, and other regions (Revilla et al., 2018). Since ASF emerged in China in 2018, it has caused significant economic losses to the pig industry of China (Ge et al., 2018). Recently, certain lower virulent ASF virus (ASFV) strains have emerged and spread in the domestic pigs of China, which has caused non-lethal, sub-acute, or chronic disease and persistent infection in the pigs (Boinas et al., 2004; Gallardo et al., 2019; Sun et al., 2021).

ASFV, the only known arbovirus DNA virus, belongs to the genus *Asfivirus* within the *Asfarviridae* family (Wilkinson et al., 1988). The virion has a complex icosahedral structure and the length of the complete genome varies from 170 to 193 kb encoding approximately 160 proteins (Andres et al., 2020). Among them, the p72 encoded by the *B646L* gene is the primary component of viral capsid proteins and can induce strong immune responses in pigs (Jolaoluwa et al., 2021). Thus, it is an ideal target antigen for developing an immunoassay to detect antibodies against ASFV in pig sera (Jia et al., 2017; Chen et al., 2021; Geng et al., 2022). The extracapsular CD2v protein, encoded by the *EP402R* gene, plays a key role in the hemadsorption phenomenon in the PAM cells and pathogenicity in pigs (Sun et al., 2021). Previous studies have documented that most of the lower virulent ASFV isolates in China exhibited complete or partial *EP402R* deletion in the genome compared to the earliest isolate in China (Boinas et al., 2004; Chen et al., 2021). In other studies, the earliest strains were usually named wild-type ASFV and it was considered that they could induce specific antibodies against the

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CD2v protein in the pigs (Jiang et al., 2022; Wang et al., 2022; Yang et al., 2022). Currently, the CD2v protein is the primary target for the design of attenuated vaccines (Pérez-Núñez et al., 2022), and most of the gene-deleted attenuated vaccine candidates had the *EP402R* gene deleted (Chen et al., 2020; Hemmink et al., 2022). These *EP402R*-deleted ASFV strains, both in the field and in vaccines, were commonly referred to as CD2v-deleted ASFV. As the CD2v protein can also induce immune responses in the pigs, it may be used as an antigen for developing immunoassays to differentiate the pigs infected by wild-type and the less virulent strain or attenuated vaccine candidates with the *EP402R* gene deleted.

To date, several assays have been developed to diagnose ASFV infection, which include real-time and conventional polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescent analysis, and hemadsorption tests amongst others (Wang D. et al., 2020; Wang Y. et al., 2020). Of these, real-time PCR is universally used as an early diagnostic method for preventing and controlling ASF outbreaks worldwide as there are no available commercial vaccines (Biagetti et al., 2018; Wang A. et al., 2020). Additionally, ELISA is also recommended for diagnosis of ASFV infection in the pigs by WOAH. In particular, ELISA is an ideal method for the diagnosis of the lower virulent ASFV infection (Lv et al., 2021). Currently, most ELISAs for detecting anti-ASFV antibodies target the viral structural proteins including p72, p30, p54, pp62, and p22 as coating antigens and polyclonal or monoclonal antibodies as the reagents (Cao et al., 2021; Caixia et al., 2022; Nah et al., 2022). However, these ELISAs were developed using traditional antibodies, which resulted in a high cost for production and complex production processes (Oura et al., 2013).

Nanobodies, the variable domains of the heavy chain (VHH) of single-domain antibodies found in camelid animals, have several advantages, including a small molecular weight (~15 kDa), recognition of cryptic epitopes of antigens, and ease of genetic engineering (Pillay and Muyldermans, 2021). They have been widely used as a tool for researching protein functions and developing diagnostic methods and treatments (Muyldermans, 2021). Compared with monoclonal antibodies or polyclonal antibodies produced by immunized mice or rabbits, the construction of phage libraries and screening of nanobodies using camels is easier, and all the genes encoding a nanobody can be easily cloned and expressed *in vitro* (Pillay and Muyldermans, 2021). Recently, two nanobody-based competitive ELISAs (cELISA) were developed using anti-p30 and p54 nanobodies as reagents (Zhao H. et al., 2022; Zhao J. et al., 2022). Compared with ELISAs developed using traditional antibodies, nanobody-based cELISA shows several advantages, such as simplicity and lower development costs, thus presenting strong market potential. Additionally, nanobodies targeting ASFV p30 and p54 serve as useful tools for studying the functions of these two proteins. Therefore, screening nanobodies against ASFV p72 and CD2v proteins, based on the characteristics of nanobodies and the functions of ASFV p72 and CD2v proteins, may also provide important tools for researching on ASFV biology, diagnosis, and treatment.

In the present study, we screened nanobodies against ASFV p72 and CD2v proteins and developed two nanobody-based cELISAs. The two assays showed high sensitivity, specificity, reproducibility, stability, and good agreement with the commercial ELISA kits. They were also ideal assays for investigating ASFV infection in pigs. Importantly, the combination of the two cELISAs could differentiate pigs infected with wild-type and CD2v-deleted ASFV.

2. Materials and methods

2.1. Cells, vectors, and viruses

HEK293T cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured at 37 °C in a humidified incubator supplied with 5% CO_2 in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Inc., USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., USA) and penicillin/streptomycin. Porcine alveolar macrophages (PAMs) were collected by bronchoalveolar lavage of the lungs of 6-week-old pigs as previously described (Zhao J. et al., 2022) and cultured in RPMI 1640 medium (Biological Industries; Sartorius AG, Germany) supplemented with 10% FBS and cultured at 37 °C in a humidified incubator.

The pET-28a vector (Novagen, USA) was used for prokaryotic expression of the ASFV-p72 and -CD2v proteins. The pMECS vector was kindly provided by Professor Muyldermans and used to construct the library of VHH as described previously (Vincke et al., 2012). The pCMV-N1-HRP vector was designed and constructed using a pCMV-N1-EGFP vector (Clontech, Japan) as a backbone to produce nanobody-HRP fusions (Sheng et al., 2019).

2.2. Serum samples

The pig serum samples used in the study were divided into five groups. Group 1: 638 negative pig sera for antibodies against ASFV were collected from different healthy pigs and were confirmed negative using a commercial ELISA kit (INgezim® PPA Compac, Eurofins INGENASA, Spain). Group 2: 24 ASFV-positive standard pig sera purchased from the Institute of Veterinary Drug Control (IVDC, China). Group 3: 122 pig sera positive for antibodies against other swine viruses including classical swine fever virus (CSFV, n = 12), porcine reproductive and respiratory syndrome virus (PRRSV, n = 21), porcine circovirus type 2 (PCV2, n = 18), porcine parvovirus (PPV, n = 25), porcine epizootic diarrhea virus (PEDV, n = 20), and porcine pseudorabies virus (PRV, n = 26) were conserved in our laboratory. Group 4: 23 pig sera positive for antibodies against ASFV were collected from the clinical recovery pigs infected by wild-type ASFV, and 36 samples positive for antibodies against CD2v-deleted ASFV were obtained from pigs experimentally infected by a two-gene-deleted attenuated vaccine candidate. These serum samples were kindly provided by New Hope Liuhe Co., Ltd. and confirmed using the commercial p72 monoclonal antibody-based ELISA kit (INgezim® PPA Compac, Eurofins INGENASA, Spain) and CD2v monoclonal antibody-based ELISA kit (Biovet Biotechnology Compac, Beijing, China), respectively. Group 5: 85 serum samples were collected from recovery pigs infected by a wild-type virulent ASFV isolate (GenBank accession no. MN393476) and 123 samples from pigs infected with the two-gene-deleted ASFV vaccine candidate (*EP402R* and *MGF360*-deleted). All the 208 pig sera were also kindly provided by New Hope Liuhe Co., Ltd.

2.3. Expression and purification of the recombinant ASFV-p72 and -CD2v proteins

The gene encoding ASFV-p72 (GenBank accession no. AYW34053.1) protein was synthesized by Genewiz, Inc (Nanjing, China). Another gene encoding the truncated ASFV-CD2v (GenBank accession no. AYW34030.1) protein, consisting of the cytoplasmic domain and extracellular domain, connect via a 15 amino acid flexible $(G4S)_3$ linker was also synthesized by Genewiz, Inc. The two genes were cloned into the pUC-56 vector to construct the plasmids pUC-56-p72 and pUC-56-CD2v. Using the two plasmids as templates, the p72 and CD2v genes were separately amplified by PCR using the respective primers ASFV-p72-F, ASFV-p72-R, and ASFV-CD2v-F, ASFV-CD2v-R (Supplementary Table S1). Then, the two genes were separately cloned into the pET-28a (+) vector with a His tag (Novagen, USA), which was then digested using the *BamH*I and *Xho*I enzymes (New England Biolabs, USA).

After sequencing, the two recombinant positive plasmids were separately transformed into *Escherichia coli* (*E. coli*) Rosetta (DE3) cells (TransGen Biotech, Co., Ltd., Beijing, China), and protein purification was performed as described previously (Sheng et al., 2019). Briefly, after the OD_{600nm} of positive bacteria cultures reached 0.6–0.8, the expression of the two recombinant proteins was induced by adding 0.5 mmol/L isopropyl-β-D-thiogalactoside (IPTG) at 37 °C for 6–8 h. Next, the

bacterial pellets were collected, resuspended in PBS (0.1 mol/L, pH = 7.2), and sonicated at 50 min (35 W, 20 kHz frequency, 3 s on and 3 s off). After centrifugation with 2000 ×*g* for 15 min, the pellets were dissolved in lysis buffer (8 mol/L Urea, 100 mmol/L NaH_2PO_4, 100 mmol/L Tris·HCl, pH = 8.0) and then the two recombinant p72 and CD2v proteins were purified using a high-affinity Ni NTA Beads 6FF column according to the manufacturer's protocol (Smart-Lifesciences, Changzhou, China).

Finally, expression, purification, and antigenicity of recombinant proteins were analyzed by SDS-PAGE and Western blotting using pig sera from the recovered pig infected by ASFV as the primary antibodies and HRP conjugated goat anti-pig IgG antibodies as the secondary antibodies (Abcam, Cambridge, UK).

2.4. Bactrian camel immunization and library construction

Animal immunization and library constructions were performed as described previously (Vincke et al., 2012). Briefly, a 4-year-old adult male Bactrian camel was immunized with the purified ASFV-p72 and -CD2v proteins five times. In the first immunization, the p72 (1 mg, 2 mg/mL) and CD2v (1 mg, 1 mg/mL) were emulsified in Freund's complete adjuvant and for subsequent immunizations, the proteins were emulsified in Freund's incomplete adjuvant. A total of 5 immunizations were performed at 2-week intervals. The serum samples from the immunized camel were collected to determine the titers of anti-p72 and -CD2v antibodies with indirect ELISA as previously described (Du et al., 2019). A total of five days after the final immunization, peripheral blood (200 mL) was collected, and lymphocytes were isolated using Leucosep® tubes according to the manufacturer's protocol (Greiner Bio-One, Germany).

Total RNA was extracted from 3×10^7 lymphocytes with the Neasy® Plus Mini RNA extraction kit (QIAGEN GmbH, Germany) and the SuperScript III First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc., USA) was used to synthesize cDNA by reverse transcription. Subsequently, the VHH genes were amplified by nested PCR using the cDNA as a template, and the primer pairs CALL001 and CALL002 (Supplementary Table S1) were used to amplify a 700 bp target band. Next, the VHH fragments with the expected size of 400 bp were amplified using the second primer sets VHH-FOR and VHH-REV (Supplementary Table S1).

Finally, the VHH gene (~400 bp) was ligated into the phagemid vector pMECS using T4 DNA ligase (New England BioLabs, Inc., USA), which were both digested with *Pst*I and *Not*I restriction enzymes. The recombinant vectors were electro-transformed into *E. coli* TG1 cells. The transformed cells were plated on LB agar plates containing 2% glucose and 100 μg/mL ampicillin and incubated at 37 °C for 6–8 h. Subsequently, the colonies on the plates were scraped off and stored at −80 °C by adding 20% glycerol. Meanwhile, the 20 μL of the transformed cells were diluted to 1×10^{-6} and plated on the plates. The clones were counted to evaluate the library's storage capacity.

The 48 single clones were randomly selected to evaluate the positive rate of the library using MP57 and GIII primers (Supplementary Table S1). To evaluate the library's diversity, the sequences of the hypervariable complementary-determining region 3 (CDR3) of positive clones were aligned.

2.5. Screening of specific nanobodies against ASFV-p72 and -CD2v proteins

To screen specific nanobodies separately against ASFV-p72 and -CD2v proteins, phage rescue, and bio-panning were performed as previously described with some modifications (Vincke et al., 2012). Briefly, the recombinant phage library was rescued by infecting TG1 cells with M13K07 phagemid. For every round of bio-panning, the ELISA plates were separately coated with the purified ASFV-p72 and -CD2v proteins (10 μg/well, four wells per round) at 4 °C overnight. After washing with PBS'T [PBS containing 0.5% (v/v) Tween-20] three times, 200 μL of the blocking buffer [PBS'T containing 5% (w/v) skimmed milk] was added to the plates and incubated at 37 °C for 1 h. This was followed by washing with PBS'T, after which the phage particles from the rescuing recombinant phage library were added to the plates and incubated at 25 °C for 2 h. Next, 100 μL of freshly prepared trimethylamine (100 mmol/L) was added to each well and incubated at 25 °C for 10 min to elute the bound phages. The eluent was collected and neutralized immediately with 100 μL Tris-HCl buffer (1 mol/L, pH = 7.4). Then, the *E. coli* TG1 was infected with the eluted phage particles, and amplified for further rounds of selection.

After three rounds of bio-panning, the enrichments of specific phage particles against ASFV-p72 and -CD2v proteins were separately determined by phage titer assay and ELISA. The 96 colonies of each protein were randomly picked from plates of the third round to produce soluble crude periplasm extracts containing nanobodies. Briefly, the 96 colonies were cultured in 96-well plates containing 100 μL LB/AMP-GLU medium [100 mL LB medium containing 1 mol/L glucose, 0.01 (W/V) ampicillin] at 37 °C for 6 h. Then, 10 μL of the culture was transferred to 24-well plates with 1 mL TB medium and the plates were cultured at 37 °C until the OD_{600nm} of the bacterial cultures reached 0.6–0.8. The bacteria were induced overnight by IPTG with a final concentration of 1 mmol/L at 37 °C. Next, the cell pellet was obtained and resuspended in PBS after freezing and thawing three times. After centrifugation, the soluble crude periplasm extracts containing nanobodies were obtained. The specificity and binding capacity of periplasm extracts to ASFV-p72 and -CD2v proteins were separately determined by indirect ELISA. The nucleocapsid protein of Newcastle disease virus (NDV-NP) was used as the coating antigens for the negative control. The protein was expressed using the same vectors and bacterial as ASFV-p72 and -CD2v proteins. Then, the positive colonies were sequenced and the obtained sequences were grouped according to their CDR3.

2.6. Expression of nanobody-HRP fusion proteins against ASFV-p72 and -CD2v proteins

Nanobody-HRP fusion proteins against ASFV-p72 and -CD2v proteins were expressed as described previously (Sheng et al., 2019). Briefly, the positive recombinant pMECS plasmids containing the genes encoding nanobodies and the pCMV-N1-HRP vectors with HA and His tags were both digested with *Pst*I and *Not*I enzymes (New England Biolabs, USA). Then, the genes were cloned into the vectors to obtain the recombinant plasmids. After sequencing, the positive plasmids were transfected into HEK293T cells using Polyethyleneimine agents (PEI, Polysciences Inc., USA). A total of 48 hour-post-transfection (hpt), the nanobody-HRP fusion proteins were identified by indirect immunofluorescence assays (IFA) in the transfected cells. The supernatants from the transfected cells containing the nanobody-HRP fusion proteins were separately collected and filtered through 0.22-μm-pore cellulose acetate membranes. The specific reaction between the nanobody-HRP fusion proteins in the supernatants and ASFV-p72 and -CD2v proteins were separately determined with the direct ELISA using the two purified proteins as coating antigens and the supernatant of the above-transfected cells as the source of the primary antibody. In addition, IFA was performed to identify nanobody-HRP fusion protein binding to ASFV-infected PAM cells. ASFV infection in PAM cells and IFA was performed in the enhanced biosafety level 3 (P3) laboratory at Spirit Jinyu Biological Pharmaceutical Co., Ltd.

2.7. ELISA

To determine the titers of antibodies in the immunized camel, periplasm extracts and the supernatants separately reacting with the ASFV-p72 and -CD2v proteins, different types of ELISA were performed as described previously (Zhao J. et al., 2022). Briefly, the ELISA plates were separately coated with the purified ASFV-p72 and -CD2v proteins (both 400 ng/well) using PBS at 4 °C overnight. After blocking and washing, the different dilutions of serum samples from immunized camel, different

periplasm extracts, and the supernatants were added to the plates and incubated for 1 h at 37 °C. For the serum samples, the rabbit anti-camel antibodies and HRP-labelled goat anti-rabbit antibodies (Beijing TransGen Biotech Co., Ltd., Beijing, China) were added in turn. For the periplasm extracts, the anti-HA monoclonal antibodies and HRP-labelled goat anti-mouse antibodies (Beijing TransGen Biotech Co., Ltd., Beijing, China) were added. For the supernatants, no further antibodies were added. After the plates were washed with PBS'T, trimethylbenzene (TMB) was added to each well to visualize the reaction. Reactions were halted using 3 mol/L H_2SO_4, and the OD_{450nm} values of each well were read using the automatic microplate reader (Bio-Rad Laboratories, Inc., USA).

2.8. Indirect IFA

To confirm that the nanobody-HRP fusion proteins were expressed in HEK293T cells and could bind to the ASFV-infected PAM cells, different types of IFA were performed as described previously (Zhao J. et al., 2022).

Briefly, the HEK293T cells were plated into 6-well cell plates and the VHH-pCMV-N1-HRP positive plasmids were transfected using PEI. At 48 hpt, the cells were fixed with 70% ethanol at 4 °C for 30 min and blocked with 1% BSA at 37 °C for 1 h. After the plates were washed with PBS, the cells were incubated with anti-His monoclonal antibodies (Beijing Transgen Biotech Co., Ltd., Beijing, China) at 37 °C for 1 h, and then incubated using FITC-labelled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. USA). Next, the nucleus was counterstained using DAPI, and the stained cells were observed using a fluorescence microscope (Leica AF6000, Leica Microsystems GmbH, Germany).

ASFV infection in PAM cells and IFA were performed in the enhanced biosafety level 3 (P3) laboratory at JINYU Group Co., Ltd. Briefly, after the PAM cells were plated for 5 h, the ASFV isolate (GenBank accession no. MW393476) was added to the wells with an MOI of 0.1. At 48 hpt, the cells were fixed with 70% ethanol at 4 °C for 30 min and blocked with 1% BSA at 37 °C for 1 h. After the plates were washed with PBS, the cells were incubated with the supernatant containing the nanobody-HRP fusion proteins at 4 °C overnight. Next, the anti-His monoclonal antibodies and FITC-labelled goat anti-mouse IgG antibodies were added sequentially. After the nucleus was stained with DAPI, the stained cells were observed using a fluorescent microscope (Leica AF6000).

2.9. Development of the two cELISAs using nanobody-HRP fusion proteins

After the nanobody-HRP fusion proteins against ASFV-p72 and -CD2v proteins were produced, two cELISAs (p72-cELISA and CD2v-cELISA) were developed as described previously (Sheng et al., 2019). First, the best nanobody-HRP fusion proteins were separately selected for developing the two ELISAs in a blocking format. Briefly, the plates were separately coated with the purified ASFV-p72 and -CD2v proteins. After blocking and washing, the sera positive and negative for anti-ASFV antibodies were separately added to the plates, incubated for 1 h, and subsequently washed. Next, the supernatants containing nanobody-HRP fusion proteins were added to the wells. TMB was added to develop the color reaction and 3 mol/L H_2SO_4 was used to stop the reaction, after which the OD_{450nm} values were measured using an automatic microplate reader. The optimal fusion proteins were selected when the ratios between the OD_{450nm} values of positive and negative sera (P/N) were the smallest.

Second, the optimized quantities of coated antigens and dilutions of the optimal fusion proteins were determined with checkerboard titration by direct ELISA. Briefly, the plates were separately coated with different amounts (10, 20, 40, 80, 160, and 320 ng/well) of the ASFV-p72 and CD2v proteins using PBS at 4 °C overnight. Then, the different dilutions (1:10, 1:100, 1:1000, and 1:10,000) of the optimal nanobody-HRP fusion proteins against ASFV-p72 and -CD2v proteins were added, respectively. The TMB and 3 mol/L H_2SO_4 were added sequentially and the OD_{450nm} values of each well were read. The optimized amount of coating antigens and dilution of fusion proteins for p72-cELISA and CD2v-cELISA were both selected when the OD_{450nm} values of the conditions were ~1.0 in the direct ELISA.

Third, the optimized dilutions of the testing pig serum samples for the two cELISAs were also determined. Each highly and weakly positive, and two negative pig sera were separately tested using the p72-cELISA and CD2v cELISAs. The ELISA plates were separately coated with the optimized amounts of ASFV-p72 and -CD2v proteins. Then, the different dilutions (1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280) of the testing pig sera were separately mixed with the optimized dilutions of fusion proteins against ASFV-p72 and -CD2 proteins. After the mixtures were added to the plates, respectively, and incubated for 1 h at 37 °C, TMB and 3 mol/L H_2SO_4 were added sequentially and the OD_{450nm} values were measured. The optimized dilution of testing pig sera for p72-cELISA and CD2v-cELISA were both determined when the P/N values were the smallest.

Fourth, the optimal incubation times between the mixtures containing the testing pig sera and fusion proteins with coated antigen and the TMB incubation times were also determined using checkerboard titrations. One weakly positive and one negative pig sera were separately tested using the p72-cELISA and CD2v-cELISAs. After the plates were separately coated with the purified ASFV-p72 and -CD2v proteins, the incubation times between the mixtures and the coated plates were 15, 20, 25, 30, 45, and 60 min and the TMB incubation times were used with 10 and 15 min. The optimized times were determined when the P/N values were the smallest.

After the above conditions were optimized, the p72-cELISA and CD2v-cELISA were both performed as follows. The ELISA plates were separately coated with the optimized ASFV-p72 and -CD2v proteins (100 μL/well) in PBS at 4 °C overnight. Then, the plates were blocked with blocking buffer (200 μL/well) for 1 h at 37 °C. After washing three times with PBST, the mixtures (100 μL/well) including the optimized dilutions of testing pig serum samples and nanobody-HRP fusion proteins were added to the plate, and incubated at 37 °C with the optimized incubation time. After washing with PBST again, TMB (100 μL/well) was added to the plate and incubated for the optimized amount of time. Finally, 3 mol/L H_2SO_4 was added to each well (50 μL/well) to stop the reaction and the OD_{450nm} values were read using an automatic microplate reader. After the serum samples were tested using the assays, the following formula was used to calculate the percent competitive inhibition (PI): $PI = [1 - (OD_{450nm}$ values of testing serum sample/OD_{450nm} value of negative ones)] × 100%.

2.10. Determination of the cut-off value for the two cELISAs

After 638 negative pig sera for anti-ASFV antibodies (Group 1) were assessed using the two assays, the cut-off values of the two cELISAs were both defined as the mean PI value of the negative samples (N) + 3 × standard deviation (SD). When the PI value of testing pig serum samples using the assays was greater than or equal to the cut-off value, it was considered as positive. Conversely, when the reverse was true, it was considered negative.

2.11. Determination of the sensitivity, specificity, reproducibility, and stability of the two cELISAs

To determine the sensitivity of the two cELISAs, 24 positive pig sera for anti-ASFV antibodies (Group 2) and 24 negative pig sera (from Group 1) were serially diluted (from 1:10 to 1:5120) with nanobody-HRP fusion proteins in PBS. Then, the mixture was tested using the assays.

To evaluate the specificities, the two cELISAs were used to test 122 positive pig sera for antibodies against other swine viruses (Group 3). In addition, 23 positive sera of antibodies against wild-type ASFV and 36 positive ones of antibodies against CD2v-deleted ASFV were both tested using the assay (Group 4).

To assess the reproducibility of the two cELISAs, the intra-assay and inter-assay variabilities were evaluated using the coefficient of variation (CV) by testing each 6 positive and negative sera for antibodies against ASFV. To determine intra-assay variabilities, each sample was tested triplicate on the same ELISA plate to calculate intra-assay CV. To determine inter-assay variabilities, each sample was tested on three different ELISA plates at different times by different operators to calculate the inter-assay CV.

To evaluate the stabilities of the two cELISAs for the production of commercial kits, the variabilities of the plates and reagents stored at 4 °C for lengths of time were analyzed. Briefly, the plates were separately coated with the optimized ASFV-p72 and -CD2v proteins. After the plates were blocked with blocking buffer, they were dried in a fume hood and stored at 4 °C under the vacuum. Then, the supernatants containing nanobody-HRP fusion proteins were also stored at 4 °C. The direct ELISA using the plates and supernatants as reagents and the cELISA described as above were performed every 30 days for 180 days.

2.12. Comparison of the two developed cELISAs using a commercial ELISA kit

To determine the coincidence rates between the two cELISAs and commercial ELISA kits, a total of 208 positive pig sera (Group 5) were simultaneously tested with the developed p72-cELISA, CD2v-cELISA, commercial p72 monoclonal antibody-based ELISA kit (INgezim® PPA Compac, Eurofins INGENASA, Spain), and commercial CD2v monoclonal antibody-based ELISA kit (Biovet Biotechnology Compac, Beijing, China).

To assess the inconsistent pig sera, Western blotting was subsequently performed. The recombination p72 and CD2v proteins were separately transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). And then, the membranes were incubated with the inconsistent pig sera as primary antibodies and HRP conjugated goat anti-pig IgG (Beijing TransGen Biotech Co., Ltd., Beijing, China) as the secondary antibodies.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, Inc., San Diego, CA, USA). All data were presented as the mean ± SD. Significant differences between samples were assessed using Student's *t*-test. The κ index values were calculated to estimate the coincidence between the two cELISA and the commercial ELISA kits using SPSS software (version 20; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Production of recombinant ASFV-p72 and -CD2v proteins

To identify the expression and antigenicity of the recombinant ASFV-p72 and -CD2v proteins, Western blotting was performed. After expression and purification, Western blotting showed that the recombinant ASFV-p72 and -CD2v proteins were successfully expressed and purified in the *E. coli* with the expected size of 75 kDa and 42 kDa (Supplementary Fig. S1A and S1B). The specific target band can be detected by the positive pig sera as primary antibodies (Supplementary Fig. S1C and S1D), indicating that both recombinant proteins exhibited antigenicity.

3.2. Construction of a phage display VHH library

After immunizing five times, the titers of antibodies against ASFV-p72 and -CD2v proteins in the immunized camel separately reached 1:256,000 and 1:128,000 (Fig. 1A and B), indicating that the camel produced a good immune response to the two proteins. Then, a phage display VHH library against two recombinant proteins was constructed, which contained approximately 3.8×10^8 individual transformant colonies. Next, 48 single colonies were randomly selected to confirm the successful insertion of the *VHH* gene. The PCR results showed that 44 colonies had an insert corresponding to the size of a *VHH* gene and the positive rate was 91.7% (44/48) (Supplementary Fig. S2). After the positive colonies were sequenced, the results showed that each clone contained a distinct VHH sequence (less than 50% amino acid similarity) indicated the library had good diversity (Supplementary Fig. S3).

3.3. Screening of specific nanobodies against ASFV-p72 and CD2v proteins

Using the purified ASFV-p72 and -CD2v as coating antigens and PBS as the control, three rounds of bio-panning were performed. By testing the titers of phages, the results showed that the specific phages against ASFV-p72 increased from 36 to 1.6×10^3, and the specific phages against ASFV-CD2v increased from 205 to 1.94×10^4 (Table 1). Both were significantly enriched after the third round compared with the control group (Table 1). Using the periplasmic extracts of 192 colonies (96 for each protein) from the third-round plates as the primary antibodies, the results of indirect ELISAs showed that 56 and 96 colonies reacted with the ASFV-p72 and -CD2v proteins, respectively (Fig. 1C and D). After these positive colonies were sequenced, alignments of these sequences showed that 8 and 10 nanobodies separately against ASFV-p72 and -CD2v proteins were obtained. Amino acid sequence analysis indicated that the conserved residues at 37, 44, 45, and 47 (located on the VH-VL interface region of VHs) from the 18 nanobodies were hydrophilic amino acids. For nanobodies against ASFV-p72, they were named ASFV-p72-Nb2, -Nb3, -Nb20, -Nb21, -Nb30, -Nb37, -Nb45, and -Nb64, and for CD2v proteins, they were named ASFV-CD2v-Nb4, -Nb22, -Nb25, -Nb34, -Nb42, -Nb55, -Nb67, -Nb70, -Nb88, and -Nb92 (Fig. 1E and F).

3.4. Production of nanobody-HRP fusion proteins against ASFV-p72 and -CD2v proteins in HEK293T cells

To produce the nanobody-HRP fusion proteins, the recombinant plasmids were transfected into HEK293T cells. After the positive plasmids containing the genes encoding nanobodies against ASFV-p72 and -CD2v proteins with HRP, HA, and His tags were transfected into HEK293T cells, the results of IFA showed that a total of 18 nanobody-HRP fusion proteins were all successfully expressed in the cells (Fig. 2A and B). Subsequently, the results of direct ELISA using the supernatants as primary antibodies showed that the eight fusion proteins against ASFV-p72 and ten against ASFV-CD2v were all expressed and secreted (Fig. 2C and D). The results also indicated that the fusion proteins still could bind to antigens and had HRP bioactivity.

To analyze the binding of the fusion proteins to ASFV-infected PAM cells, the results of IFA showed that four nanobody-HRP fusion proteins against ASFV-p72 (ASFV-p72-Nb2-HRP, -Nb21-HRP, -Nb37-HRP, and -Nb64-HRP) and two nanobody-HRP fusion proteins against ASFV-CD2v (ASFV-CD2v-Nb22-HRP, and -Nb67-HRP) could bind to the ASFV-p72 and- CD2v proteins in the ASFV-infected PAM cells (Fig. 2E and F).

3.5. Development of the two cELISAs for the detection of anti-ASFV antibodies

To select the best nanobodies for developing the two cELISAs, blocking ELISA was performed and the results showed that the blocking rates of ASFV-p72-Nb2-HRP and ASFV-CD2v-Nb22-HRP fusion proteins as the blocked antibodies were the highest, respectively (Fig. 3A and B). Thus, the two fusion proteins were separately selected for developing the p72-cELISA and CD2v-cELISA.

According to the results of the checkerboard titration, the optimal amount of ASFV-p72 for the p72-cELISA was 160 ng/well and the optimal dilution of ASFV-p72-Nb2-HRP was 1:1000. For the CD2v-cELISA, the optimized quantity of ASFV-CD2v was 80 ng/well and the dilution of ASFV-CD2v-Nb22-HRP was also 1:1000 (Table 2).

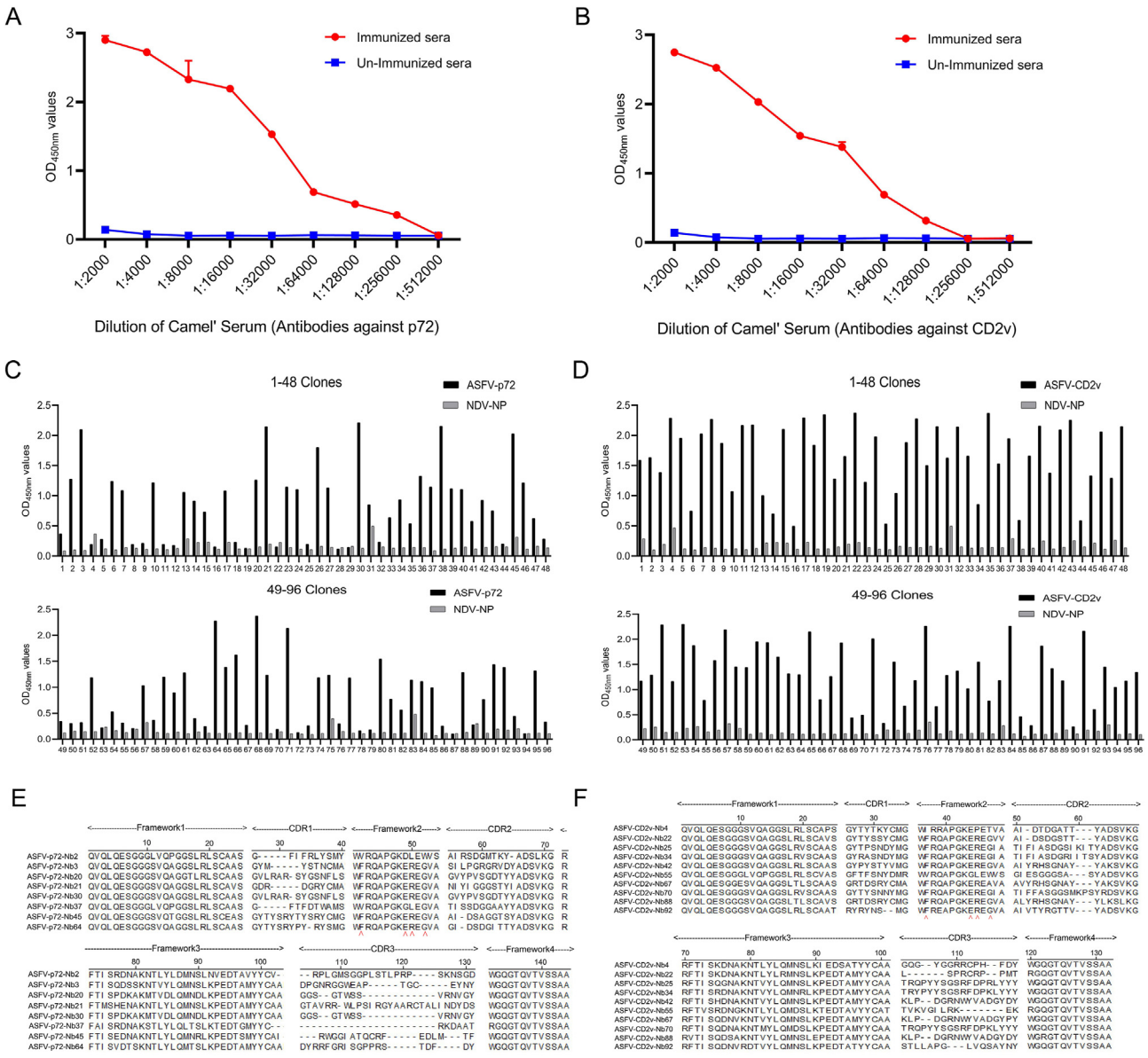

Fig. 1. Screening and identification of nanobodies against the ASFV-p72 and -CD2v proteins. **A** Titration of the sera from the immunized camel against the recombinant ASFV-p72 protein. Data are presented as means ± SD. **B** Titration of the sera from the immunized camel against the recombinant ASFV-CD2v protein. **C** Indirect ELISA using periplasmic extracts from the 96 clones to identify specific binding to ASFV-p72. A total of 56 clones were positive. **D** Indirect ELISA using periplasmic extracts from the 96 clones to identify specific binding to ASFV-CD2v. All the 96 clones were positive. NDV-NP was used as the negative control and expressed using the same system as the two proteins. **E** Amino acid alignment of the eight nanobodies against the ASFV-p72 protein. **F** Amino acid alignment of the ten nanobodies against the ASFV-CD2v protein. The hydrophilic amino acids residues at positions 37, 44, 45, and 47 are indicated by red arrows.

Table 1
Enrichment of specific phage particles against ASFV-p72 and -CD2v during three rounds of panning.

Antigen	Round of panning	Input phage (PFU/well)	P output phage (PFU/well)	N output phage (PFU/well)	Recovery (P/input)	P/N
ASFV-p72	1st round	5×10^{10}	3.6×10^{4}	1×10^{3}	7.2×10^{-7}	36
	2nd round	5×10^{10}	8.5×10^{5}	6.1×10^{3}	1.7×10^{-5}	140
	3rd round	5×10^{10}	2.5×10^{6}	1.6×10^{3}	5.0×10^{-5}	1.6×10^{3}
ASFV-CD2v	1st round	5×10^{10}	4.3×10^{5}	2.1×10^{3}	8.6×10^{-6}	205
	2nd round	5×10^{10}	9.5×10^{7}	5.5×10^{4}	1.9×10^{-3}	1.73×10^{3}
	3rd round	5×10^{10}	3.3×10^{7}	1.7×10^{3}	6.6×10^{-4}	1.94×10^{4}

Input: The number of phage particles added to each well; P output: The number of phage particles eluted from positive well; N output: The number of phage particles eluted from negative well.

For the p72-cELISA, the optimal dilution of testing pig sera was 1:40 (Table 3) and the mixtures and the TMB incubation times were 50 min and 10 min, respectively (Table 4). For the CD2v-cELISA, the optimized dilution of testing pig sera was 1:20 (Table 3) and the mixtures and the TMB incubation times were 40 min and 10 min, respectively (Table 4).

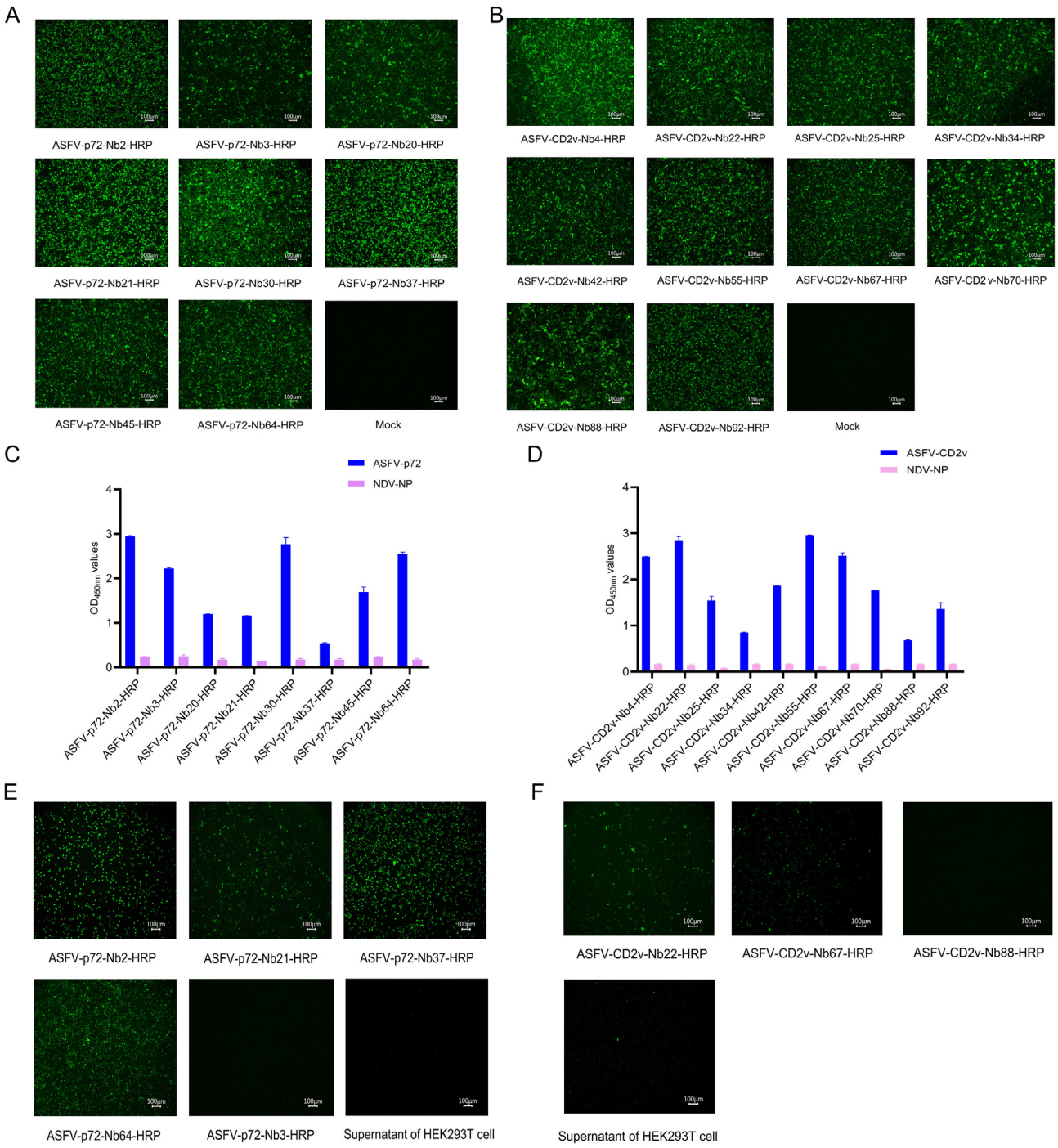

Fig. 2. Expression of the nanobody-HRP fusion proteins in HEK293T cells. **A** Identification of the eight nanobody-HRP fusion proteins against the ASFV-p72 protein expressed in HEK293T cells using indirect immunofluorescence assay (IFA). **B** Identification of the ten nanobody-HRP fusion proteins against the ASFV-CD2v protein expressed in HEK293T cells using IFA. **C** Direct ELISA to identify the specific reactions between the eight nanobody-HRP fusion proteins with ASFV-p72. **D** Direct ELISA to identify the specific reactions between the ten nanobody-HRP fusion proteins with ASFV-CD2v. Data are presented as means ± SD. **E** Identification of the eight nanobody-HRP fusion proteins against the ASFV-p72 protein binding to the ASFV-infected PAM cells by IFA. A representative image of ASFV-p72-Nb3-HRP is shown here, which is the same as the other three nanobody-HRP fusion proteins that do not target the ASFV-p72 protein. **F** Identification of the 10 nanobody-HRP fusion proteins against ASFV-CD2v protein binding to the ASFV-infected PAM cells by IFA. A representative image of ASFV-CD2v-Nb88-HRP is shown here, which is the same as the other seven nanobody-HRP fusion proteins that do not target the ASFV-CD2v protein. Scalar bar, 100 μm.

3.6. Cut-off values of the two cELISAs

To determine the cut-off values of each cELISA, a total of 638 negative pig sera were evaluated using the p72-cELISA and CD2v-cELISA, respectively. The results showed that the mean PI of these negative sera was 10.5% and 8.35% and the SD values were 5.1% and 4.7%, respectively. Thus, the cut-off value of p72-cELISA was 25.8% (10.5% + 3 × 5.1%) and the cut-off value of CD2v-cELISA was 22.45% (8.35% + 3 × 4.7%) (Fig. 4A and B). When the PI (%) values of testing pig sera were ≥25.8% using p72-cELISA and PI (%) values ≥22.45% using CD2v-cELISA, the samples were considered positive for the two assays, respectively. When the opposite was true, they were considered negative.

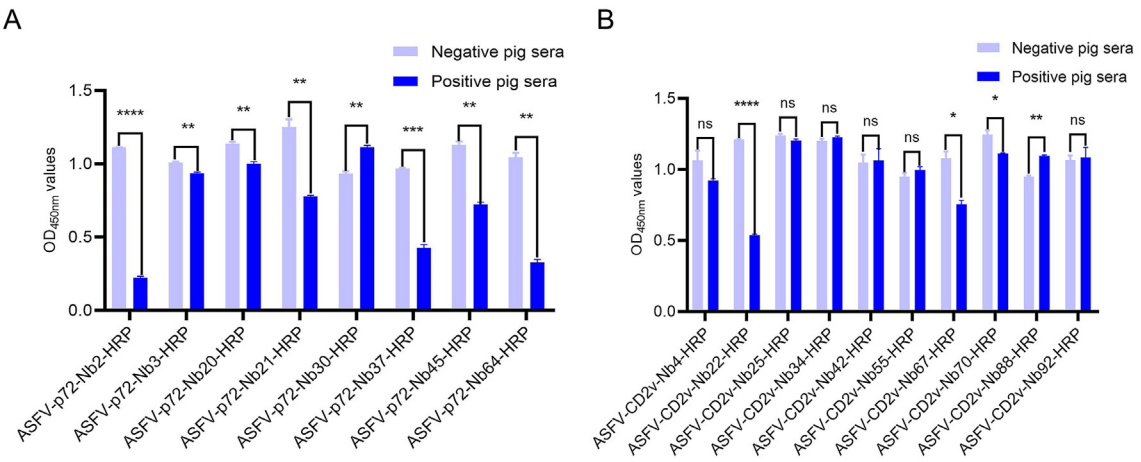

Fig. 3. Optimization of the best nanobody-HRP fusion proteins for separately developing p72-cELISA and CD2v-cELISA. **A** Analysis of the negative and positive pig sera for anti-ASFV antibody blocking the reaction between ASFV-p72 and the eight nanobody-HRP fusion proteins. **B** Analysis of the negative and positive pig sera blocking the reaction between ASFV-CD2v and the 10 nanobody-HRP fusion proteins. Data are presented as means ± SD. Statistical analyses were performed using a *t*-test with GraphPad Prism software. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant.

Table 2
Optimized amounts of ASFV-p72 and -CD2v proteins as coating antigens and dilutions of nanobody-HRP fusion proteins for the two cELISAs.

cELISA	Nanobody-HRP fusion proteins	Amounts of coated antigens (ng)	Dilutions of nanobody-HRP fusion proteins			
			10^{-1}	10^{-2}	10^{-3}	10^{-4}
p72-cELISA	ASFV-p72-Nb2-HRP	10	0.231	0.237	0.158	0.058
		20	0.758	0.562	0.185	0.059
		40	1.822	0.835	0.370	0.051
		80	1.556	1.628	0.768	0.185
		160	2.778	2.421	1.112	0.223
		320	1.908	2.443	1.585	0.303
CD2v-cELISA	ASFV-CD2v-Nb22-HRP	10	0.226	0.143	0.141	0.052
		20	0.342	0.451	0.125	0.053
		40	0.822	0.672	0.564	0.052
		80	1.662	1.448	0.981	0.072
		160	2.572	2.257	1.517	0.127
		320	3.108	2.846	1.854	0.157

Table 3
Optimized dilutions of tested pig sera for the p72-cELISA and CD2v-cELISA.

cELISA	Sera type	Dilutions of the pig serum samples							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
p72-cELISA	Highly positive	0.12	0.11	0.12	0.24	0.33	0.57	0.78	0.94
	Negative	0.78	0.93	1.06	1.06	1.02	1.09	1.02	0.97
	P/N	0.15	0.12	0.11	0.23	0.32	0.52	0.76	0.97
	Weakly positive	0.31	0.25	0.27	0.44	0.63	0.67	1.01	1.05
	Negative	0.93	1.02	1.03	1.02	0.97	1.01	1.14	1.05
	P/N	0.33	0.25	0.27	0.44	0.65	0.67	0.89	1.00
CD2v-cELISA	Highly positive	0.46	0.45	0.51	0.42	0.89	0.89	1.16	1.15
	Negative	1.12	1.25	1.07	1.19	1.41	1.15	1.22	1.10
	P/N	0.41	0.36	0.48	0.52	0.63	0.77	0.95	1.04
	Weakly positive	0.55	0.46	0.61	0.77	0.75	1.04	1.15	1.07
	Negative	1.071	1.24	1.23	1.02	1.10	1.05	1.23	1.09
	P/N	0.51	0.37	0.50	0.75	0.75	0.99	0.93	0.98

3.7. Sensitivity, specificity, reproducibility, and stability of the two cELISAs

To determine the sensitivity of those two cELISA methods, 24 pig sera positive for anti-ASFV antibodies and 24 negative samples (Group 2) were serially diluted (1:10 to 1:5120) and tested by the two cELISAs. The results showed that the detection limit of p72-cELISA was approximately 1:320 and for the CD2v-cELISA, it was about 1:160 (Fig. 4A and B).

To evaluate the specificity of the two cELISAs, 122 pig sera positive for antibodies against other swine viruses (Group 3) were tested with the two cELISA. The results showed that all the sera were negative for ASFV (Fig. 4C and D), and both p72-cELISA and CD2v-cELISA showed high specificity. Additionally, 23 sera from wild-type ASFV-infected pigs and 36 sera positive for anti-CD2v-deleted ASFV antibodies were tested with p72-cELISA and CD2v-cELISA. The results showed that all the pig sera were positive in the p72-cELISA, but only 23 sera samples from wild-type ASFV-infected pigs were positive using the CD2v-cELISA. These results suggested that the combination of p72-cELISA and CD2v-cELISA could be used to differentiate antibodies against wild-type and CD2v-deleted ASFV (Fig. 4E and F).

Table 4
Optimized incubation times of the mixtures including nanobody-HRP fusion proteins and tested pig sera with the coated antigens and color reaction times for the p72-cELISA and CD2v-cELISA.

cELISA	Times (min) of color reaction	Sera type	Incubation times (min) of tested pig sera, nanobody-HRP fusion proteins and coated antigens					
			10	20	30	40	50	60
p72-cELISA	10	Positive	0.88	0.81	0.58	0.34	0.26	0.31
		Negative	1.09	1.11	1.07	0.95	1.00	1.07
		P/N	0.81	0.73	0.54	0.36	0.26	0.31
	15	Positive	1.05	0.64	0.52	0.37	0.30	0.41
		Negative	1.10	1.05	0.93	1.02	0.97	1.10
		P/N	0.95	0.61	0.56	0.36	0.31	0.37
CD2v-cELISA	10	Positive	0.93	0.81	0.46	0.44	0.53	0.55
		Negative	1.01	1.06	0.97	1.05	0.96	1.10
		P/N	0.92	0.76	0.47	0.42	0.55	0.50
	15	Positive	0.95	0.83	0.71	0.58	0.43	0.62
		Negative	0.97	1.07	0.96	1.02	0.91	1.10
		P/N	0.98	0.78	0.74	0.57	0.47	0.56

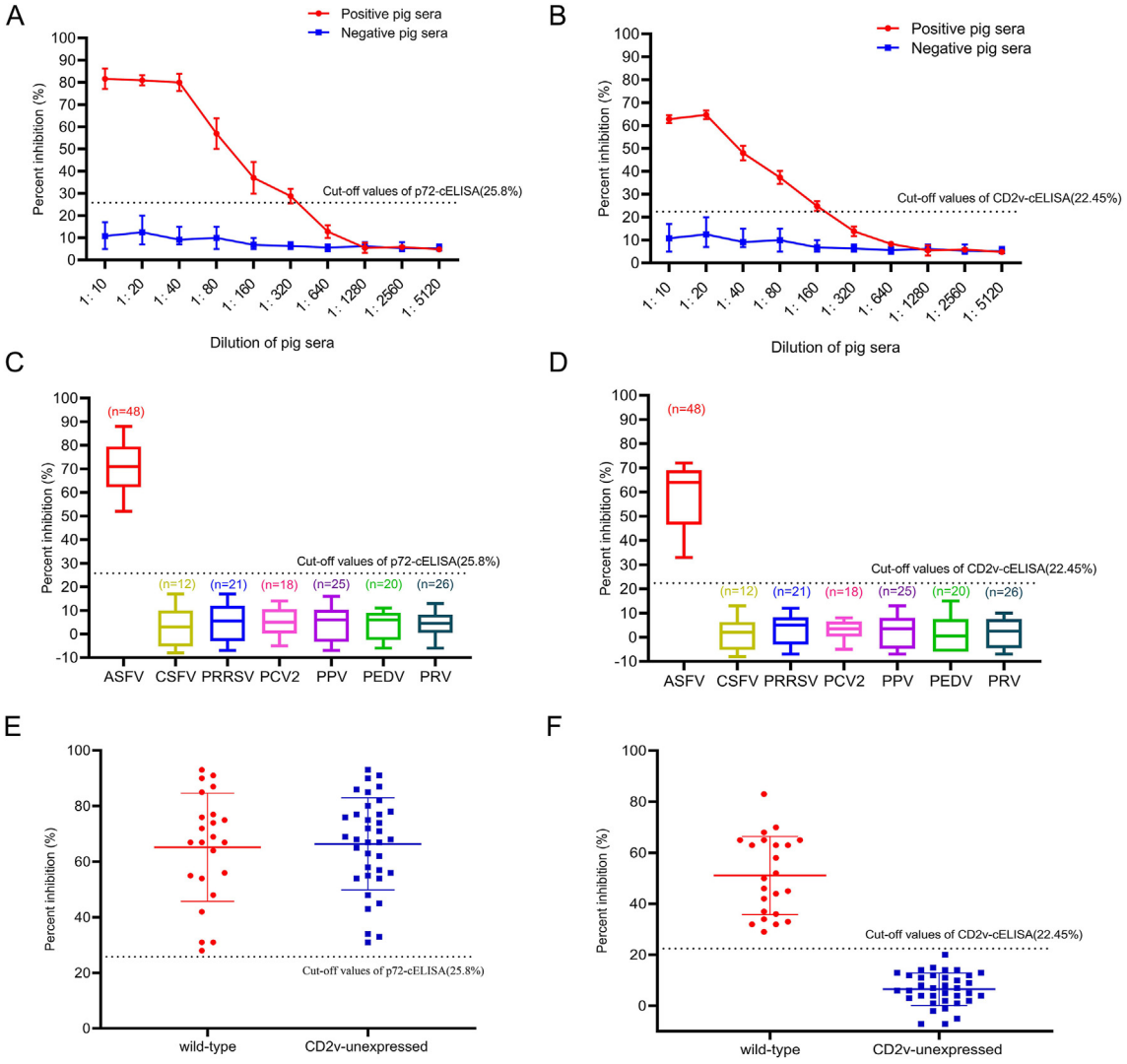

Fig. 4. Sensitivity and specificity analysis of the two developed cELISAs. **A** Determination of the limit of detection of the p72-cELISA. Data are presented as means ± SD. **B** Determination of the limit of detection of the CD2v-cELISA. **C** Analysis of positive samples for antibodies against other pig pathogens by p72-cELISA. **D** Analysis of the positive samples for antibodies against other pathogens by CD2v-cELISA. **E** Detection of the 23 positive sera for anti-wild-type ASFV (red) antibodies and 36 positive sera for anti-CD2v-deleted ASFV (blue) antibodies by p72-cELISA. **F** Detection of the 23 positive sera for anti-wild-type ASFV (red) antibodies and 36 positive sera for anti-CD2v-deleted ASFV (blue) antibodies by CD2v-cELISA.

Assessing the reproducibility of the p72-cELISA, the results showed that the intra-assay CV of the PI ranged from 1.42% to 5.58% and the inter-assay CV of the PI ranged from 1.71% to 7.35% (Table 5). For the CD2v-cELISA, the intra-assay CV of the PI ranged from 1.55% to 6.23% and the inter-assay CV of the PI ranged from 1.47% to 7.52% (Table 5). The above results indicated that the two cELISAs had good repeatability.

Table 5
Reproducibility of the two cELISAs determined by CV% value of intra- and inter-assay.

cELISA	Intra- or Inter-assay	CV % value range of six serum simples	Median value
p72-cELISA	Intra-assay	1.42–5.58	3.50
	Inter-assay	1.71–7.35	4.53
CD2v-cELISA	Intra-assay	1.55–6.23	3.89
	Inter-assay	1.47–7.52	4.50

Assessing the stability of the p72-cELISA and CD2v-cELISA, the results of direct ELISA showed that the OD_{450nm} values were still approximately 1.0 (CV = 7.36% and 2.17%) when the plates and supernatants were stored at 4 °C for 180 days. The results of the two cELISAs also showed that the competitive rates did not significantly decrease after 180 days (CV = 7.70% and 7.44%) (Fig. 5A and B).

3.8. Agreement between the two developed cELISAs and commercial ELISA kits

To evaluate the agreement between the developed cELISAs and commercial kits, 208 pig sera (85 serum samples collected from recovery pigs infected by a wild-type virulent ASFV isolate and 123 samples from pigs infected with the *EP402R* and *MGF360*-deleted ASFV vaccine strain) were tested using p72-cELISA, CD2v-cELISA, and two commercial ELISA kits, respectively. For the p72-cELISA, the results showed that 205 were positive. For the commercial p72 monoclonal antibody-based ELISA kit (INgezim® PPA Compac, Eurofins INGENASA, Spain), 208 were positive. Three pig sera that provided inconsistent results were assessed using Western blotting and the results showed they were positive (Data not shown). The coincidence rate between the two assays was 98.6% (κ = 0.93) (Table 6). The results of the commercial CD2v monoclonal antibody-based ELISA kit (Biovet Biotechnology Compac, Beijing, China) showed 83 were positive and 125 were negative; the results of the CD2v-cELISA developed in the present study showed that 78 of 83 pig sera were positive and another 130 were negative. The 5 pig sera that provided inconsistent results were also assessed using Western blotting and the results showed they were positive (data not shown). The coincidence rate between the two assays was 97.6% (κ = 0.95) (Table 6).

4. Discussion

ASFV infection was reported in China in 2018 and spread rapidly throughout the country. The disease caused severe economic losses to the pig industry and the early ASFV isolate was characterized as a highly virulent genotype II strain (Ge et al., 2018). The viral DNA can be detected during the early stages of infection in mouth and nose swabs, fecal swabs, and blood samples from pigs infected by the highly virulent ASFV isolates (Gallardo et al., 2019; Zhu et al., 2019). However, certain less virulent ASFV strains were isolated in China in 2020 (Sun et al., 2021). These less virulent strains usually showed a non-hemadsorbing (non-HAD) phenotype and the deletion of the *EP402R* gene. Additionally, compared to the highly virulent ASFV strain, it is harder to detect the less virulent or *EP402R*-deleted ASFV isolates from oral, rectal, and blood samples, which results in a very complicated situation of ASFV infection in pig farms (King et al., 2003; Sun et al., 2021). Thus, ELISAs to detect anti-ASFV antibodies are important to monitor ASFV infection in pig farms. ELISA can differentiate antibodies against wild-type and CD2v-deleted ASFV (Lv et al., 2021).

In this study, two ASFV-p72 and -CD2v nanobody-based cELISAs were developed to detect anti-ASFV antibodies. These two cELISAs also showed ease of operation, low cost, and a simple production process. Importantly, the combination of the two cELISAs could differentiate pigs infected with wild-type and CD2v-deleted ASFV. All wild-type and CD2v-deleted ASFV strains contain p72 proteins, allowing the p72-cELISA to detect all ASFV antibodies in the pig sera. However, the CD2v proteins are only expressed in the wild-type ASFV, limiting the CD2v-cELISA to detect wild-type ASFV antibodies but not anti-CD2v-deleted ASFV antibodies. Recently, some partial CD2v-deleted ASFV strains were detected in pig farms in China. Due to limited resources, the ability of the developed CD2v-cELISA to detect antibodies against the partial CD2v-deleted ASFV strain was not evaluated in this study. In future studies, the

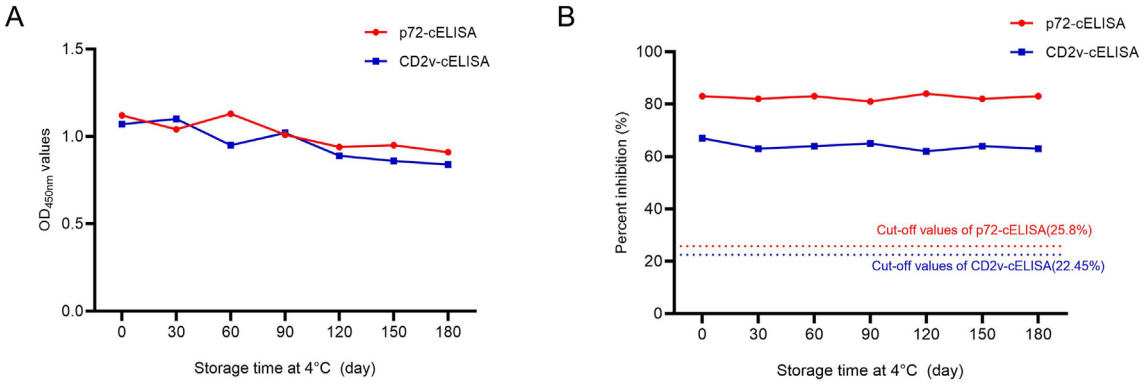

Fig. 5. Stability analysis of the two developed cELISAs stored at 4 °C for 180 days. **A** Binding analysis of the nanobody-HRP fusion proteins to ASFV-p72 or ASFV-CD2v by direct ELISAs. **B** Analysis of the positive pig sera blocking the nanobody-HRP fusion protein to react with the ASFV-p72 and ASFV-CD2v proteins by cELISAs. The nanobody fusion proteins and coated plates were stored at 4 °C for 180 days.

Table 6
Comparisons of the developed two cELISAs with commercial ELISA kits.

cELISA	Positive (+) or negative (−)	Number	Commercial ELISA kits (p72/CD2v)		Agreement (%)	Kappa value
			+	−		
p72-cELISA	+	205	205	0	98.6%	0.93
	−	3	3	0		
CD2v-cELISA	+	78	78	0	97.6%	0.95
	−	130	5	125		

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

referenced positive pig sera from pigs infected with the partial CD2V-deleted ASFV strain will be collected and assessed using the CD2v-cELISA.

At present, certain gene-deleted ASFV strains as live attenuated vaccines have shown significant prospects (Chen et al., 2020; Liu et al., 2023). For example, the 7 and 5 gene-deleted ASFV strains have been developed by researchers and have been proven to provide reliable protection against the parental ASFV strain (Chen et al., 2020; Liu et al., 2023). Interestingly, most of these live attenuated ASFV vaccine candidate strains have the *EP402R* gene deleted. Therefore, if these vaccines are commercialized in the future, the two cELISAs may also be used to effectively distinguish between wild-type ASFV infection and *EP402R* gene-deleted ASFV immunization. However, due to limited resources, only the *EP402R* gene-deleted attenuated vaccine candidate strain was evaluated in the present study.

As the primary component of the viral icosahedral capsid, the p72 protein encoded by the *B646L* gene accounts for ~32% of all of the virion's proteins (Liu et al., 2019). Previous studies showed that the p72 protein is one of the most immunogenic proteins and can induce high levels of specific antibodies after ASFV-infected pigs. Thus, the p72 protein is universally used as the coating antigen to develop ELISAs for the detection of anti-ASFV antibodies in pig sera (Freije et al., 1993; Sastre et al., 2016; Heimerman et al., 2018). Additionally, there are 24 known genotypes of ASFV based on genetic differences of the p72 protein. The strain reported in China was identified as genotype II (Jolaoluwa et al., 2021). In the present study, the two cELISAs were only used to test pig sera from pig farms in China. Therefore, it is uncertain whether antibodies against other genotypes of ASFV can be detected using the two cELISAs.

The developed p72-cELISA exhibited lower sensitivity for detecting anti-ASFV antibodies in challenged pig sera compared to the developed p30-cELISA, which was based on the expression of p30 in the bacterial system and nanobody-HRP fusions as previously reported (Zhao J. et al., 2022). The seropositivity was first observed at 7 dpi in challenged pigs using the p30-cELISA, while with the p72-cELISA, it was observed at 9 dpi. Additionally, sensitivity analysis results indicated low percent competitive inhibition for detecting highly positive serum in both assays. This may be attributed to the single-domain nature of nanobodies compared to traditional antibodies. In the future, different anti-ASFV-nanobodies can be fused and expressed the poly-nanobody, and then the sensitivity and the percent competitive inhibition of the cELISA may be improved using the poly-nanobody as reagents., The nanobody-HRP fusion protein designed in the present study had His and HA tags, facilitating easy purification from the HEK293T supernatant. Furthermore, establishing recombinant HEK293T cell lines with stable expression of nanobody-HRP fusion proteins in the future will eliminate the complexity and variability of transfection, ensuring reproducibility from batch to batch. Moreover, the titers of nanobody-HRP fusion proteins from the recombinant HEK293T cell lines will surpass those from a transient transfection system.

Previous studies reported that the complete glycosylated CD2v protein was approximately 89 kDa in ASFV-infected cells (Goatley and Dixon, 2011). However, when the complete *EP402R* gene encoding CD2v protein was cloned into the pET-28a vector, the CD2v protein was not expressed. Subsequently, the gene encoding the transmembrane region of CD2v was deleted, and the truncated ASFV-CD2v protein was successfully expressed. Furthermore, the sensitivity of the CD2v-cELISA for detecting anti-ASFV antibodies in pig sera was lower than that of the p72-cELISA and CD2v monoclonal antibody-based ELISA. This may be due to the ASFV-CD2v expressed with the prokaryotic system in the study lacking important posttranslational modifications of the antigenic epitope (Jia et al., 2022). Therefore, in the future, full-length ASFV-CD2v will be expressed using the eukaryotic system, potentially improving the sensitivity of cELISA using the complete ASFV-CD2v protein as the coating antigen.

5. Conclusions

In the present study, eight nanobodies against ASFV-p72 and ten against ASFV-CD2v were successfully screened. Subsequently, using ASFV-p72-Nb2-HRP and ASFV-CD2v-Nb22-HRP as probes, p72-cELISA and CD2v-cELISA were successfully developed for detecting anti-ASFV antibodies in the pig sera. Importantly, the combinations of two cELISAs could differentiate the pigs infected by wild-type and CD2v-deleted ASFVs. The two cELISAs offer a cost-effective solution with shorter detection time than commercial ELISA kits, and thus provide a valuable tool for monitoring ASFV infection in pig farms.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

Animal experiments (Camel immunization) 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 animal experimental procedures were carried out in accordance with 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 (approval no. 20220036/03).

All the positive serum samples for anti-ASFV antibodies were from the clinical pigs infected by different ASFV strains, confirmed by the commercial ELISA kits kindly provided by the New Hope Liuhe Co., Ltd. The clinical pigs infected by the ASFV were further confirmed by real-time RT-PCR.

Author contributions

Jiahong Zhu, Qiangyuan Liu: investigation, data curation, writing-original draft. Liuya Li, Runyu Zhang, Yueting Chang, Jiakai Zhao: investigation, validation. Siyu Liu: investigation. Xinyu Zhao: data curation. Xu Chen: data curation. 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.04.002.

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