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METHODS AND PROTOCOLS

HRP-conjugated-nanobody-based cELISA for rapid and sensitive clinical detection of ASFV antibodies

Huijun Zhao[1,2] · Jiahui Ren[1,2] · Shuya Wu[1,2] · Haoran Guo[1] · Yongkun Du[1,2] · Bo Wan[1,2] · Pengchao Ji[1,2] · Yanan Wu[1,2] · Guoqing Zhuang[1,2] · Angke Zhang[1,2] · Gaiping Zhang[1,2,3]

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Abstract

African swine fever (ASF), which is caused by the ASF virus (ASFV), is a highly contagious hemorrhagic disease that causes high mortality to domestic porcine and wild boars and brings huge economic losses to world swine industry. Due to the lack of an effective vaccine, the control of ASF must depend on early, efficient, and cost-effective detection and strict control and elimination strategies. Traditional serological testing methods are generally associated with high testing costs, complex operations, and high technical requirements. As a promising alternative diagnostic tool to traditional antibodies, nanobodies (Nb) have the advantages of simpler and faster generation, good stability and solubility, and high affinity and specificity, although the system is dependent on the immunization of Bactrian camels to obtain the specific VHH library of the target protein. The application of Nbs in the detection of ASFV antibodies has not yet been reported yet. Using a phage display technology, one Nb against the ASFV p54 protein that exhibited high specificity and affinity, Nb8, was successfully screened. A HEK293T cell line stably expressing Nb8-horseradish peroxidase (HRP) fusion protein was established using the lentiviral expression system. Following the optimization of the reaction conditions, the Nb8-HRP fusion protein was successfully used to establish a competitive enzyme-linked immunosorbent assay (cELISA) to detect ASFV-specific antibodies in pig serum, for the first time. There was no cross-reaction with healthy pig serum, porcine pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine epidemic diarrhea virus (PEDV), and classical swine fever virus (CSFV) positive sera. The optimal cut-off value for the cELISA by ROC analysis was 52.5%. A total of 209 serum samples were tested using the developed cELISA and a commercial ELISA kit. The results showed that the relative specificity of the cELISA was 98.97%, and the relative sensitivity of the cELISA was 93.3%, with the percent agreement between the two ELISA methods being 98.56%. In conclusion, a specific, sensitive, and repeatable cELISA was successfully developed based on the Nb8 as a probe, providing a promising method for the detection of anti-ASFV antibodies in clinical pig serum.

Key points

- *We successfully screened a specific, high affinity nanobody against ASFV p54 protein.*
- *We establish a method for continuous and stable expression of Nb-HRP fusion protein using a lentiviral packaging system.*
- *We establish a nanobody cELISA detection method that can monitor an ASF infection.*

Keywords Nanobody · cELISA · African swine fever virus · Serum antibody

Huijun Zhao and Jiahui Ren contributed equally to this work.

✉ Angke Zhang
zhangangke1112@126.com

✉ Gaiping Zhang
zhanggaiping2003@163.com

[1] College of Veterinary Medicine, Henan Agricultural University, Zhengzhou 450046, Henan, China

[2] International Joint Research Center of National Animal Immunology, College of Veterinary Medicine, Henan Agricultural University, Zhengzhou 450046, Henan, China

[3] Key Laboratory of Animal Immunology of the Ministry of Agriculture, Henan Academy of Agricultural Sciences, Zhengzhou 450002, Henan, China

Introduction

African swine fever (ASF), which is caused by ASF virus (ASFV), is a highly contagious hemorrhagic viral disease afflicting domestic pigs and wild boars. The infection of ASFV usually results in a high mortality in pigs, rendering ASF the most significant threat to the global pig industry. In fact, ASF has been classified as a notifiable disease by the World Organization for Animal Health (OIE) (Dixon et al. 2013; Sanchez-Vizcaino et al. 2012). Since the first reported outbreak in China in August 2018, ASF has been detected in more than 16 other countries in Asia and has resulted in the death or culling of > 1,192,000 pigs, with losses accounting for > 10% of the total pig population in China, Mongolia, Vietnam, and other 16 countries (OIE-WAHIS). The most recent outbreak of ASF in China and Southeast Asia, along with the infection of wild boar in Belgium and Germany, has created a sense of urgency for the development and use of cost-effective approaches that can prevent the entry of ASFV into countries and areas where the virus has not spread.

ASFV is an enveloped, icosahedral virus that contains a double-stranded DNA genome with a length of 170–194 kb (Alonso et al. 2018; Dixon et al. 2013); it is the only member of the Asfarviridae family and *Asfivirus* genus. Thus far, > 150 unique proteins have been identified from ASFV-infected pig macrophage tissue culture, of which ≥ 50 have been found to react with serum from pigs that have recovered from ASF (Dixon et al. 2004). In the acute form of the ASFV infection, particularly in naive populations, death usually occurs prior to the seroconversion to a detectable level (Blome et al. 2013; Mur et al. 2016). However, in enzootic areas affected by ASF, particularly sub-acute infections, surviving animals may maintain a detectable level of antibodies post-infection and serve as carriers of the virus (de Carvalho Ferreira et al. 2013; Penrith et al. 2009). Since there is currently no commercially available vaccine for ASF, the presence of antibodies in the serum is a definitive indicator of infection, and their detection is critical for the control of viruses in infected herds, as well as for surveillance to track the absence of infection.

The effective control of ASF is based on early diagnosis and the enforcement of strict sanitary measures. Molecular diagnostic technologies, including polymerase chain reaction (PCR) or quantitative PCR (qPCR), are very effective in the early diagnosis of ASF (Aguero et al. 2003; Basto et al. 2006; Wang et al. 2020a, b, c). However, despite PCR and qPCR being the commonly used methods for ASFV detection in the laboratory, they require thermal cycling instruments and skilled operators, which is not ideal for resource-limited situations. In addition, other molecular diagnostic methods, including the invader assay (Hjertner et al. 2005), loop-mediated isothermal amplification (James et al. 2010; Wang et al. 2020a, b, c), recombinase polymerase amplification (Wang et al. 2017), and methods of detecting ASFV antigens based on the CRISPR system (Tao et al. 2020; Wang et al. 2020a, b, c), have been developed. Although these methods exhibit high sensitivity and specificity, the majority of them are laborious and costly, which are limitations that seriously hinder their clinical application.

Field investigations of ASF outbreaks need to couple serologic and PCR testing, suggesting that serological diagnostic in ASF diagnosis and control is very important. At present, the routine, OIE-approved, diagnostic method for ASF is enzyme-linked immunosorbent assay (ELISA) after preliminary screening, followed by western blotting (Pastor et al. 1989, 1990). The viral antigens in the OIE-approved detection methods are derived from live viruses, a process that requires a level 3 biosafety laboratory (Arabyan et al. 2019; Gallardo et al. 2015). In addition, numerous ELISA-based serological tests using the structural and highly immunogenic protein p30, the major capsid protein p72, and other antigens that can induce higher levels of antibodies can be used for the detection of ASFV antibodies as early as 7 days after infection (dpi) (Cubillos et al. 2013; Gallardo et al. 2009; Perez-Filgueira et al. 2006). In addition, the structural and immunogenic p54 protein is used for serological diagnosis, since anti-p54 antibodies have been shown to appear as early as 8 dpi and persist for several weeks (Alcaraz et al. 1995; Perez-Filgueira et al. 2006). There are few commercial ASFV ELISAs exhibiting good diagnostic performance, such as some commercial indirect ELISA diagnostic kit using p72 or p30 protein as antigen (Carlson et al. 2018; Freije et al. 1993; Luis et al. 2016; Sastre et al. 2016a, b). However, limitations in sensitivity, specificity, simplicity, and expenditure continue to restrict the use of traditional ELISA for research and clinical purposes.

Monoclonal antibodies (mAbs) comprise the largest and most widely used type of diagnostic antibodies in the serological diagnosis of viral diseases. Nevertheless, their clinical application is hampered by the time-consuming and costly process of antibody manufacturing using eukaryotic systems. A well-known reason for this is the fact that the large-scale production of mAbs typically takes > 3–6 months, making timely production difficult in an epidemic setting, such as that of ASF.

One of the most promising alternatives to mAbs is single-domain antibodies, also known as heavy-chain variable domains (VHH) or nanobodies (Nbs), which are produced in camels. Unlike ordinary IgG antibodies, Nbs have a small molecular weight (~ 15 kDa) and are highly soluble and stable. They are also readily bioengineered into bi-/multivalent forms, have relatively low production costs, and can be

rapidly and efficiently produced in prokaryotic expression systems (Vincke et al. 2012). Nbs might be better suited to access hidden targets and cryptic sites than normal antibodies (Zhu et al. 2014). In addition, Nbs bind to their targets with high affinity and specificity, due to having an extended antigen-binding region (Muyldermans 2013; Steeland et al. 2016). Based on their advantages over conventional antibodies, Nbs are promising candidates for various biomedical applications, such as disease diagnosis and treatment (Salvador et al. 2019; Wu et al. 2020; Xiang et al. 2020; Zhao et al. 2018). Furthermore, certain studies have shown that Nbs are superior to traditional antibodies in the development of new viral antigen or antibody detection methods, such as the Nb-based rapid single-molecule detection of coronavirus disease 2019 and Middle East respiratory syndrome antigens (Guo et al. 2021).

Thus far, to the best of our knowledge, no specific Nbs against ASFV structural or non-structural proteins have been reported. Considering the broad application prospects of Nbs in pathogen detection, a novel Nb against p54 protein, Nb8, was generated in the present study, through the immunization of a Bactrian camel with recombinant ASFV p54 protein and phage display technology. Nb8 was then conjugated with horseradish peroxidase (HRP) to create the Nb8-HRP recombinant protein in HEK293T$^{Nb8-HRP}$ cell line, which was further used as a probe to establish the cELISA for the detection of ASFV antibodies in inactivated pig serum samples (Fig. 1). The results showed that the established cELISA exhibited high specificity and repeatability. It also exhibited comparable sensitivity to the commercial ELISA kit; however, the time-consuming was less, thus demonstrating a promising application potential in future clinical pig serum detection.

Materials and methods

Serum samples

To establish the cELISA, a total of 216 ASFV-positive serums were collected from pigs with obvious clinical syndrome from 3 different farms of Henan province (93 samples), Hubei province (58 samples), and Anhui province (65 samples), and the serum samples were inactivated immediately at 60 °C for 30 min after each collection. According to the time of infection, 35 serums were collected at 7-day post-infection (dpi), 47 serums at 10 dpi, 38 serums at 13 dpi, 53 serums at 16 dpi, and 43 serums at 23 dpi. The 295 ASFV-negative serums were collected from swine diseases decontamination pig farm and 216 inactivated ASFV-positive serums were used to determine the cut-off value of the developed cELISA.

Expression and purification of recombinant ASFV p54 protein

To prepare the antigen used for camel immunization, a pET-30 prokaryotic expression system was used to express recombinant p54 protein of the ASFV China/2018/AnhuiXCGQ strain (GenBank accession no. AYW34096.1). Following the construction of the recombinant plasmid, pET-30-p54-His, the plasmids were sequenced, and the correct plasmids were transferred into Transetta (DE3) expression competent cells (TransGen Biotech, Beijing, China). A single clone was selected and treated with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 12 h. Following ultrasonication, the lysates were centrifuged at 12,000 × g for 30 min at 4 °C, and precipitates and supernatants were subjected to SDS-PAGE and western blot analysis, respectively.

The ASFV p54 protein was purified using cOmplete His-Tag Purification Resin (Roche, Basel, Switzerland). Before protein purification, the resin was equilibrated with 10 times column volumes of buffer A (50 mM NaH_2PO_4 pH = 8, 300 mM NaCl). Then the contaminated proteins were eluted with buffer A containing 20 mM imidazole, and the p54 protein was eluted with buffer A containing 250 mM imidazole. The eluted product was collected and analyzed by SDS-PAGE.

Immunization and construction of the Nb library

A Bactrian camel was first immunized with a mixture of 5 ml p54 protein (5 mg) and an equal volume of Freund's complete adjuvant (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA). For the subsequent immunizations, p54 protein was mixed with an equal volume of Freund's incomplete adjuvant (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA); immunizations were performed at 2-week intervals, four times. A week after the fourth immunization, 300 ml whole blood was collected, and peripheral blood lymphocytes (PBLs) were separated from 200 ml whole blood using Ficoll-Paque PLUS (Cytiva) with Leucosep™ tubes (Greiner Bio-One GmbH). The remaining 100 ml whole blood was used for serum isolation and immunization titer detection. All camel experiments were performed according to the guidelines approved by the Animal Care and Use Committee of Henan Agricultural University (Zhengzhou, China).

For library construction, total RNA from PBLs was extracted using a RNeasy® Plus Mini kit (Qiagen AB). cDNA was reverse transcribed using a SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.). By using the primers CALL001 and CALL002 (Table S1), an ~ 700-bp target band spanning the VHH-CH2 exons was cloned during the first round of PCR. The VHH encoding sequences (~ 400 bp) were amplified using the products

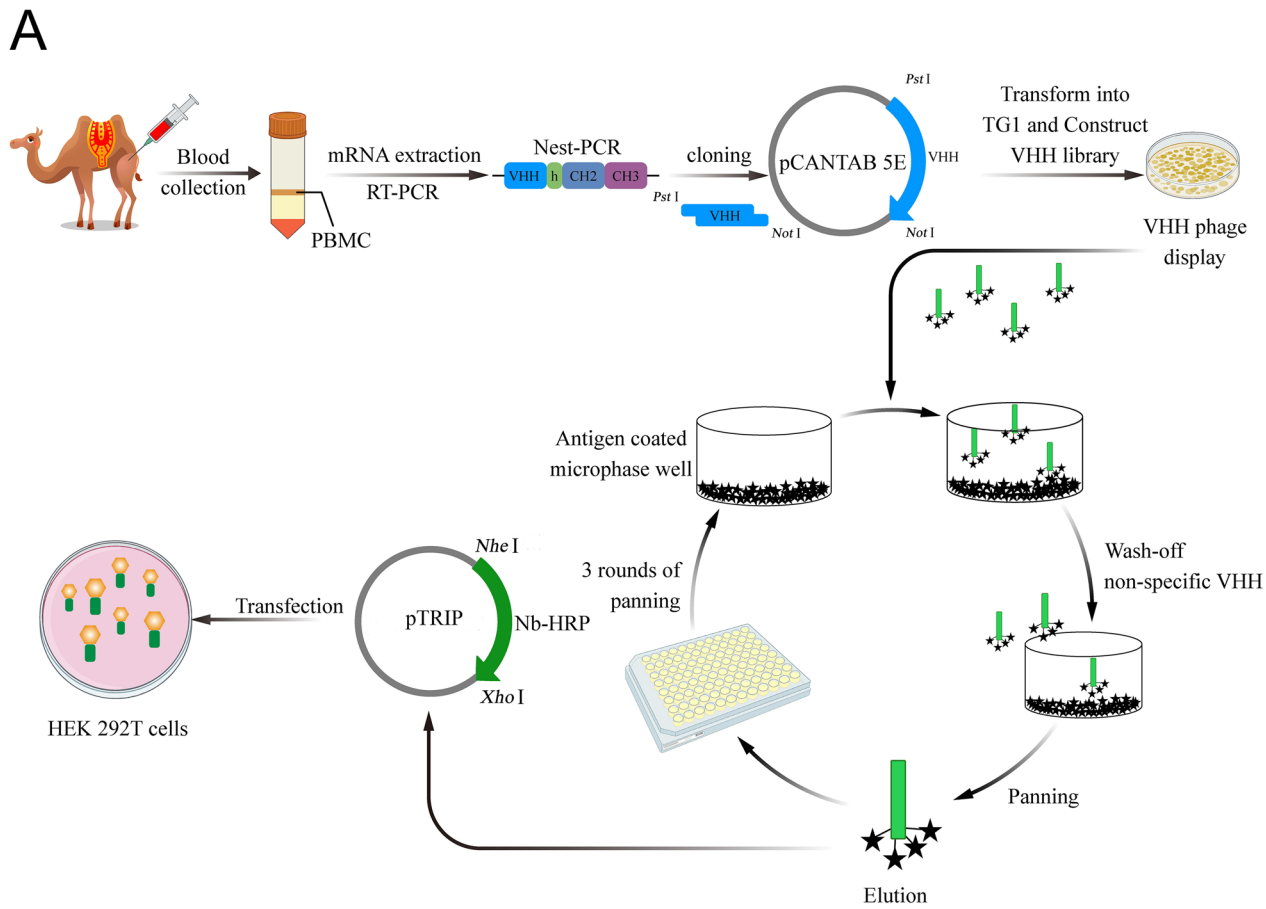

Fig. 1 Schematic representation of developing the cELISA to detect ASFV serum antibody. **A** Schematic representation of screening the nanobodies from the immunized Bactrian camel and the platform for expressing nanobody-HRP. **B** Detection of pig serum with nanobody-HRP fusions

from the first PCR as a template (primers: VHH-For and VHH-Rev, Table S1) and then purified using agarose gel electrophoresis. Following digestion with *Pst*I and *Not*I, the target segments were cloned into the phage display vector pCANTAB-5E (Cytiva) and then electro-transformed into freshly prepared *E. coli* TG1 competent cells. The transformation products were cultured in solid 2X YT medium containing 100 µg/ml ampicillin and 2% (w/v) glucose overnight at 37 °C. The colonies were scraped from the plates, placed into 3-ml liquid Luria–Bertani (LB) medium (Oxoid) supplemented with 20% (v/v) glycerol, and stored at − 80 °C. Following a gradient dilution, the capacity of the constructed library was detected by counting the number of colonies.

Library screening using phage display

The specific Nbs against ASFV p54 protein were screened via three consecutive rounds of bio-panning with the p54 protein. Briefly, ~ 1×10^{10} TG1 cells (Beyotime Biotech, Shanghai, China) from the library stock were recovered and cultured in 2X YT medium containing 100 µg/ml ampicillin and 2% (w/v) glucose for 2 h at 37 °C. Then, the TG1 cells were infected with M13K07 Helper Phage (New England Biolabs, lpswich, MA, USA) (1.8×10^{13} pfu/ml) and incubated at 37 °C for 1 h without shaking. Cells were collected using centrifugation at 3,000 × g for 10 min at room temperature, followed by resuspending into 2X YT medium supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin, which were cultured overnight at 37 °C at 220 rpm. The phages in the supernatant were precipitated using PEG 6000/NaCl for 3 h on ice, and were then centrifuged at 12,000 × g for 30 min at 4 °C and resuspended in sterile PBS. The phages were quantified using phage titration. For every round of bio-panning, ~ 5×10^{11} pfu/ml phages were incubated in ninety-six-well plates (Thermo Fisher Scientific, Inc.) coated with p54 protein (10 µg/well). The enrichment of specific phage particles was monitored using an anti-M13 HRP-conjugated antibody (Sino Biological, 1:2000) for ELISA and phage titration, as previously reported (Zhu et al. 2014). After three consecutive rounds of bio-panning, the enrichment of specific phage particles was calculated, and 96 individual colonies were randomly selected and treated with 1.0 mM IPTG. The positive clones expressing E-Tag p54-specific Nbs were identified using periplasmic extract ELISA (PE-ELISA) with an anti-E-Tag antibody (GenScript, 1:2000). If the absorbance in the antigen-coated well was > threefold higher than that of the well containing PBS, the colony was regarded as positive. The identified positive clones were then sequenced, and the amino acid sequences of Nbs were analyzed and classified into different groups, based on their sequence diversity in third complementarity-determining (CDR3) regions.

Specificity and affinity analysis of Nbs against p54 protein

For specificity analysis, ninety-six-well plates were coated with 400 ng/well of ASFV p54 protein, porcine reproductive and respiratory syndrome virus (PRRSV) N protein, porcine epidemic diarrhea virus (PEDV) N protein, and ASFV p30 protein at 4 °C overnight, respectively. Then the periplasmic PE-ELISA was performed using an anti-E-Tag antibody.

For affinity analysis, ninety-six-well plates coated with ASFV p54 protein (400 ng/well) were incubated at 4 °C overnight. After washing with 0.5% PBS'T (PBS with 0.5% Tween 20) for three times, the plates were incubated with serial dilutions of crude extract of Nbs (1:1, 1:10, 1:100, and 1:1000) for 1 h at 37℃. Then PE-ELISA was conducted with an anti-E-Tag antibody.

Production of Nb-HRP recombinant protein against p54 protein

HRP-conjugated recombinant Nb was prepared, according to previous reports with some modifications (Yamagata et al. 2018). Specific DNA sequences, including a signal sequence for protein secretion derived from the human Ig kappa chain, which promotes the extracellular secretion of fusion proteins, and a codon-optimized HRP gene sequence (Yamagata et al. 2018), were synthesized and fused with the Nb gene amplified from the recombinant pCANTAB-5E vector using overlap extension PCR. Following digestion with *Eco*RI and *Nhe*I, the fusion fragments were inserted into the multiple cloning sites of the pCAGGS-hemagglutinin (HA) eukaryotic expression vector, termed pCAGGS-Nb-HRP in the following experiment. Following sequencing, the recombinant plasmid was transfected into HEK293T cells using polyetherimide reagents (Polysciences Inc.). At 60 h following transfection, cells and supernatants containing the Nb-HRP recombinant protein were harvested and analyzed using western blotting or an indirect fluorescence assay (IFA) to determine the Nb-HRP recombinant protein expression. Supernatants were filtered using a 0.22-µm filter membrane for further use.

For western blotting, the HEK293T cells were harvested 60-h post-transfection for analysis using mouse anti-HA mAb (Beyotime Institute of Biotechnology, Shanghai, China; 1:1000) as the primary antibody and HRP-conjugated goat anti-mouse IgG (H&L) (Jackson ImmunoResearch Laboratories, Inc.; 1:2000) as the secondary antibody. For IFA, at 36-h post-transfection, the HEK293T cells were fixed with − 20 °C pre-cooled 70% alcohol and probed with mouse anti-HA mAb, followed by incubation with Alexa Fluor 594-conjugated goat anti-mouse IgG (H&L). The cells were then observed under a fluorescence microscope (Leica AF6000; Leica Microsystems GmbH, 200×). For

ELISA analysis of the Nb-HRP recombinant protein in the supernatant, 100 and 200 µl supernatant with or without plasmid transfection, respectively, was directly incubated in ninety-six-well ELISA plates at 4 °C overnight. Following washing with 0.5% PBS'T three times, 100 µl tetramethylbenzidine (TMB) was added to each well and incubated at 37 °C for 15 min in the dark. Next, 50 µl/well of 3 M H_2SO_4 was added to stop the reaction, and the absorbance of the plate was measured at OD450 nm using a spectrophotometer (PerkinElmer, Inc.).

Specificity analysis of Nb-HRP recombinant protein against p54 protein

For specificity analysis, direct ELISA developed by our laboratory was used. Ninety-six-well plates were coated with ASFV p54 protein, PRRSV N protein, PEDV N protein, and ASFV p30 protein at a density of 200 ng/well at 4 °C overnight. Following blocking with 2.5% dried milk at 37 °C for 1 h, supernatants collected from pCAGGS-Nb-HRP-transfected HEK293T cells were added (100 µl/well) and incubated at 37 °C for 1 h, followed by washing with 0.5% PBS'T three times. A total of 100 µl/well TMB was added and incubated at 37 °C for 15 min in the dark. Finally, 50 µl/well 3 M H_2SO_4 was added to stop the reaction and absorbance was measured at 450 nm (PerkinElmer, Inc.). The specificity of Nb-HRP against p54 protein was analyzed based on the OD450 value.

Analysis of the affinity of Nb-HRP recombinant protein against p54 protein

The kinetic characteristics of Nb-HRP in cell culture supernatants bound to recombinant p54 protein were determined using direct ELISA developed by our laboratory. Briefly, ninety-six-well plates coated with the purified recombinant p54 protein (200 ng/well) were incubated at 4 °C overnight. The plates were washed with 0.5% PBS'T three times and incubated with serial dilutions of Nb-HRP supernatants (1:5, 1:10, 1:20, 1:40, 1:80,1:160, 1:320, 1:640, and 1:1280) for 1 h at 37 °C. After washing three times with 0.5% PBS'T, 100 µl/well of TMB was added, followed by incubation at 37 °C for 15 min in the dark. The reaction was stopped by the addition of 50 µl/well of 3 M H_2SO_4 and absorbance was measured at OD450 nm. The affinity of Nb-HRP against p54 protein was analyzed based on the OD450 value.

Construction of HEK293T cell line stably expressing Nb-HRP recombinant protein

To establish HEK293T cells stable expression of Nb-HRP protein, Nb-HRP gene was cloned into the pTRIP to construct pTRIP-Nb-HRP recombinant expression vector. After sequencing, the recombinant plasmid (650 ng/well) was co-transfected into HEK293T cells with envelope plasmid pMD2.G (900 ng/well) and packaging plasmid psPAX2 (500 ng/well) using polyetherimide transfection reagents (Polysciences Inc.). At 48-h post-transfection, cells were selected with 0.4 mg/ml puromycin and puromycin-selective medium was replaced every 2 days. Two weeks later, cells were harvested and analyzed using western blotting and indirect fluorescence assay (IFA) to determine the expression of Nb-HRP recombinant protein. Supernatants were harvested for direct ELISA analysis.

Development of the cELISA using Nb-HRP recombinant protein as a probe

Based on the specific Nb-HRP recombinant protein, a cELISA was established for the detection of ASFV p54 antibodies in the serums. To optimize the detection effect, the optimal concentration of p54 protein and dilution ratio of supernatant Nb-HRP were first determined using direct ELISA via the checkerboard method by mixing a serial dilution of p54 protein (10, 20, 40, 80, 160, 320, and 640 ng/well) and supernatant Nb-HRP ($1:2^4$, $1:2^5$, $1:2^6$, $1:2^7$, $1:2^8$, $1:2^9$, $1:2^{10}$, and $1:2^{11}$). The combination at an OD450 of 1.0 was determined to be the optimal antigen coating amount and Nb-HRP supernatant dilution ratio.

Next, the optimal dilution ratio of porcine serums, incubation time of the mixture of the serum and Nb-HRP supernatants, and colorimetric reaction times to be tested was determined. According to the determined optimal working concentration of p54 protein and Nb-HRP supernatants, Nb-HRP supernatants (100 µl/well) together with 5 inactivated ASFV antibody-positive serum samples diluted at 1:5, 1:10, 1:20, and 1:40 (100 µl/well) were used for cELISA. Another 5 negative serum samples were analyzed the same as positive serum samples did. The mixture mentioned above was then added to the wells and incubated at 37 °C for different durations (15, 30, 45, and 60 min). Following three times washing with 0.5% PBS'T, 100 µl freshly prepared TMB solution was added to the plate, which was incubated at room temperature for 5, 10, or 15 min. The reaction was stopped by the addition of 50 µl 3 M H_2SO_4, and the absorbance was measured at OD450 nm using a microplate reader. The smallest ratio of OD450 values between the positive and negative sera (P/N) was selected as the optimal incubation and colorimetric reaction times.

Based on the optimized reaction conditions described above, a cELISA was developed as follows: ninety-six-well plates were coated with the optimal concentration of purified p54 protein overnight at 4 °C. After discarding the coating buffer and washing three times with 0.5% PBS'T (300 µl/well), the plates were blocked with 2.5% dried milk (200 µl/well) for 1 h at 37 °C. Following washing three times with

0.5% PBS'T (300 µl/well), a mixture of Nb-HRP supernatants (100 µl/well) and serum samples (100 µl/well) was added to the wells at the optimal dilution ratio and incubated at 37 °C for the optimal duration and followed by washing with 0.5% PBS'T for three times. 100 µl/well fresh TMB was added to the wells and incubated at 37 °C for the optimal duration. Next, 50 µl/well 3 M H_2SO_4 was added to each well to stop the reaction, and the absorbance value was detected at OD450 nm. The P/N value was calculated.

The procedure of the commercial ELISA kit (Beijing Jinnuobaitai Biotechnology) was briefly described as following: ASFV p30 protein was used as the coating antigen. All of the detection reagents were placed at room temperature for at least 1 h before use. Then the serum samples to be tested were 50-fold diluted using sample diluent and added to the reaction well, 100 µl/well, followed with incubation at 25 °C for 30 min. Undiluted negative control serum (NC) and inactivated positive control serum (PC) were added simultaneously. After washing three times with washing solution, 100 µl/well HRP-conjugated antibody was added and incubated at 25 °C for 30 min and followed by washing three times with washing solution. 100 µl/well TMB was added and incubated at 25 °C for 15 min, then 50 µl/well stop solution was added and the absorbance values of all samples were detected at 450 nm. The S/P value was calculated using the following formula: S/P = (sample OD value-NC OD mean value)/(PC OD mean value-NC OD mean value). The commercial ELISA kit did not cross-react with positive serums of PRRSV, PEDV, pseudorabies virus (PRV), and classical swine fever virus (CSFV), exhibiting good specificity.

Determination of the cut-off value of cELISA

A total of 295 ASFV-negative and 216 inactivated ASFV-positive serum samples were used to determine the cut-off values for a positive and negative result for the cELISA. The percentage of inhibition (PI) was then calculated: PI = 100% × [negative serum OD450 value—(test serum OD450 value / negative reference serum OD450 value)]. The results were analyzed using the ROC analysis by SPSS software version 20. The calculated PI value was used to generate the ROC curve (the X-axis represents 1-specificity; the Y-axis represents sensitivity) and the area under the curve (AUC) was calculated. The maximum value of the Youden's index which corresponds to the critical value is the optimal cut-off value: Youden's index = sensitivity + specificity—1.

Specificity, sensitivity, and repeatability of cELISA

Five hundred seventy-one samples collected from pig farms of different districts were detected using RT-PCR and specific commercial ELISA kit firstly to determine the specific pathogen-positive serum fractions. The 571 samples showed 147 PRRSV antibody-positive, 132 PRV antibody-positive, 173 PEDV antibody-positive, and 119 CSFV antibody-positive samples according to the detection results. Then, the 571 and 156 inactivated ASFV-positive serums were used to determine the specificity of the developed cELISA. Ninety-six-well plates were coated with the optimal ASFV p54 protein concentration at 4 °C overnight, and the cELISA was conducted as described previously.

To test the sensitivity of the cELISA, 156 serum samples confirmed as ASFV antibody-positive using RT-PCR and the commercial ELISA kit were tested using the developed cELISA.

The established cELISA method was used to perform a repeatability test six times on the same test plate and different test plates from those used for the three inactivated ASFV antibody-negative serum samples and three inactivated ASFV antibody-positive serum samples (23 dpi; sample 1 titer: 1:128,000; sample 2 titer: 1:144,000; sample 3 titer: 1:134,000). For the intra-assay repeatability test, the swine serum samples were detected using a cELISA. The same batch of p54 protein-coated ELISA plates was tested three times in triplicate wells for each sample, and the OD450 value was detected. For the inter-assay repeatability test, different batches of plates were tested separately three times in triplicate wells for each sample, the OD450 value was detected, and the PI value was calculated.

Detection of field serum samples

A total of 209 clinical porcine field serum samples (193 ASFV-negative and 16 ASFV-positive serums by RT-PCR) collected from three farms in the Henan and Hubei provinces between 2019 and 2020 were tested using the developed cELISA and commercial ELISA kit, as previously described and following the manufacturer's instructions, respectively. The percent agreement between the developed cELISA and the commercial ELISA kit (Beijing Jinnuobaitai Biotechnology) was calculated using Microsoft Excel's Correl function (Microsoft Corporation).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 software (GraphPad Software, Inc.). Data are expressed as the mean ± SD. Kappa values were calculated to measure of agreement between the developed cELISA and the commercial ELISA kit using SPSS software version 20. For ROC analysis, a sufficient number of positive and negative samples of precisely known ASFV status were detected and analyzed using SPSS software version 20 (IBM Corp.; http://www.spss.com.cn).

Results

Expression and purification of recombinant ASFV p54 protein

As shown in Fig. 2A, SDS-PAGE results showed that the p54 protein with the expected size 16 kDa was successfully expressed. Moreover, after ultrasonication, most p54 protein distributed in supernatant, and only small part of protein distributed in precipitation. Thus, the supernatants were further purified using Ni–NTA column. After purification, most irrelevant proteins from *E. coli* were removed, and the purity of p54 protein was high (Fig. 2A). The purified p54 protein was further identified using western blotting with inactivated ASFV-positive pig serum. The results showed that specific target band was detected (Fig. 2B). Finally, after buffer exchange to PBS, about 50 mg of purified p54 protein was obtained. The p54 protein was aliquoted and stored at − 80 °C for further use.

Construction and verification of phage display VHH library

The purified p54 protein was used to immunize a 4-year-old male Bactrian camel. A total of four immunizations were conducted, and dose was 5.0 mg for each immunization (Fig. 1). One week after the last immunization, polyclonal antiserum was extracted and the titer was evaluated by indirect ELISA. ELISA results showed that the titer of the antiserum was 1:256,000, indicating that the immunization was successful (Fig. 3A). Then the whole blood was collected, PBLs were separated, and total RNA was extracted and transcripted into cDNA, which was used as the template of PCR. After one round of PCR, about 700 bp of target band was obtained (Fig. 3B). Then the target band was harvested for the second round of PCR. As shown in Fig. 3C, about 400 bp of VHH target band was amplified.

Bio-panning and specific Nb screening

To screen specific Nbs against ASFV p54 protein from the phage display VHH library, three consecutive rounds of bio-panning were conducted using phage ELISA coated with p54 protein as the antigen. Following three rounds of bio-panning, phage particles carrying the specific VHH genes were found to be markedly enriched (Table S2). Next, to obtain soluble Nbs with high specificity and affinity, 96 individual colonies were selected from the culture plate used in the third round of bio-panning and treated with IPTG (Fig. 3D and E). The periplasmic extracts from the 96 clones were subjected to indirect PE-ELISA to identify whether they could bind to the p54 protein. The results showed that, among the 96 clones, 93 could specifically react with p54 protein compared with the negative control (Fig. 4A). These specific clones were sequenced for VHH genes, and sequence analysis results indicated that a total of 13 specific Nbs (Nb8, Nb9, Nb10, Nb11, Nb13, Nb39, Nb45, Nb46, Nb56, Nb79, Nb81, Nb83, and Nb90) were screened and identified based on the CDR3 region of VHH genes (Fig. 4B). Amino acid sequence analysis revealed that conserved residues at the 37, 44, 45, and 47 positions (located on the VH-VL interface region of VHs) from the 13 Nbs were identified to be hydrophilic amino acids (Fig. 4B). To further confirm which Nb had the best reaction activity and specificity, whether the 13 Nbs reacted with PRRSV N, PEDV N, and ASFV p30 proteins was determined firstly using PE-ELISA. The results showed that none of the 13 Nbs reacted with these unrelated proteins. Nevertheless,

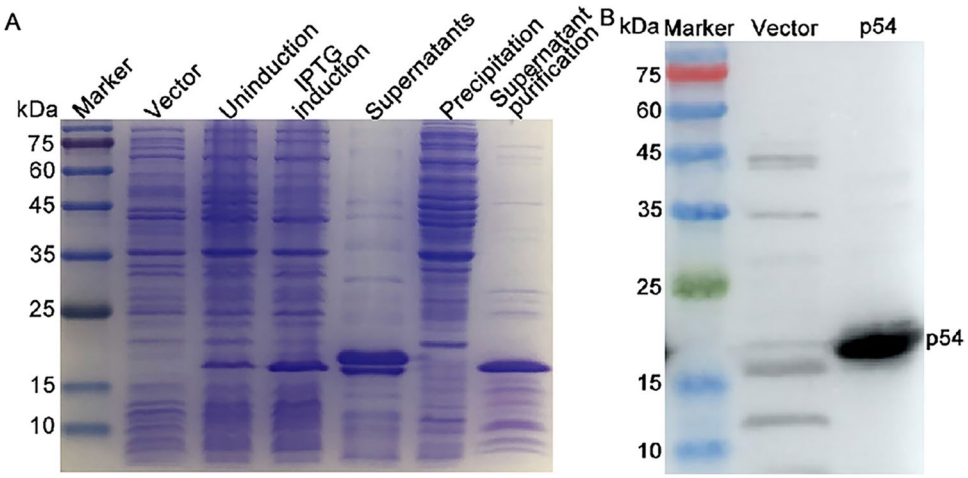

Fig. 2 Expression and purification of p54 protein. **A** Transetta DE3 competent cells transformed with pET-30-p54-His plasmids were induced or not with 1.0 mM IPTG for 12 h. After ultrasonication and centrifugation, supernatants or precipitation were subjected to SDS-PAGE analysis. **B** Purified p54 protein was verified using western blotting with inactivated ASFV-positive pig serum. Abbreviations: ASFV, African swine fever virus; IPTG, isopropyl β-D-thiogalactoside

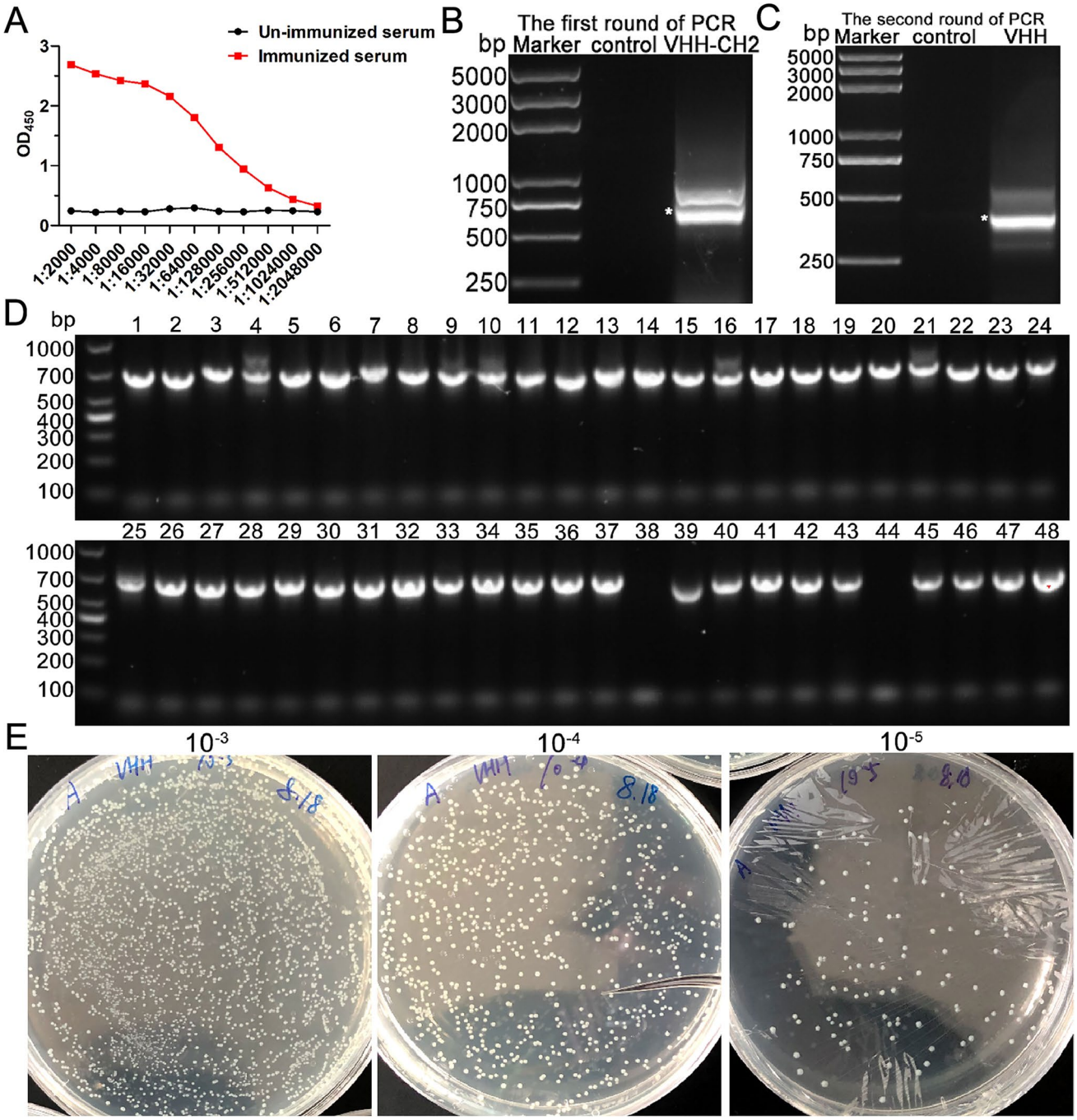

Fig. 3 Bactrian camel immunization and construction of phage library of VHH. **A** A male Bactrian camel was immunized with purified p54 protein (5 mg per immunization) together with Freund's adjuvant. One week after the forth immunization, serum was separated and the titer was determined using indirect ELISA, with which the p54 protein was coated as the antigen, the immunized/unimmunized camel serum as the first antibody, the mouse anti-camel serum as the second antibody, and the HRP-conjugated goat anti-mouse IgG (H&L) as the third antibody. **B**, **C** Total RNA extracted from camel PBLs was reversed transcripted into cDNA and used as the templates of the first round of PCR, and VHH gene was obtained by the second round of PCR. **D** After pCANTAB 5E-Nbs were electroporated into the TG1 competent cells, 100 µl of the transformation products was used to coat on the LB plate and 48 clones were picked randomly 16 h later and identified with PCR; **E** the resting bacteria were serially diluted and coated on the LB plate for analysis of library size. Abbreviations: PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PBLs, peripheral blood lymphocytes

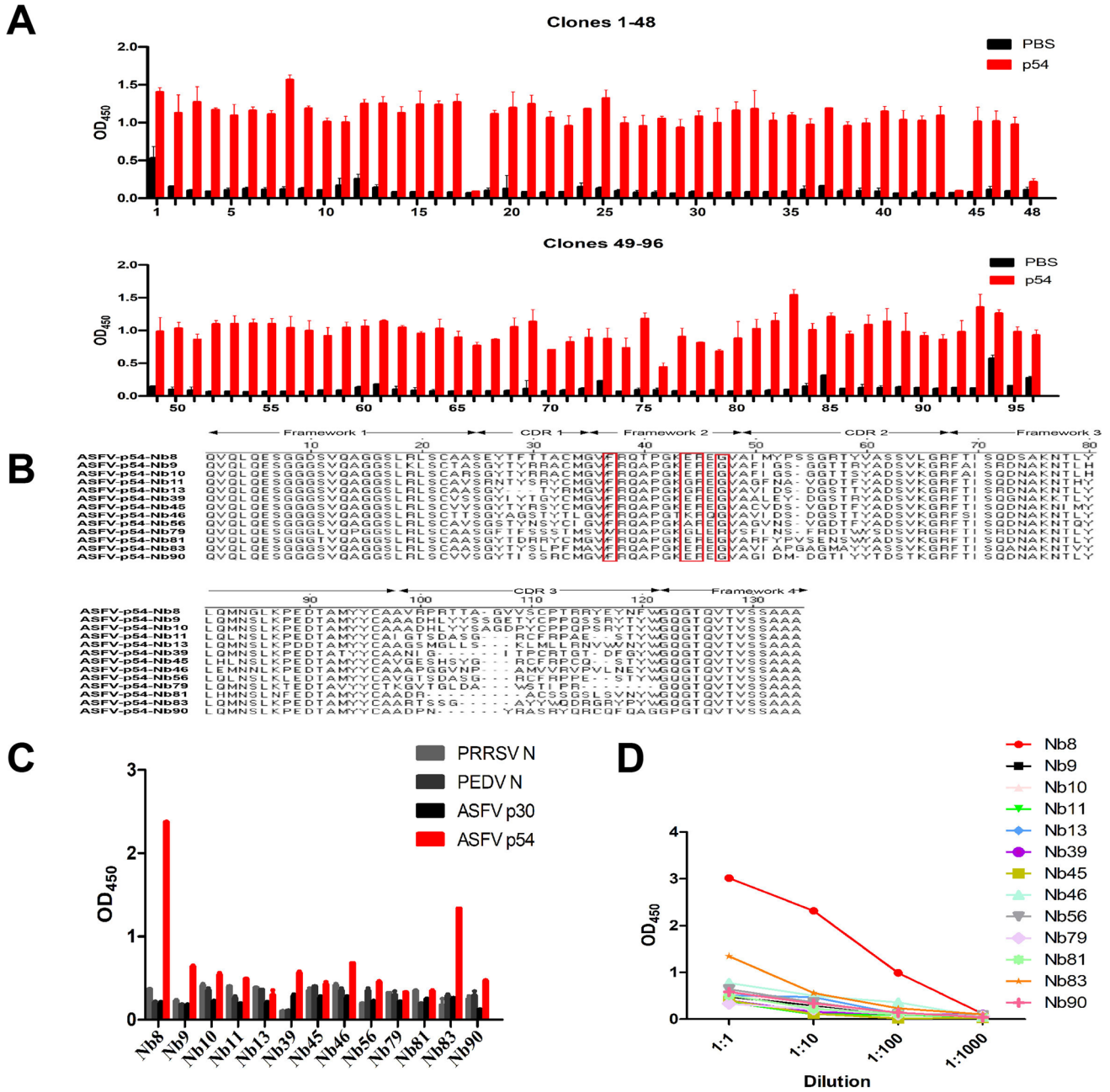

Fig. 4 Specificity and reactivity of isolated Nbs against the ASFV p54 protein. **A** PE-ELISA analysis of periplasmic extracts reacted with the ASFV p54 protein from 96 clones. **B** Amino acid sequence alignment of isolated Nbs. Numbering and CDRs were determined as previously described (Liu et al. 2015). **C** Specificity detection of 13 Nbs against p54 protein using a PE-ELISA. **D** The affinity of the 13 Nbs to the p54 protein was detected using a PE-ELISA. ASFV, African swine fever virus; Nbs, nanobodies; CDRs, complementary-determining regions; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Ig, immunoglobulin

when the PE-ELISA was performed using p54 protein, Nb8 and Nb83 exhibited the higher reactivity with p54 protein (Fig. 4C). The affinity of these Nbs to p54 protein was further investigated through the serial dilution of supernatants containing them. As shown in Fig. 4D, Nb8 and Nb83 exhibited the higher affinity.

Expression of HRP-conjugated recombinant Nb8 and Nb83 in HEK293T cells

The Nb8 (GenBank accession no. OM459819) and Nb83 (GenBank accession no. OM459818) genes were amplified from the pCANTAB-5E vector and fused with the HRP

gene, and then cloned into the pCAGGS-HA vector to construct the pCAGGS-Nb8-HRP and Nb83-HRP recombinant plasmid (Fig. 5A–C). As shown in Fig. 6A, western blotting results showed that the Nb8-HRP and Nb83-HRP fusion protein was expressed in HEK293T cells with the expected size of 65 kDa. IFA results showed that both

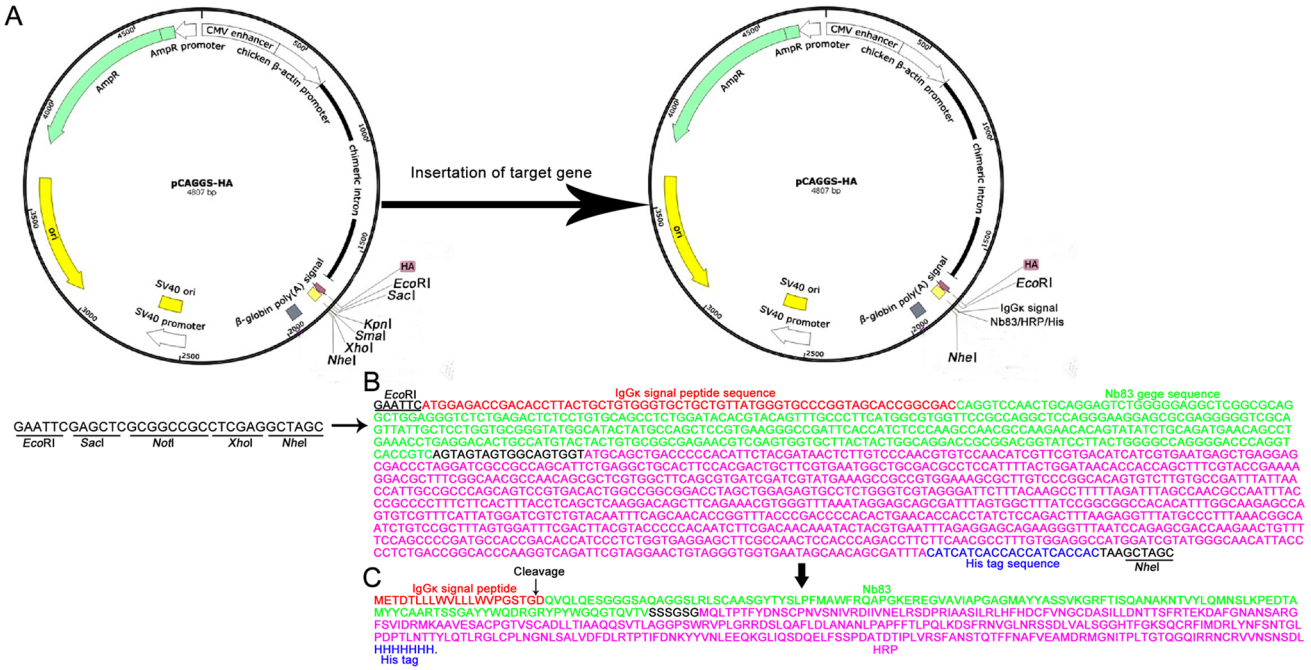

Fig. 5 Construction of Nb-HRP eukaryotic expression system. **A** Schematic of eukaryotic expression vector expressing Nb-HRP. **B** Nucleotide base sequence of IgGκ and Nb-HRP. **C** Amino acid sequence of IgGκ and Nb-HRP encoded by eukaryotic expression vector pCAGGS-HA. Abbreviations: IgGκ, human immunoglobulin G kappa chain; HRP, horseradish peroxidase

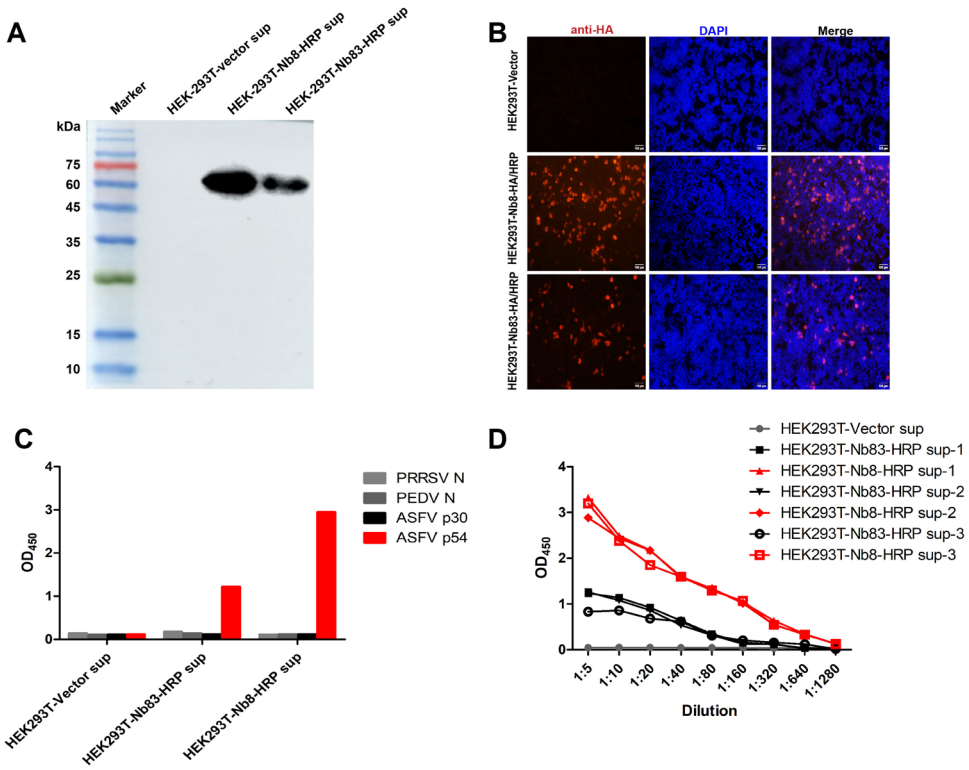

Fig. 6 Expression and identification of Nb8-HRP and Nb83-HRP fusion protein in HEK293T cells. pCAGGS-Nb8-HRP and Nb83-HRP recombinant plasmids were transfected into HEK293T cells for 36 h, respectively. Cells were harvested for protein analysis using western blotting (**A**) and IFA (**B**). At 36-h post-pCAGGS-Nb8-HRP and Nb83-HRP transfection, HEK293T cell culture supernatants were harvested for Nb8 and Nb83 specificity (**C**) and affinity (**D**) assay using a direct ELISA. Nb, nanobody; IgG, immunoglobin G; ASFV, African swine fever virus; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; mAb, monoclonal antibody; HA, hemagglutinin

Nb8-HRP and Nb83-HRP were successfully expressed (Fig. 6B). Direct ELISA analysis of cell culture supernatants revealed that Nb8 and Nb83 was secreted into cell culture supernatants, and both Nb8 and Nb83 could specifically react with p54 protein, but not with other unrelated proteins (Fig. 6C). In addition, direct ELISA analysis of the Nb8 and Nb83 affinity suggested that the Nb8 had a higher binding affinity for the p54 protein than Nb83 from the supernatants of 3 separate groups (Fig. 6D). Based on the specificity and affinity analysis results, Nb8 was selected for the development of the cELISA.

Generation of HEK293T cell line stably expressing Nb8-HRP fusion protein

After selecting using 0.4 mg/ml puromycin for 2 weeks, HEK293T cells were harvested and analyzed using western blotting and IFA to determine the expression of Nb8-HRP recombinant protein. As shown in Fig. 7A, western blotting results showed that Nb8-HRP fusion protein was expressed in the HEK293T and IFA yielded similar results (Fig. 7B), indicating that the recombinant cell line was successfully established, which named HEK293T$^{Nb8-HRP}$. Direct ELISA analysis of cell culture supernatants revealed that the amount of Nb8-HRP fusion protein secreted by HEK293T$^{Nb8-HRP}$ is much higher than that of the transient transfection cells (Fig. 7C).

Development of cELISA using the Nb8-HRP as a probe

Based on the optimized reaction conditions, the cELISA procedure was performed as follows: ninety-six-well plates were coated with 200 ng/well purified p54 protein overnight at 4 °C (Table S3). After washing three times with 0.5% PBS'T, the plates were blocked with 200 µl/well of 2.5%

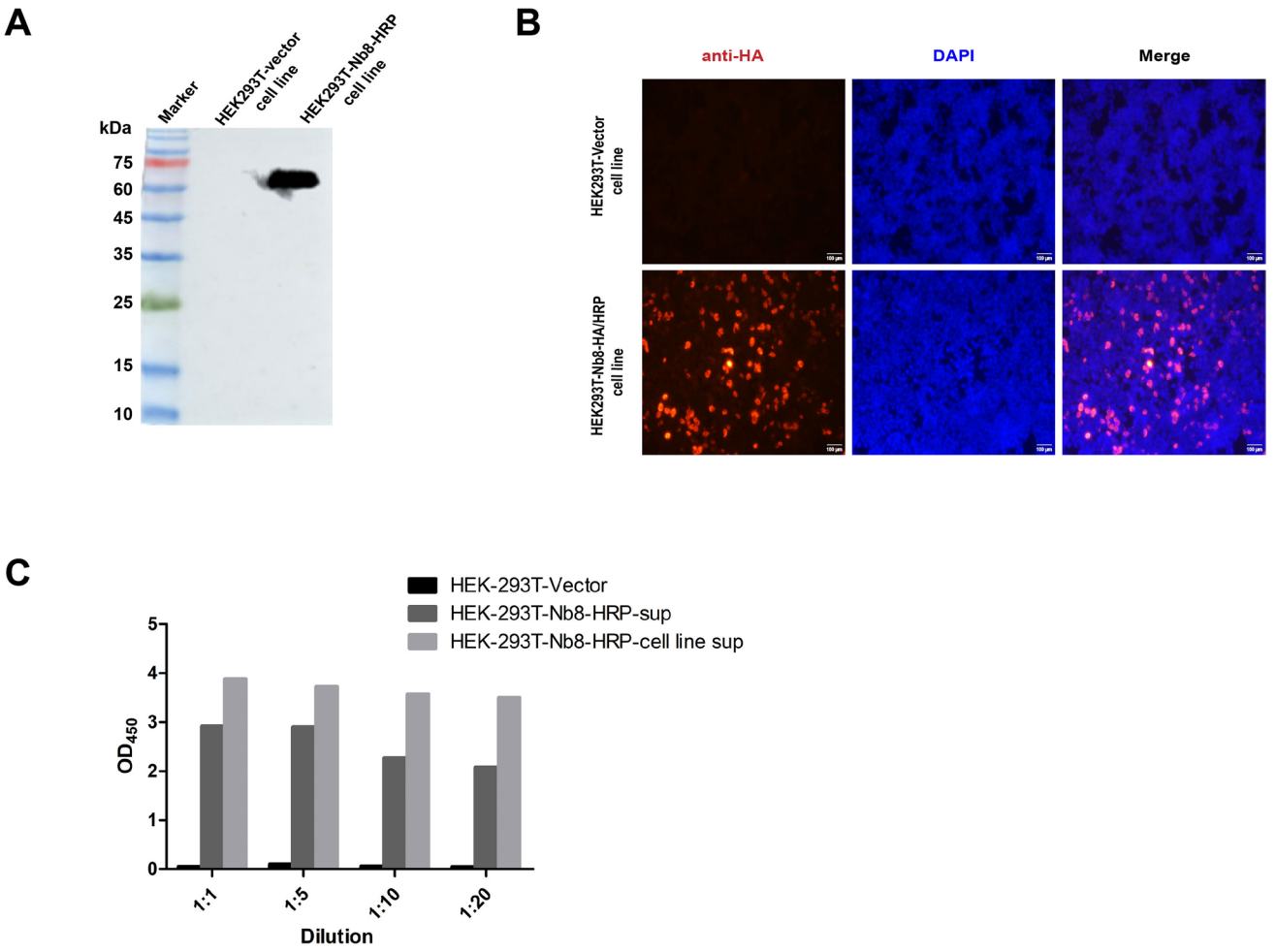

Fig. 7 Generation of HEK293T cell line stably expressing Nb8-HRP fusion protein. **A** Western blotting identification of HEK293T$^{Nb8-HRP}$ recombinant cell line. **B** IFA identification of HEK293T$^{Nb8-HRP}$ recombinant cell line using mouse anti-HA monoclonal antibody. **C** Direct ELISA analysis of supernatants Nb8-HRP fusion protein affinity from HEK293T$^{Nb8-HRP}$ cell line and transient transfection cells. ELISA, enzyme-linked immunosorbent assay; IFA, immunosorbent fluorescence assay

dried milk for 1 h at 37 °C and followed by further washing three times with 0.5% PBS'T. Serum samples to be tested (1:5, 100 µl/well, Table S4) and Nb8-HRP supernatant ($1:2^5$, 100 µl/well, Table S3) were added to the wells and incubated at 37 °C for 45 min (Table S5). The plates were washed three times with 0.5% PBS'T, and then 100 µl/well fresh TMB was added and incubated at 37 °C for 10 min (Table S5). Finally, 50 µl/well 3 M H_2SO_4 was added to each well to stop the reaction, and the absorbance value was detected at OD450 nm (Fig. 1B).

Determination of cut-off value for cELISA

To determine the cut-off value of the cELISA, 295 standard ASFV-negative and 216 inactivated ASFV-positive pig serum samples were detected using a cELISA. The ROC curve was shown in Fig. 8. The results of area under the curve (AUC) showed that the area under the ROC curve was 0.984 (95% confidence interval: 0.974–0.993, $P < 0.001$) (Table S8). As shown in Table S9, a series of sensitivity and 1-specificity values according to the results of coordinates of the curve were obtained. The maximum of Youden's index was calculated to be 0.909 and the specificity was 91.50% and the sensitivity was 99.40%. Thus, the cut-off value for cELISA was 52.50%. The sample was regarded as ASFV antibody-positive at a PI ≥ 52.50% and ASFV antibody-negative at a PI < 52.50%.

Specificity, sensitivity, and repeatability of cELISA

To detect specificity of the developed cELISA, PRRSV, PRV, PEDV, and CSFV antibody-positive serums were used. A standard negative serum and 156 inactivated standard ASFV antibody-positive serums were used simultaneously as the control. The 571 serum samples and control serum samples were detected using the developed cELISA. The PI of each sample was calculated based on the OD450 value. The results suggested that no serum, except the inactivated ASFV antibody-positive serum, exhibited a competitive effect (PI < 52.50%; Fig. 9), suggesting that the cELISA was specific to antiserum against ASFV.

For determination of the sensitivity of the cELISA, the 156 clinical inactivated ASFV-positive serums were all positive via detection with the developed cELISA and the PI values ranging 52.5–95% (Fig. 10). The PI values of 139 serum samples were greater than 65%, and only 17 samples had PI values from 52.5 to 65% (Fig. 10). Thus, the sensitivity of cELISA for the tested clinical pig serum was 100%.

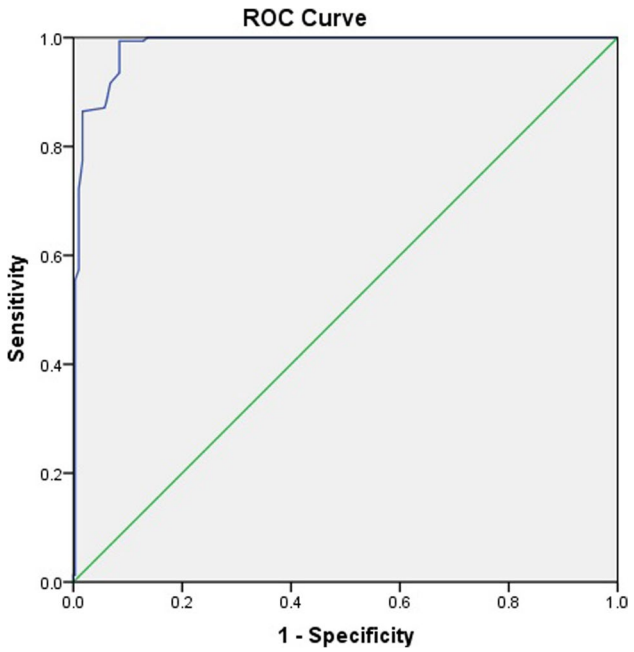

Fig. 8 ROC curve analysis of optimal cut-off value. 295 standard ASFV-negative and 216 inactivated ASFV-positive pig serum samples of precisely known ASFV status were detected by cELISA and analyzed using SPSS software version 20 (IBM Corp.; http://www.spss.com.cn). ASFV, African swine fever virus; cELISA, competitive enzyme-linked immunosorbent assay

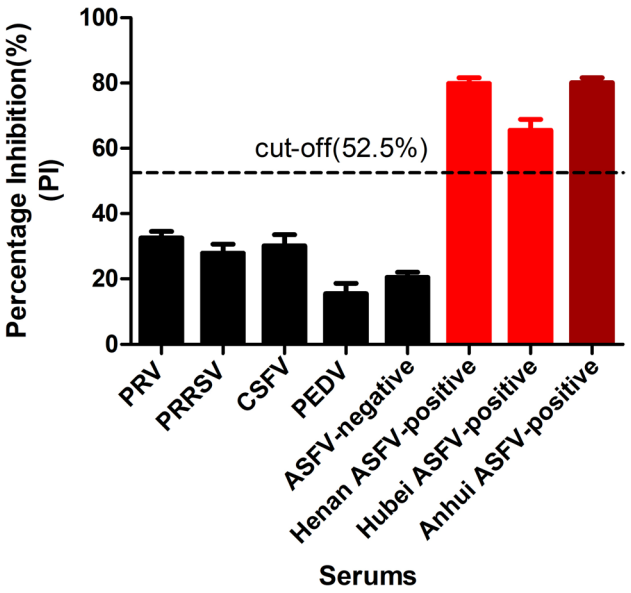

Fig. 9 Specificity verification of the developed cELISA. PRRSV-, PRV-, PEDV-, CSFV- and inactivated ASFV antibody-positive serums were reacted with p54 protein (200 ng/well) and used to coat ninety-six-well plates to determine the specificity of the developed cELISA. A negative serum and 156 inactivated standard ASFV antibody-positive serums were used simultaneously as the control. PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; PEDV, porcine epidemic diarrhea virus; CSFV, classical swine fever virus; ASFV, African swine fever virus; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase

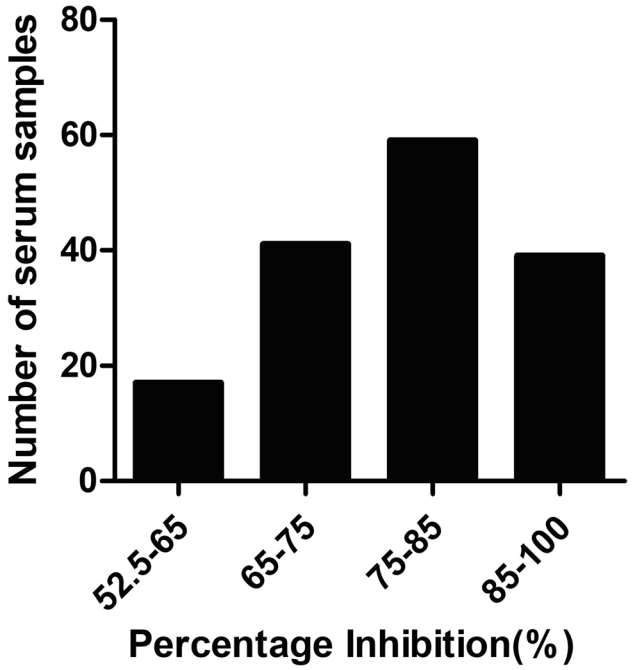

Fig. 10 Sensitivity analysis of the developed cELISA. A total of 156 different inactivated ASFV antibody-positive serums were incubated in ninety-six-well plates coated with ASFV p54 protein (200 ng/well) to evaluate the sensitivity of the developed cELISA. Percentage inhibition was determined according to the OD450 value. Three replicate wells were used for each sample. The data are presented as the mean ± SD. ELISA, enzyme-linked immunosorbent assay; ASFV, African swine fever virus; OD, optical density

To further determine the repeatability of the cELISA, the intra-assay variability of the PI was calculated by testing three inactivated ASFV antibody-positive and three ASFV antibody-negative serum samples; the test was repeated three times. The results showed that the intra-assay coefficient of variation (CV) of the PI was 0.75–6.20%, with a median value of 3.475%. To determine the inter-assay variability, the six samples were tested in three different plates at different time points, and the inter-assay CV of the PI was 1.49–10.12%, with a median value of 5.805% (Table S6). These data suggested that the developed cELISA displayed good repeatability.

Detection of field serum samples

To confirm whether the cELISA could be used for field serum detection, a total of 209 pig serum samples from pig farms (individual pigs have clinical symptoms) in the Henan and Hubei provinces were inactivated and tested using the developed cELISA (Table S7) and the results were compared with those of the commercial ELISA kit. The cELISA results revealed 16 ASFV antibody-positive and 193 ASFV antibody-negative serum samples, while the commercial ELISA kit revealed 15 positive and 194 negative serum samples (Table S7). When comparing the cELISA with the commercial ELISA kit, the former was found to have a positive coincidence rate of [14/(14 + 1)] = 93.3% and a negative percent agreement of [192/(2 + 192)] = 98.97%. The sensitivity coincidence rate was [14/(14 + 1)] = 93.3% and the specificity percent agreement was [193/(3 + 193)] = 98.97%. Therefore, the overall percent agreement was [(14 + 192)/(14 + 2 + 1 + 192)] = 98.56%, which was quite consistent with the commercial kit. Furthermore, Kappa index values revealed a high degree of coincidence between the cELISA and the commercial ELISA kit (kappa value = 0.993), with the results of the developed cELISA being highly similar to those of the commercial kit.

Discussion

ASF is a serious threat to the pig farming industry worldwide, including in China (Zhou et al. 2018). Considering that ASF spreads to surrounding areas, continuous improvements in ASF diagnosis and surveillance are critical for disease containment. Pigs that survive natural infection develop antibodies against ASFV 7–10 days after infection, which are detectable for a long period of time (Sanchez-Vizcaino et al. 2012). Therefore, despite the critical role of ASFV surveillance, a simpler, more cost-effective approach should be developed based on efficient, low-cost, and accurate diagnostic testing. Prior to the development of effective vaccines and treatments against ASF, molecular diagnostic methods and serological detection techniques are considered to be the main means of identifying infected animals and eradicating the potential risk of ASFV infection. The ASFV antibody detection methods recommended by the OIE mainly include ELISA, western blotting, and IFA (Gallardo et al. 2019). Among them, ELISA is simple, low-cost, and more suitable for large-scale field epidemiological investigations (Gallardo et al. 2019, 2015). Polyclonal antibodies or mAbs are commonly used in traditional ELISA, but the quality can be inconsistent among the different batches of polyclonal antibodies, and the industrialized large-scale production process of mAbs is complicated and costly (Rodrigues et al. 2010). By contrast, Nbs, the variable domains of heavy chain-only antibodies that were first discovered in camelids and sharks (Pírez-Schirmer et al. 2017), can be easily produced in prokaryotic and eukaryotic expression systems, including the low-cost and rapid HEK293T expression system described herein. Of course, the system is dependent on the immunization of camel to obtain the specific VHH library of the protein of interest. In the present study, a novel cELISA was developed to detect anti-ASFV antibodies on the basis of

HRP-conjugated Nb (Zhao et al. 2021). Nb, as a probe, exhibited the same specificity as that of the mAb that acted as the specific probe in the commercial ELISA kit, as well as higher sensitivity and simpler operation. In general, high affinity and high specificity nanobodies can be successfully screened by immunizing Bactrian camels with soluble proteins. For inclusion of body proteins, the relative success rate may be lower. The nanobody with the best affinity and specificity will be used for further experimental or clinical application studies. However, of the 13 Nbs, only Nb8 exhibited high affinity and specificity to the p54 protein, with low affinity and specificity observed in the other 12 Nbs (Fig. 2C and D). In addition, the cELISA revealed that Nb8 had a significant competitive effect. We hypothesized that Nb8 may recognize the same epitope(s) on the p54 protein with p54 antibody in ASFV antibody-positive serum, and the other 12 Nbs may recognize different epitopes from those of Nb8, or no epitopes at all.

The ASFV proteins commonly used for antigen detection are p10, p30, p54, p72, and p73 (Geoffrey et al. 2006; Neilan et al. 2004; Oviedo et al. 1997). ASFV p54 protein is a type of structural protein that appears in the early stage of viral replication, plays an important role in maintaining the stability of the virus, and can induce cell apoptosis (Hernáez et al. 2004). The p54 protein is present throughout the life cycle of the virus and is often used as a tracer protein to study the replication, assembly, and invasion of ASF viruses (Salas et al. 2013). In addition, the dynamic protein-binding domain of ASFV p54 protein is the main neutralization site of serum antibodies (Neilan et al. 2004). Following the comparison of the amino acid sequences of p54 protein with those of other viral proteins, no similar sequences were identified, and the amino acid sequences of p54 protein of the China/2018/AnhuiXCGQ strain exhibited 96.42% homology compared to other China ASFV strains, which suggested that p54 protein is unique to ASFV and is an ideal antigen for the development of diagnostic reagents for ASF and the establishment of specific detection methods. Besides, the p54 protein was able to react with inactivated positive serums of different ASFV strains from China (Fig. 9), exhibiting good applicability. It is worth noting that the p54 epitope peptide sequence possesses a high degree of variation among the different ASFV genotypes across Europe (Petrovan et al. 2020), suggesting that the Nb-based cELISA developed herein using p54 as an antigen may not be suitable for serological detection in pigs infected with other ASFV strains in Europe, although our results showed that the p54 protein could react with some other ASFV strains' positive serums. In the present study, a p54-specific Nb could recognize the p54 protein successfully, but the precise recognition site and whether this site is conserved between different ASFV strains remain unclear. Our future studies will focus on analyzing the p54 epitope map in detail to clarify the molecular mechanism of the recognition of p54 by Nb and optimizing the cELISA so that it can be used for the serological detection of different ASFV strains. In addition to p54, there are several ELISA-based serological tests incorporating p72 and p30 antigens, which are the basis for existing commercial assays (Gimenez-Lirola et al. 2016; Sastre et al. 2016a, b). A p54 ELISA-based serological test showed 93.3% sensitivity and 98.97% specificity compared with the OIE-approved ELISA (Gallardo et al. 2009), indicating that p54, p30, and p72 comprise an ideal set of antigen targets for the detection of ASFV antibodies. However, the present study used p54 as the antigen, instead of p30 or p72. Whether p30 or p72 are more specific and can overcome the strong variability between p54 strains in establishing an Nb cELISA using p30 or p72 as the targets remains unclear and requires further study.

The clinical symptoms and pathological changes of ASF are closely associated with PRRSV, CSFV, PCV, salmonella, erysipelas, and other swine diseases. These similarities increase the difficulty of the clinical diagnosis of ASF (Oura et al. 2013). The cELISA developed herein exhibited excellent specificity and no cross-reactivity with PRRSV-, PRV-, PEDV-, TGEV-, PPV-, and CSFV-positive serums (Fig. 8). The newly developed cELISA was also evaluated in terms of its sensitivity, which was proven to be excellent. An intra- and inter-assay comparison also revealed good repeatability. Compared with the commercial ELISA kit, the developed cELISA exhibited lower production costs and less preparation time for antibody production. Taken together, a novel, rapid, specific, and low-cost cELISA was developed based on an HRP-conjugated Nb, which can be used to assess clinical serum samples. To the best of our knowledge, this was the first report of an Nb against ASFV and its practical application; therefore, a cELISA may represent a promising diagnostic method for the detection of ASFV antiserum in pig farms.

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Author contribution A.Z. and G.Z. conceived and designed the experiments. H.Z. and J.R. performed most of the experiments and data analyses. S.W., H.G., Y.D., B.W., and G.Z. contributed to data analysis. P.J. and Y.W. provided technical support for experiment implementation. All authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in the article.

Declarations

Ethics approval The animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Henan Agricultural University (ND19-12). We declare that current research is in full compliance with ethical standards.

Consent for publication All authors listed on this manuscript have read and agreed to the publication of this research.

Competing interests The authors declare no competing interests.

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