



Identification and Characterization of Nanobodies from a Phage Display Library and Their Application in an Immunoassay for the Sensitive Detection of African Swine Fever Virus

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ABSTRACT African swine fever (ASF) is one of the most lethal and devastating diseases of domestic and wild swine. The continual spread and frequent outbreaks of ASF have seriously threatened the pig and pig-related industries, causing great socioeconomic losses at unprecedented proportions. Although ASF has been documented for a century, no effective vaccine or antiviral treatment is currently available. Nanobodies (Nbs) derived from heavy-chain-only antibodies in camelids have been discovered to be effective as therapeutics and robust biosensors in imaging and diagnostic applications. In the present study, a high-quality phage display library containing specific Nbs raised against ASFV proteins was successfully constructed, and 19 nanobodies specific to ASFV p30 were preliminarily identified by phage display technology. After extensive evaluation, nanobodies Nb17 and Nb30 were employed as immunosensors and applied to develop a sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of ASFV in clinical specimens. This immunoassay showed a detection limit of approximately 1.1 ng/mL target protein and $10^{2.5}$ hemadsorption ($\text{HAD}_{50}/\text{mL}$) of ASFV and exhibited high specificity with no cross-reaction with the other porcine viruses tested. The performances of the newly developed assay and a commercial kit in testing 282 clinical swine samples were very similar (93.62% agreement). However, the novel sandwich Nb-ELISA showed higher sensitivity than the commercial kit when serial dilutions of ASFV-positive samples were tested. The present study describes a valuable alternative technique for the detection and surveillance of ASF in endemic regions. Furthermore, additional nanobodies specific to ASFV may be developed using the generated VHH library and employed in different biotechnology fields.

KEYWORDS African swine fever, nanobody, phage display, biosensor, immunoassay

In recent years, the frequent occurrence of emerging and reemerging infectious diseases has posed a great threat to human health and livestock industries. Indeed, the current COVID-19 pandemic and African swine fever outbreaks are critical concerns to the World Health Organization (WHO) and World Organization for Animal Health (WOAH) (1, 2). Emerging infectious diseases are often characterized by the sudden appearance of widespread outbreaks incurring enormous losses in livestock and productivity due to the delayed implementation of control measures and/or lack of sufficient diagnostic tools or vaccines. This is particularly true in the early stages of an outbreak or the first-time appearance of an infectious agent.

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African swine fever (ASF) is considered the most devastating and economically significant disease of wild and domestic swine (3). The causative agent of ASF is the African swine fever virus (ASFV), a large icosahedral DNA virus and the sole member of the *Asfarviridae* family (3). ASFV is currently the only known DNA arbovirus, and it can be efficiently transmitted by soft ticks in the genus *Ornithodoros* (4). The soft tick *Ornithodoros moubata* has been shown to be both a biological vector and a reservoir host of ASFV and is involved in a sylvatic cycle in Africa (5), while *Ornithodoros erraticus* is a competent vector and reservoir in Europe (6). The disease is characterized by high contagiousness, high fever, hemorrhage in internal organs, and high mortality in domestic pigs; however, the clinical symptoms are indistinguishable from those of other swine virus diseases, such as classical swine fever and porcine reproductive and respiratory syndrome (7). ASF is an “old” and reemerging infectious disease of swine since the causative agent was first identified in Kenya in the 1920s (8). The transcontinental transmission of ASFV occurred first from Africa to Portugal in 1957 and then later to South America and the Caribbean. After great losses, eradication of ASF outside Africa was eventually achieved, with the exception of Sardinia in Italy (3). However, in 2007, ASF spread again to the Republic of Georgia in the Caucasus and subsequently to other neighboring countries and to the European Union in 2014 (3). Unfortunately, the threat posed by ASF was exacerbated after its identification in August 2018 in China, which contains the largest pig breeding industry and reports the highest pork consumption rate in the world (2). Nowadays, further outbreaks and spread of ASF in Asia and Europe are still under way to ongoing, presenting a serious global threat to swine industries (3, 4). Although the disease has been recognized for over a century, neither effective vaccines nor treatments against ASF are available, and control measures have to rely on early diagnosis and culling of herds exposed to the virus (7).

Nanobodies (Nbs), also known as single-domain antibodies (sdAbs), are derived from the variable region of heavy-chain antibodies (HCAbs) found naturally in camelids and sharks, and they lack light chains and the CH1 domain in the heavy chains (9, 10). They are the smallest antibodies discovered thus far, with a molecular mass of approximately 15 kDa, and are the smallest known functional antigen-binding fragments (9). Due to their single-domain nature, nanobodies exhibit many specialized characteristics distinguishing them from conventional antibodies, including good solubility and stability, high specificity and affinity, low immunogenicity, and high tissue penetration ability (11, 12). Moreover, because of their small size and relatively long CDR3 loop, they can access intracellular targets and cryptic sites otherwise inaccessible to conventional antibodies (12). In particular, nanobodies can be genetically engineered and easily expressed in different microbial systems with high-yield production and low cost (13). These special and beneficial properties make nanobodies ideal candidates for multiple biomedical applications in the fields of basic research, therapeutics, and diagnostics (13).

In the present study, a nanobody phage display library was constructed from Bactrian camels immunized with ASFV proteins, and specific nanobodies against ASFV were isolated by taking advantage of phage display technology. Furthermore, the identified nanobodies were evaluated for their use in the development of immunosensors and applied to a sandwich enzyme-linked immunosorbent assay (ELISA) for the sensitive and specific detection of ASFV in clinical practice.

MATERIALS AND METHODS

Facility and ethics statements. The experiments with live ASFV were carried out in a biosafety level 3 facility at the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (LVRI, CAAS), and authorized by the Ministry of Agriculture and Rural Affairs of China. The animal treatments and sample collection were approved by the animal owners, and all procedures complied with animal ethics procedures and guidelines and were approved by the Animal Ethics Committee of LVRI, CAAS.

Cells, virus, animals, and samples. Primary porcine alveolar macrophages (PAMs) were prepared by bronchoalveolar lavage and cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco, USA) at 37°C with 5% CO₂ (14). The ASFV CN/SC/2019 strain was stored and provided by the African Swine Fever Regional Laboratory of China (Lanzhou). Classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine parvovirus (PPV), porcine circovirus type 2 (PCV2), and foot-and-mouth disease virus

serotype-O (FMDV-O) were provided by LVRI. Adult Bactrian camels (*Camelus bactrianus*) used in this study were rented from a livestock farm located in Jinchang, Gansu Province, China. All clinical samples were collected and provided by the ASF Regional Laboratory of China (Lanzhou) and were tested and verified by WOH-preserved real-time PCR assay (15).

Expression and purification of ASFV recombinant proteins. The CP204L (p30), K205R, and partial B646L (p72, 1 to ~1,080 bp) genes of ASFV (genotype II, Georgia 2007/1; GenBank accession no. FR682468) were synthesized and cloned into the expression vector pET-28a with a His tag. The recombinant plasmid was transformed into *Escherichia coli* Rosetta (DE3) competent cells. The expression of p30, K205R, and truncated p72 recombinant proteins was induced at 37°C for 6 h with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the cultures reached an optical density measured at 600 nm (OD_{600}) of approximately 0.6. The bacterial cells were harvested and centrifuged at $8,000 \times g$ for 20 min at 4°C, and the pellet was resuspended in phosphate-buffered saline (PBS). After sonication and centrifugation, p30 and truncated p72 recombinant proteins expressed as inclusion bodies were dissolved in 8 M urea and then purified by nickel affinity chromatography (GE Healthcare, USA) according to the manufacturer's instructions. The denatured protein was refolded and dialyzed in PBS. The soluble expressed K205R recombinant protein was also purified by nickel affinity chromatography. The expression and purification of p30, p72, and K205R were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These proteins were further analyzed by Western blotting with ASFV-positive serum and rabbit anti-pig antibody conjugated to horseradish peroxidase (HRP; Sigma, USA) as primary and secondary antibodies, respectively.

Camel immunization. Three 4-year-old healthy Bactrian camels were immunized with 0.5 mg ASFV p30, p72, and K205R recombinant protein emulsified with equivoluminal Freund's complete adjuvant through a subcutaneous route. On days 21, 35, 49, and 63 after the first immunization, four additional injections were performed with 0.3 mg of protein emulsified with equivoluminal Freund's incomplete adjuvant. Then, 1 week after the final injection, the sera were collected from the immunized camels, and the antibody titers against p30, p72, and K205R of ASFV were analyzed by indirect ELISA. Briefly, recombinant protein (250 ng/well) was coated overnight at 4°C and blocked with 2% bovine serum albumin (BSA) in PBST (0.5% Tween 20 in PBS) at 37°C for 1 h. Serial dilutions of the serum were added and incubated at 37°C for 1 h, and the preimmune serum was used as the negative control. HRP-conjugated rabbit anti-camel IgG (Solarbio, Beijing, China) was used as the secondary antibody.

VHH library construction. Peripheral blood lymphocytes were isolated from the camel blood sample taken 1 week after the final immunization. Total RNA was extracted from lymphocytes and used to synthesize cDNA by reverse transcription. Nested PCR was employed for the amplification of variable domain of heavy chain of heavy-chain antibody (VHH) gene fragments with two primer sets as described previously (16). The final PCR products (approximately 400 bp) were recovered and cloned into the phagemid vector PHEN2 with BSSH II and NheI restriction enzyme sites. The recombinant phagemids were electroporated into freshly prepared *E. coli* TG1 competent cells. The transformed cells were cultured on LB agar plates supplemented with 2% (vol/vol) glucose and 100 μ g/mL ampicillin at 37°C for 12 h. The capacity of the constructed library was measured by counting the number of colonies on the plate after gradient dilution, and the insertion rate and diversity were evaluated by PCR and sequencing. Colonies were harvested and stored in LB after the addition of 25% glycerol at -80°C.

Screening and identification of nanobodies against ASFV p30. The phage display VHH library was rescued by infection with helper phage M13K07. Three consecutive rounds of bio-panning were performed to obtain nanobodies with high affinity for ASFV p30 as described previously with minor modification (17). During bio-panning, VHHs specific for p30 were isolated from the library using a gradient of decreasing concentrations of coating antigens and increasing concentrations of Tween 20 in washing buffer. Briefly, microtiter ELISA plates were coated with 100 μ L of recombinant p30 protein at concentrations of 10, 5, and 2 μ g/mL for three rounds of bio-panning, respectively. The plates were incubated overnight (~16 h) at 4°C and washed three times with PBST (0.05% Tween 20 in PBS). The wells were blocked by incubation in 200 μ L of a blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) at 37°C for 1 h and washed with PBST. To decrease the off-target background, the phage display VHH library was incubated first with 2% skim milk in uncoated plates for 30 min and subsequently transferred to plates coated with antigen. After incubation for 1 h at room temperature (RT), the plates were washed nine times with 0.1% PBST (PBS containing 0.1% Tween 20) for the first round of bio-panning. In the following two rounds of bio-panning, the plates were washed with 0.2% and 0.3% PBST to remove free and weakly binding phages, respectively. The bound phages were eluted via digestion with 100 μ L of trypsin (1 mg/mL) for 10 min at RT, and 100 μ L of PBS with 4% skim milk was immediately added. The eluted phages were transferred to infect fresh growing *E. coli* TG1 cells for amplification and titration. The degree of specific phage enrichment was evaluated on the basis of the input and output phages of each round. Individual colonies were randomly selected from the final round of bio-panning and were tested against ASFV p30 by monoclonal phage ELISA as previously described (18). The obtained clones were cultured in 5 mL of $2 \times$ YT-AG medium (supplemented with 100 μ g/mL ampicillin and 1% glucose) and incubated overnight at 37°C. Next, 100 μ L of culture was inoculated into 20 mL of $2 \times$ YT-AG medium until the OD_{600} reached 0.4 to ~0.5 and was then infected with M13K07 helper phage at a multiplicity of infection (MOI) of 20. The phages were incubated with p30 in a microtiter plate, and capacity binding to the target protein was tested with rabbit anti-M13 and HRP-conjugated goat anti-rabbit antibodies. Finally, the VHH genes from positive clones identified by phage ELISA were sequenced (Sangon Biotech Co. Ltd., Shanghai, China) and classified according to the amino acid sequences of the CDR3 (Fig. 1).

Expression and purification of nanobodies. The VHH gene fragments were cloned into a pET-30a expression vector with EcoR I and XhoI restriction enzyme sites. The positive recombinant plasmids that

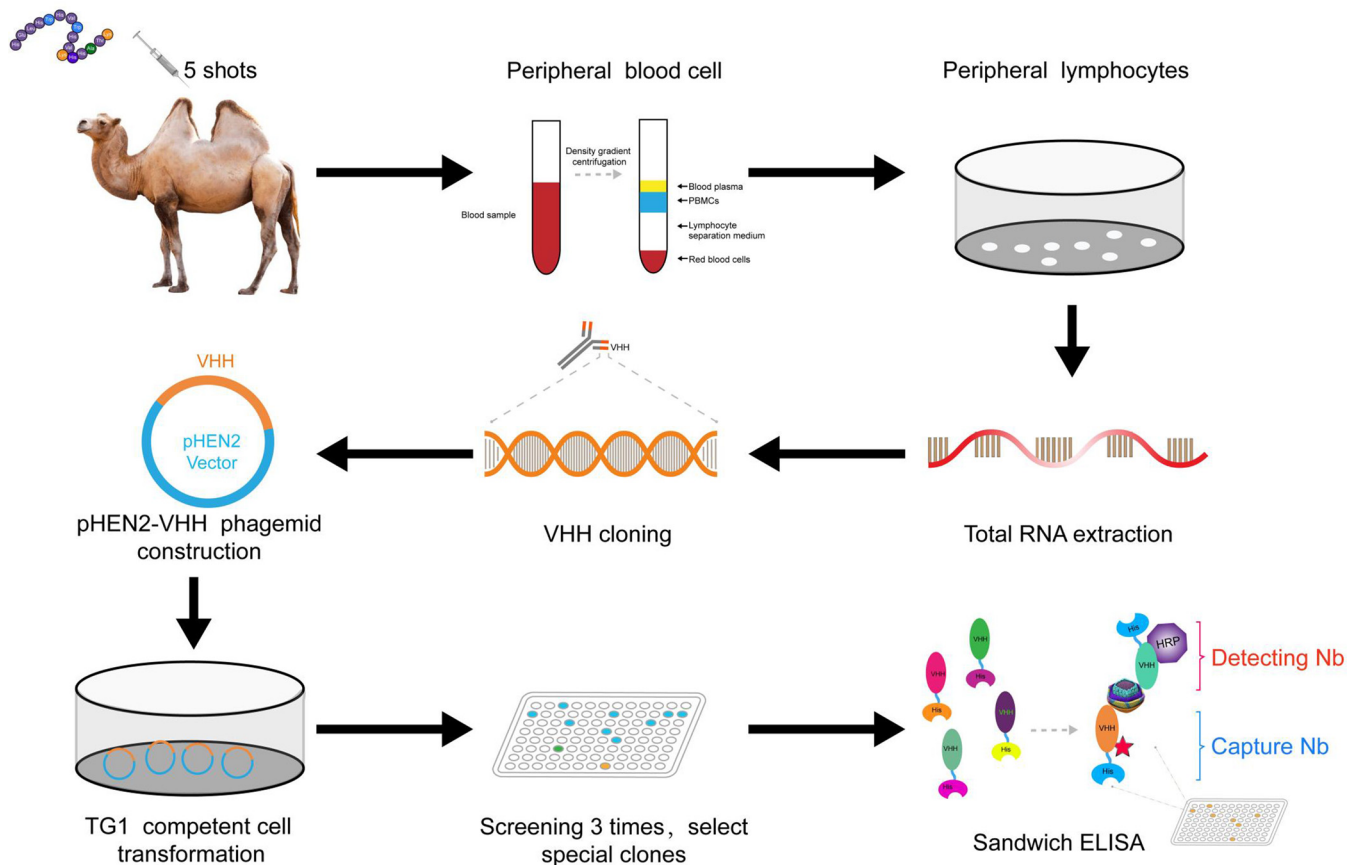


FIG 1 Schematic representation showing the isolation of specific nanobodies from the immunized phage display library and the development of the nanobody-based sandwich ELISA. The VHH genes were amplified from camels hyperimmunized with ASFV proteins and used for the construction of the phage display library. The specific nanobodies were identified using the phage display technique and further used in a sandwich ELISA for detection of ASFV.

had been verified by sequencing were transformed into *E. coli* BL21(DE3) competent cells to express the specific nanobodies. Briefly, the cells were cultured in LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ kanamycin at 37°C and 200 rpm/min. After the OD_{600} of the culture reached approximately 0.6, the expression of the nanobodies was induced by the addition of 0.1 mM IPTG and incubation for 6 h at 37°C with rotation at 200 rpm/min. The cells were collected by centrifugation, followed by resuspension in PBS and ultrasonication. The nanobodies were purified by nickel affinity chromatography (GE Healthcare, USA) according to the manufacturer's instructions. The purity of the obtained nanobodies was measured by SDS-PAGE and Western blotting with HRP-conjugated rabbit anti-His IgG (Abcam, UK). The concentration of the nanobodies was determined by fluorometric quantification (Qubit, Thermo Fisher Scientific) using a protein assay kit following the manufacturer's protocols, and they were stored at -80°C at a concentration of 1 mg/mL until further use.

Analysis of nanobody specificity. To verify the specificity and affinity of nanobody binding to the ASFV p30 antigen, porcine alveolar macrophages (PAMs) were infected with ASFV at an MOI of 0.2. Then, 48 h postinfection (hpi), the cells were harvested and lysed with ice-cold RIPA lysis buffer (Thermo Fisher Scientific, China) supplemented with protease inhibitors (protease inhibitor cocktail [PIC] and phenylmethylsulfonyl fluoride [PMSF]) by incubation for 30 min in an ice bath to extract proteins. The protein concentration was determined by fluorometric quantification using a protein assay kit (Qubit, Thermo Fisher Scientific) according to the manufacturer's protocol. The protein was electrophoresed on a 12% polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane, followed by blocking with 5% (wt/vol) BSA for 2 h at RT and washing three times with TBST. Then, the membranes were incubated overnight with nanobodies at 4°C and washed again. The reactivity of the nanobodies toward the viral p30 protein was tested with HRP-conjugated rabbit anti-His IgG (Abcam, UK).

Pairwise selection for sandwich Nb-ELISA. An indirect ELISA was designed to detect the binding activity of each nanobody with the ASFV p30 antigen. PAMs were infected with ASFV at an MOI of 1.0. Then, 96 h postinfection, both the cells and supernatants were harvested and lysed by three freeze-thaw cycles through incubation at -80°C and room temperature. Cell debris was removed by centrifugation at 4°C and $12,000 \times g$ for 15 min. The cell-free supernatant was collected and coated on microtiter ELISA plates at 4°C and incubated overnight, and PAM cell culture was used as the negative control. The plates were blocked with 2% BSA in PBST and washed three times. Serial dilutions of nanobodies

(100 ng/mL, 10 ng/mL, 1 ng/mL, and 0.1 ng/mL) were added and incubated with ASFV particles for 1 h at 37°C. After washing, HRP-conjugated rabbit anti-His IgG (Abcam, UK) was added and incubated for 1 h at 37°C. The plates were washed again, and tetramethylbenzidine (TMB) peroxidase substrate (KPL, Gaithersburg, MD) was added for colorimetric quantification. The enzymatic reaction was stopped by the addition of 0.3 M H₂SO₄, and the absorbance of each well was measured at 450 nm with a microtiter plate reader (Thermo Fisher, USA). The nanobody with optimal antigen-binding capacity was quantified at a concentration of 1 mg/mL and conjugated with HRP using a labeling kit (Proteintech, China) according to the manufacturer's instructions.

To detect the capture capacity of the nanobodies, the wells of ELISA plates were coated with 10 µg/mL nanobody and incubated at 4°C overnight. Blocking and washing of the plates was carried out under the conditions as described above. Serial dilutions of the p30 recombinant protein (1:200, 1:400, 1:800, and 1:1,600) were added to each nanobody-coated well and incubated for 1 h at 37°C. Following three washes with PBST, the optimized HRP-conjugated nanobody was added and incubated for 30 min at 37°C. After extensive washing, the HRP enzymatic reaction was developed and stopped by the addition of TMB and 0.3 M H₂SO₄, respectively. The OD_{450nm} of each well was immediately measured. The nanobody exhibiting optimal capture activity was selected for further analysis.

Development of the nanobody-based sandwich ELISA. One pair of nanobodies was employed in sandwich ELISA for the detection of ASFV. One nanobody, exhibiting a higher capture capacity, and the other, conjugated with HRP, served as the capturing and detecting antibodies in this assay, respectively. To achieve optimal performance, checkerboard titration was performed to optimize the concentrations of the capture and detection nanobodies. Briefly, 96-well microtiter plates were coated with different amounts of capture nanobodies (1.0 µg, 0.75 µg, 0.5 µg, and 0.25 µg in each well) and incubated at 4°C overnight. The plates were blocked with 200 µL of 1% BSA at 37°C for 1 h and then washed three times with PBST. ASFV-infected or uninfected cell culture supernatants prepared previously were separately added to nanobody-coated wells and incubated at 37°C for 1 h. After washing three times, serial dilutions of the HRP-conjugated nanobody used as the detection antibody (1:1,000, 1:2,000, 1:4,000, and 1:8,000) were added and incubated at 37°C for 30 min. After repeated washing, the enzymatic reaction was developed and measured as described above. All assays were conducted in duplicate. The optimal concentrations of capture nanobody and HRP-conjugated detecting nanobody were determined at the highest ratio of OD₄₅₀ between the virus-infected and uninfected samples.

To minimize interference and nonspecific binding, various blocking buffers were tested to determine their ability to block residual binding sites and decrease nonspecific signaling. The capture and detection antibodies were used at the optimal concentrations for the subsequent tests. The coated wells were blocked with 1% and 2% BSA, 0.25% casein, 5% skim milk, or 5% horse serum at 37°C for 1 h. Furthermore, the incubation times for testing the samples (30, 45, and 60 min), the HRP-conjugated detecting nanobody with antigen (30, 45, and 60 min), and the colorimetric reaction (5, 10, and 15 min) were optimized. The optimal blocking buffer and incubation time yielding the highest ratio of positive-to-negative (P/N) value were determined.

Sandwich Nb-ELISA cutoff value. To determine the cutoff value for the sandwich Nb-ELISA, 184 samples (120 whole-blood with EDTA, spleen, liver, kidney, and lung samples from 16 animals) were collected individually from healthy swine that had tested negative for ASFV, as determined with a real-time PCR assay performed as recommended by WOA and a commercial ELISA kit (INGEZIM PPA DAS; Ingenasa, Spain), were assessed using the developed sandwich Nb-ELISA. The whole-blood samples in EDTA were initially frozen and thawed three times before use, and the tissue samples were weighed and homogenized in PBS buffer (0.01 M, pH 7.2) to obtain a 10% suspension (wt/vol, 1 g of tissue in 10 mL of PBS). The whole-blood in EDTA samples were used without dilution in this assay. The ELISAs were performed according to the conditions and procedures described above. The cutoff value was determined by the mean OD₄₅₀ value of 184 negative samples plus three standard deviation (SD) values (95% confidence interval). The test samples with OD₄₅₀ values less than $\bar{X} + 3SD$ (with $\bar{X}$ representing the mean value of the OD₄₅₀ value of 184 negative samples) were considered ASFV negative, and the others were considered ASFV positive.

Cross-reaction and detection limit of the sandwich Nb-ELISA. To analyze the specificity of the sandwich Nb-ELISA, inactivated suspensions of CSFV, PRRSV, PRV, PPV, PCV2, and FMDV-O were selected for testing. Likewise, ASFV suspensions and the negative-control samples obtained from uninfected PAM cell debris were tested with this assay. The detection limit of the sandwich Nb-ELISA was evaluated. First, 2-fold serial dilutions of the ASFV p30 protein were tested to determine the sensitivity of this assay, and PBS was used as the blank control. Moreover, ASFV stocks (CN/SC/2019) were titrated on PAM cell culture in 96-well plates using the hemadsorption (HAD) assay according to a previous report (19), and 50% HAD doses (HAD₅₀) were calculated using the Reed and Muench method (20). The virus was serially diluted 10-fold using the PAM culture medium and detected by the sandwich Nb-ELISA. The cross-reaction and detection limits were determined according to the cutoff value. All analyses were performed in triplicate.

Comparison of the sandwich Nb-ELISA and a commercial ELISA kit. The performance of the sandwich Nb-ELISA was evaluated and compared with that of a commercial kit (INGEZIM PPA DAS; Ingenasa, Spain) with clinical swine samples which had been tested by the WOA real-time PCR. In total, 162 blood and 120 tissue samples (spleen, liver, lung, heart, and kidney) provided by the ASF Regional Laboratory of China (Lanzhou) were processed in accordance with the requirements described above and analyzed simultaneously using both the developed sandwich Nb-ELISA and a commercial ELISA kit. This kit is also based on a double antibody sandwich ELISA; sample with an OD₄₀₅ value higher than 0.300 is considered positive to ASFV, sample with an OD₄₀₅ value lower than 0.150 is considered negative, and sample with an OD₄₀₅ value between both values is considered doubtful. The consistency between the sandwich Nb-ELISA and the commercial kit was evaluated on the basis of the test results of each sample. Moreover, tissue

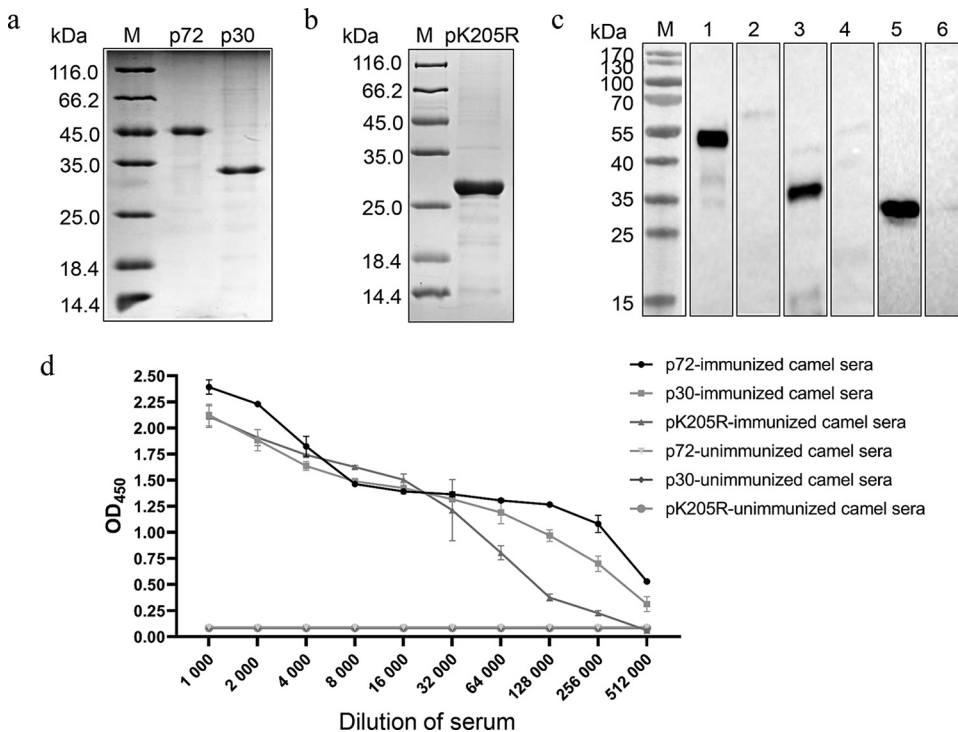


FIG 2 Preparation of ASFV recombinant proteins and their immune responses in camels. (a) SDS-PAGE analysis of the purification of ASFV p72 and p30. (b) SDS-PAGE analysis of the purification of ASFV K205R. (c) Antigenic analysis of recombinant proteins by Western blotting with ASFV-positive serum and HRP-labeled rabbit anti-pig antibody as primary and secondary antibodies, respectively. M, protein molecular marker; lane 1, p72; lane 3, p30; lane 5, K205R; lanes 2, 4, and 6, negative controls. (d) The antibody titers against ASFV proteins in the serum from immunized camel samples.

(spleen, kidney, liver, and lung) and whole-blood samples collected from a sick pig, which were confirmed to be ASFV-positive by quantitative PCR (qPCR), were tested with the two methods. The samples were prepared and serially diluted 10- or 2-fold with PBS and further analyzed with the sandwich Nb-ELISA described here. Correspondingly, the samples were processed and detected with the commercial kit according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed with Excel 2010 software (Microsoft, Inc., USA) or Prism software version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between two groups were performed using paired *t* tests, and differences were considered statistically significant at a *P* value of <0.05 . The kappa index value was calculated to compare the coincidence rates of the results obtained with the sandwich Nb-ELISA, a commercial kit, and the WOA real-time PCR with SPSS software version 25.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Preparation of recombinant proteins. Three recombinant ASFV proteins (p30, K205R, and truncated p72) were prepared and used for camel immunization. SDS-PAGE analysis revealed that these proteins were expressed and purified successfully, as indicated by the bands representing the expected molecular weights (Fig. 2a and b). In addition, a Western blot analysis showed that the p30, K205R, and p72 recombinant proteins were specifically recognized by the positive swine sera against ASFV (Fig. 2c), indicating that the antigens used to immunize the animals were appropriate.

Construction of a phage display VHH library. After primary immunization and four booster injections, the antibody titers against the target proteins were tested in camel sera by indirect ELISA. The antibody titers of the sera reached 1:512,000, 1:512,000, and 1:128,000 for the p30, p72, and K205R proteins, respectively (Fig. 2d), suggesting robust immune responses against the corresponding proteins in camels. Total RNA extracted from peripheral blood lymphocytes of the immunized camels was used to construct a VHH phage display library (Fig. 1). The VHH genes with expected sizes of approximately 400 bp were amplified from synthesized cDNA by nested PCR (see Fig. S1a and b in the supplemental material). The purified VHH fragments were inserted into the phagemid vector

TABLE 1 Degree of enrichment of the nanobodies in the phage display library as detected through three rounds of bio-panning

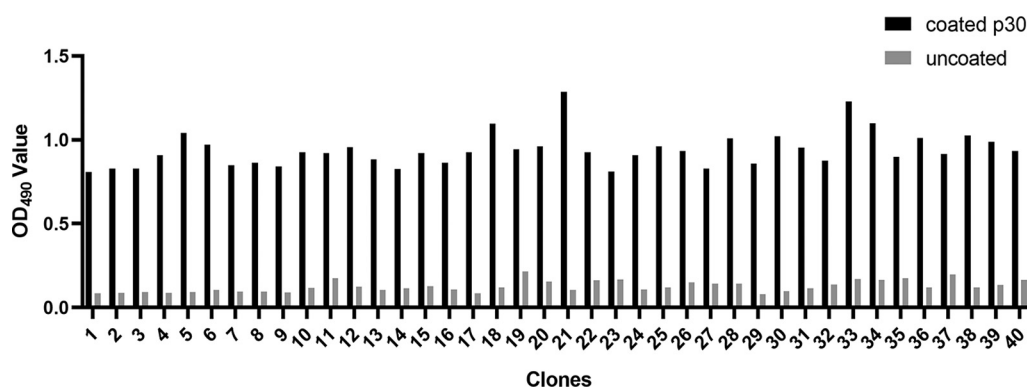
Round	Input	Output	Enriching factor (input/output)
1st	3.80×10^{11}	6.40×10^5	5.94×10^5
2nd	2.89×10^{12}	1.40×10^8	2.06×10^4
3rd	2.10×10^{11}	6.30×10^8	3.33×10^2

PHEN2 and electroporated into *E. coli* TG. The phage display VHH library against the p30, K205R, and p72 proteins of ASFV was constructed successfully with a library capacity of 2.2×10^8 CFU/mL. Individual clones ($n = 60$) were randomly selected and screened by PCR analysis, 55 (91.2%) of which contained the inserted fragment with the VHH gene of the expected size (Fig. S1c). The positive clones were sequenced, and each clone contained distinct complementary determining regions (CDRs), indicating the rich diversity of the generated library (Fig. S2).

Library screening and identification of specific nanobodies against the p30 protein. In the present study, nanobodies against the ASFV p30 protein were screened, and three consecutive rounds of phage bio-panning were conducted. The results showed that the enrichment of the binding phages against target protein increased remarkably during the bio-panning (Table 1). A total of 40 clones were randomly selected from the final round of bio-panning and subjected to monoclonal phage ELISA to identify p30-binding phages, and all of them exhibited a positive immune response with the target protein, in contrast to the negative control (Fig. 3). Sequence alignment based on the amino acid sequences of highly variable CDR1, CDR2, and CDR3 ultimately revealed 19 individual nanobodies (Fig. S3). According to the reactivity against the target protein and frequency of the VHH sequence, four nanobodies (Nb17, Nb30, Nb33, and Nb35) were selected and used in further research (Fig. S4).

Expression and purification of nanobodies. The nanobody was expressed in a prokaryotic expression system and purified using an Ni-NTA affinity column. The VHH genes of Nb17, Nb30, Nb33, and Nb35 were subcloned into a pET-30a expression vector and transformed into *E. coli* BL21(DE3). After expression and purification, SDS-PAGE revealed that the four purified nanobodies exhibited the expected molecular weights (Fig. S5a and b). Moreover, Western blot assays revealed that the nanobodies were recognized by the anti-His monoclonal antibody, shown by the specific band presented in Fig. S5c.

Specificity analysis of the nanobodies. The specificity and reactivity of the nanobodies with ASFV were evaluated by Western blotting. The ASFV genotype II strain CN/SC/2019 was cultured and applied to assess the binding reactivity of Nb17, Nb30, Nb33, and Nb35, and the immunoreactivity of these nanobodies toward ASFV was detected with the anti-His monoclonal antibody. The Western blotting results revealed

**FIG 3** Identification of ASFV p30 protein-binding phages by monoclonal phage ELISA.

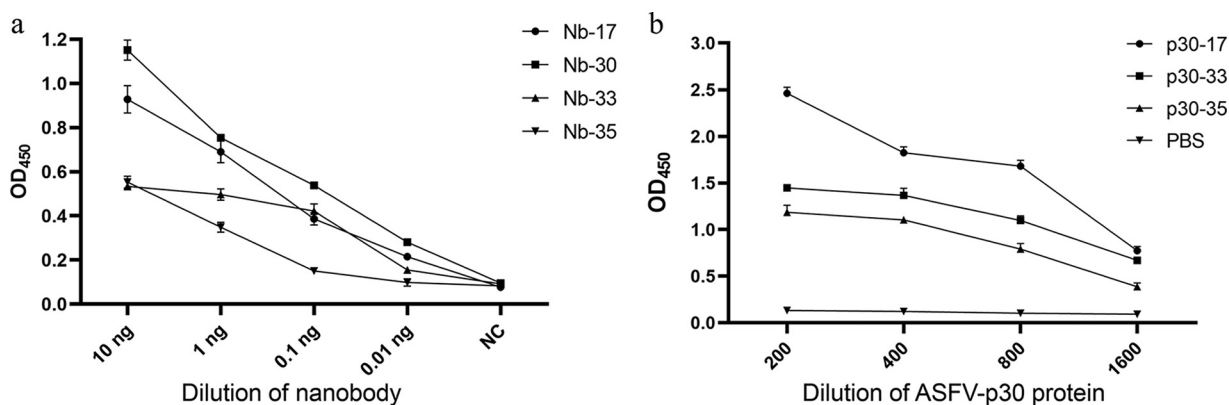


FIG 4 Specificity and binding activity of four nanobodies against ASFV p30 by ELISA. (a) The ASFV p30 protein-binding activity of the four nanobodies at different dilutions. The different amounts of Nb17, Nb30, Nb33, and Nb35 nanobodies were incubated individually with coated ASFV particles, and the immunoreaction was detected and analyzed using the HRP-conjugated anti-His monoclonal antibody. Nb30 at different concentrations exhibited the highest reactivity with ASFV. (b) The capture capacity of the Nb17, Nb33, and Nb35 nanobodies at different dilutions when paired with Nb30 for use with the sandwich Nb-ELISA. Serial dilutions of the p30 recombinant protein were incubated individually by coating the Nb17, Nb33, and Nb35, and the immunoreaction was detected and analyzed using the HRP-Nb30. The Nb17 paired with HRP-Nb30 exhibited the highest capture efficiency.

that all four selected nanobodies specifically bound with ASFV, and Nb17 and Nb30 showed higher binding activity than Nb33 and Nb35 (Fig. S5d).

Pairwise selection for sandwich Nb-ELISA. The selection of paired detection and capture antibodies is critical for the performance of double antibody sandwich ELISA. In this study, an indirect ELISA was employed to select the detection antibody used in this assay. The different amounts of Nb17, Nb30, Nb33, and Nb35 nanobodies were incubated individually with coated ASFV particles, and the immunoreaction was detected and analyzed with the HRP-conjugated anti-His monoclonal antibody. The results showed that, compared with other nanobodies, Nb30 at different concentrations exhibited the highest reactivity with ASFV particles (Fig. 4a). Nb30 was subsequently conjugated to HRP and used as the detection antibody in the sandwich ELISA. The optimum capture antibody paired with detection antibody Nb30 was then determined. The Nb17, Nb33, and Nb35 nanobodies were tested separately at the same concentrations to determine their capture effects when paired with Nb30. The results revealed that Nb17 paired with Nb30 exhibited the highest capture efficiency (Fig. 4b). Thus, the antibody pair Nb17 and Nb30 was used for the development of the sandwich ELISA for the detection of ASFV.

Development of the sandwich Nb-ELISA. The optimal concentrations of capture antibody Nb17 and HRP-labeled detection antibody Nb30 for the sandwich Nb-ELISA were determined using a checkerboard titration assay. The results showed that the optimal concentration of Nb17 was 5.0 μ g/mL, and the optimal dilution of HRP-Nb30 was 1:2,000, as shown by a higher P/N value (Fig. 5a). Under these conditions, the absorbance value of the positive sample was closest to 2.0 and that of the negative sample was less than 0.1 (data not shown). Using the determined dilutions of the capture and detection antibodies, a suitable blocking buffer was identified for this assay. The results showed that 2% BSA contributed to the highest blocking efficiency compared to the other tested blocking reagents (Fig. 5b). Moreover, the incubation times for each step of the assay were optimized; they were 30, 30, and 10 min at 37°C for antigen capture by Nb17, the HRP-labeled Nb30, and the substrate, respectively (Fig. 5c to e).

Cutoff value for the sandwich Nb-ELISA. The cutoff value for the developed sandwich Nb-ELISA was determined by testing 184 negative samples which had been detected and verified by qPCR and a commercial ELISA kit. The OD₄₅₀ values of these samples were measured as described above, and they ranged from 0.0507 to 0.2311, with an average OD₄₅₀ value of 0.1337 with an SD of 0.0392. The cutoff value for the assay was thus determined to be 0.2513 (0.1337 + 3 SD). As a result, the samples detected by sandwich ELISA with OD₄₅₀ values of ≥ 0.2513 were determined to be positive for ASFV.

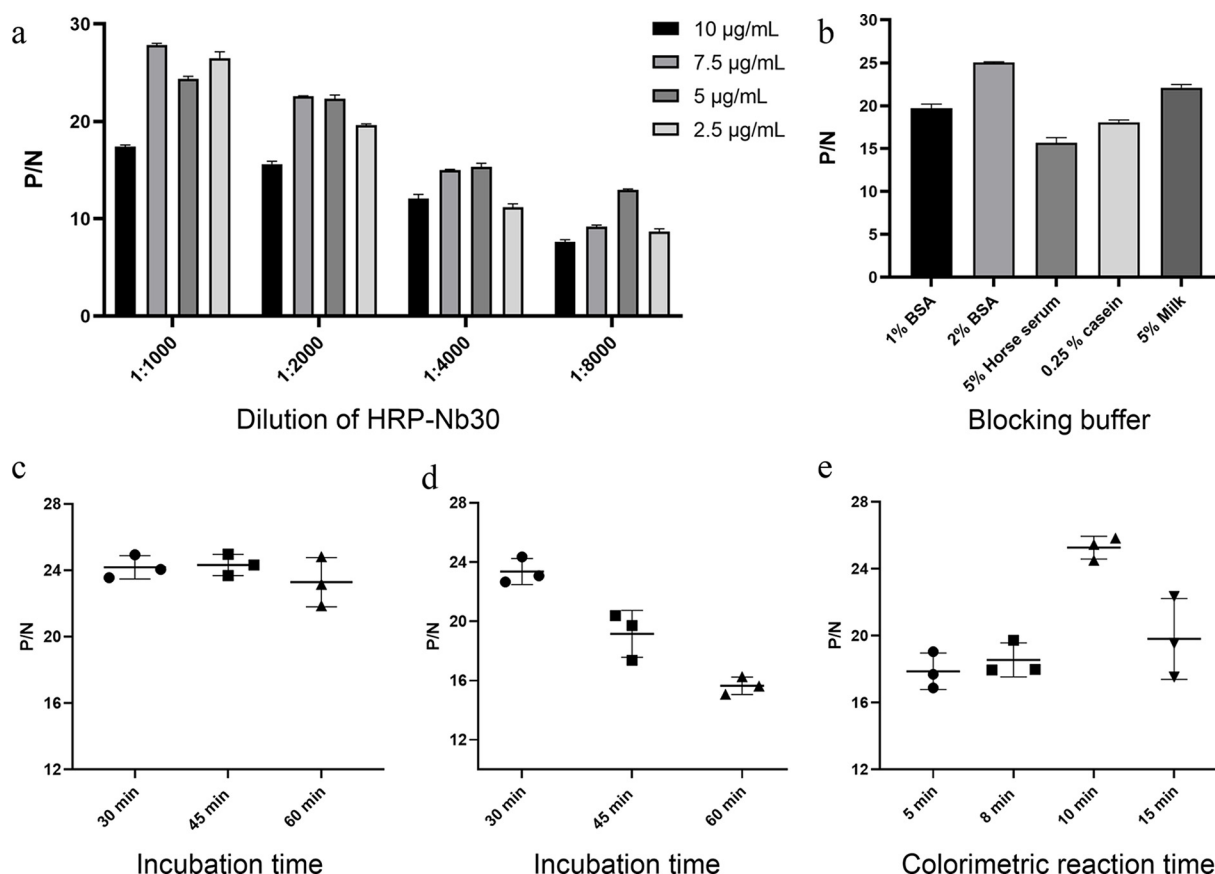


FIG 5 Determination of the optimal conditions for the sandwich Nb-ELISA. (a) Determination of the optimal concentrations of capture antibody Nb17 and detection antibody HRP-Nb30 as determined by checkerboard titration. (b) The blocking efficiency of different blocking buffers. (c) Determination of the sample incubation time. (d) Determination of the HRP-Nb30 detection antibody incubation time. (e) Determination of the colorimetric reaction time.

Cross-reaction and detection limit of the sandwich Nb-ELISA. The cross-reaction of the sandwich Nb-ELISA toward other swine disease viruses was assessed. Six viruses (CSFV, PRRSV, PRV, PPV, PCV2, and FMDV-O) were tested in triplicate with this assay, and the mean OD₄₅₀ values ranged from 0.0909 to 0.1086; the detection value for ASFV was 1.9461 (Fig. 6a). These results indicated that the nanobody-based sandwich ELISA was specific for the detection of ASFV. The sandwich Nb-ELISA was further assayed with 2-fold serially diluted p30 protein. The detection limit of this technique was approximately 1.1 ng/mL (41.97 pmol/L) target protein (Fig. 6b). In addition, the different dilutions of ASFV were tested using the sandwich Nb-ELISA, and the results showed that the detection limit of this technique was estimated at 10^{2.5} HAD₅₀/mL of ASFV (Fig. 6c).

Validation of the proposed sandwich Nb-ELISA. To assess the diagnostic efficacy of the proposed sandwich Nb-ELISA, 282 clinical swine samples were tested with the sandwich Nb-ELISA in parallel with a commercial kit. Among these samples, 62 samples were positive and 220 samples were negative as determined by the WOAH real-time PCR. Using the proposed sandwich Nb-ELISA, 54 samples were determined to be positive and 228 samples were negative. The commercial kit analysis revealed that 51 samples were positive, 216 samples were negative, and 15 samples were considered doubtful. The results of both the proposed sandwich Nb-ELISA and the commercial kit were consistent in 264 of the 282 clinical samples, with a coincidence rate of 93.62% (Table 2). The comparison between the Nb-ELISA and the WOAH real-time PCR exhibited a coincidence rate of 97.16% (Table 2). Moreover, statistical analysis revealed a high degree of coincidence between the Nb-ELISA and the commercial kit (kappa coefficient, 0.816) and the WOAH real-time PCR (kappa coefficient, 0.913) (Table 2). Taking

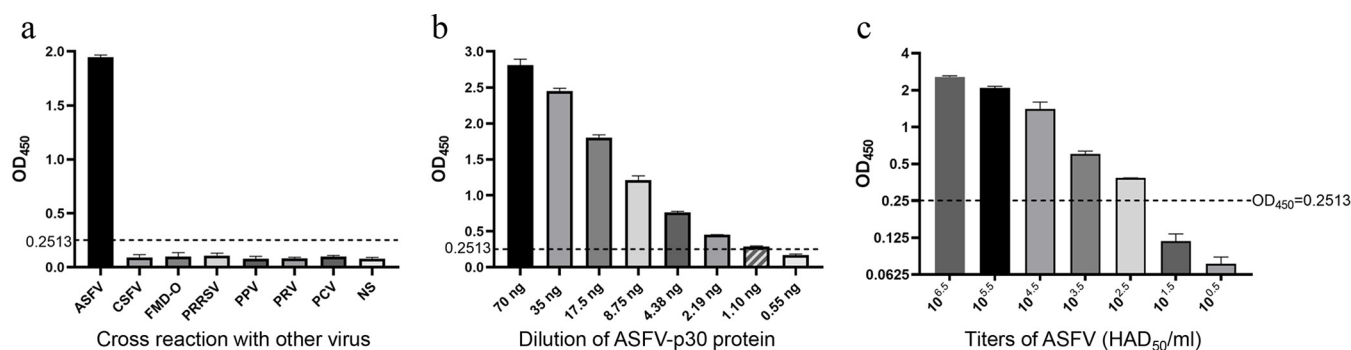


FIG 6 Specificity and detection limits of the sandwich Nb-ELISA. (a) Specificity analysis of the sandwich Nb-ELISA. The inactivated suspensions of CSFV, PRRSV, PRV, PPV, PCV2, and FMDV-O were tested with the developed sandwich Nb-ELISA. (b) The detection limit of the sandwich Nb-ELISA by testing recombinant p30 protein. Two-fold serial dilutions of the recombinant p30 protein were tested to determine the analytical sensitivity of the developed sandwich Nbs-ELISA. (c) The detection limit of the sandwich Nbs-ELISA by testing the ASFV at different titers. Serial dilutions (10-fold) of the ASFV suspensions ($10^{6.5}$ HAD₅₀/mL) were tested to determine the analytical sensitivity of the developed sandwich Nbs-ELISA.

the WOH real-time PCR as a reference assay, eight false negatives were detected out of 62 ASFV-positive samples with the Nb-ELISA (Table 2), which gave a diagnostic sensitivity (Dse) of 87.1% and a diagnostic specificity (Dsp) of 100%. The detection sensitivity of the sandwich Nb-ELISA was further assessed using ASFV-positive clinical samples at different dilutions and was compared with the results obtained with a commercial kit. Blood and tissue samples (spleen, kidney, liver, and lung) collected from a sick pig were used in the assays. The clinical detection limit of the proposed sandwich Nb-ELISA reached 1:320 for the blood, liver, and kidney samples and 1:80 for the spleen and lung samples. However, the commercial kit detection limits were 1:160 for the liver, 1:80 for the spleen, and 1:40 for the blood, lung, and kidney samples (Fig. 7). These findings suggested that the sandwich Nb-ELISA showed higher detection sensitivity than the commercial kit ($P < 0.05$).

DISCUSSION

Since the introduction of ASF into Georgia in 2007, the widespread and frequent outbreaks of the disease caused by a highly virulent p72 genotype II have occurred in many countries in Europe and Asia, particularly after its identification in China in 2018 (7). The high morbidity and mortality of ASFV genotype II isolates, coupled with a large-scale endemic in the pig-raising countries, have already led to substantial socio-economic impacts, and many relevant industries have been involved. The extraordinary resistance of ASFV under various environmental conditions facilitates the transmission and spread of the disease, making the control and eradication even harder to achieve in endemic regions (21). Considering the historical impact of ASF in Europe in the past century, it is still a serious threat to the pig industry worldwide, and controlling the disease has been becoming a great challenge.

The recognition of nanobodies in camels is more recent and has provoked great interest in their potential applications in various research fields (12). Over the past 2 decades,

TABLE 2 Comparison of the developed sandwich Nb-ELISA with a commercial ELISA kit and the WOH real-time PCR by detecting clinical samples

Methods and determination indexes	Commercial kit				WOAH-qPCR					
	Positive (n)	Negative (n)	Doubtful (n)	Total	Agreement (%)	Kappa	Positive (n)	Negative (n)	Total (n)	Agreement (%)
Nbs- ELISA										
Positive	51	3	0	54	93.62	0.816	54	0	54	97.16
Negative	0	213	15	228			8	220	228	
Total	51	216	15	282			62	220	282	

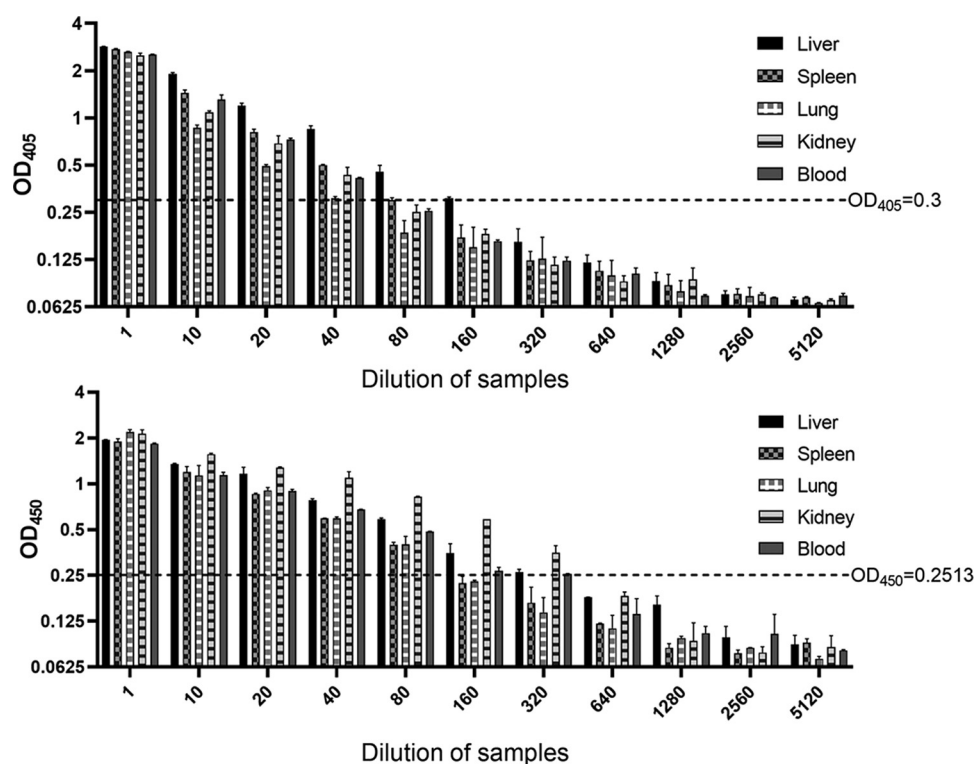


FIG 7 Evaluation and comparison of the sensitivity of the sandwich Nbs-ELISA (bottom) and that of a commercial ELISA kit (top) with diluted ASFV-positive samples.

great effort has been directed to exploring and improving the performance of nanobodies, which exhibit many advantages over traditional antibodies when applied as diagnostics, therapeutics, and research reagents (22–24). In the present study, a phage display VHH library was successfully constructed from camels hyperimmunized with the recombinant proteins p30, p72, and K205R of ASFV. It has been well documented that the size and sequence diversity of the VHH library are considered to be crucial factors for screening high-quality nanobodies, and they can be frequently obtained from a large immune library (25). As described here, the generated VHH library exhibited high capacity and genetic diversity, suggesting its competence for further selection of high-affinity binders with the target antigens of ASFV.

Primarily, nanobodies against ASFV p30 were screened and identified from the camelid immune VHH library by bio-panning. The viral phosphoprotein p30 is an important structural protein located in the inner membrane of the ASFV particle (26, 27). As one of the most immunogenic viral proteins, p30 is abundantly expressed in the cytoplasm of infected cells during the early stage of infection, and its expression persists throughout the viral life cycle (27–29). In this study, 19 (up to 47.5%) individual nanobodies against p30 were classified from 40 randomly selected clones on the basis of sequence alignment (Fig. S3). The framework regions of these nanobodies were highly conserved, and the CDR regions were distinct. Moreover, typical amino acid substitutions were observed at positions 37, 44, 45, and 47 in the framework region 2 (FR2) of the nanobodies, indicating that they were camelid-derived heavy-chain antibodies (30). These results verified the high capacity, rich diversity, and superior quality of the constructed phage display library, and the specific nanobodies against ASFV p72 and K205R can likely be further acquired from the library through the phage display technique. Furthermore, due to the previously mentioned intrinsic characteristics, the p30-specific nanobodies obtained in this study are valuable alternatives to use in a wide variety of applied and basic research areas, such as tracing antigens or viruses in living cells and determining a neutralizing effect, or in antiviral research and as biosensors in diagnostic applications.

Since no commercial vaccine or treatment is available, the prevention and control of ASF currently rely on the implementation of early detection and strict sanitary measures (3). Because of the acute characteristics and severe viremia of ASFV genotype II that has occurred internationally (7), virological tests are vital for identification of viral infection timely, and the effective control measures could be therefore implemented rapidly in virus-affected areas. Even though virus isolation is the WOA-recommended gold standard for the diagnosis of ASF, this process is time-consuming, labor-intensive, and requires highly skilled personnel and sophisticated facilities (a high-level biosafety laboratory) (7). Various PCR-based technologies, especially real-time PCR, and isothermal amplification assays have been widely used in the detection of viral genomes in clinical samples. Although these methods are robust and suitable for high-throughput analysis for disease surveillance, their applications are still constrained by the equipment needed, costs, operator training requirements, and frequent aerosol contamination (31). In addition to these techniques, double-antibody sandwich ELISA for the detection of viral antigens is a cost-effective, rapid, and convenient method for diagnosis (32). The sandwich format assay requires two antibodies specific for different epitopes of the antigen, and conventional polyclonal and monoclonal antibodies are the most commonly employed. In this study, ASFV p30-specific nanobodies obtained by the phage display technique were evaluated and employed for the first time in the development of a sandwich ELISA for the detection of ASFV infection. According to comparative tests, the high-affinity nanobody Nb17 was designated as the captured antibody, and HPR-coupled Nb30 served as the detector antibody in this assay. The reaction conditions of the novel proposed nanobody-based sandwich ELISA have been optimized, and the cutoff value was determined by testing 184 negative samples. With the cutoff value, this assay exhibited high sensitivity and specificity, and no cross-reaction was observed when other porcine viruses were tested. Generally, the preferable performance of nanobody-based immune assays is guaranteed by the high affinity and epitope-specific recognition of the nanobodies (13). Attempted applications of nanobodies in laboratory tests, especially for use in the diagnosis of infectious diseases, have been well documented. Numerous nanobody-based ELISA methods have been described for the detection of specific etiological agents, such as SARS-CoV-2, Zika virus, PCV2, PEDV, and Newcastle disease virus (NDV), and have exhibited superior performance in clinical practice (24, 32–35). The specific nanobodies obtained in this study, combined with the sensitive ELISA method, suggest a valuable and alternative technique for the large-scale detection and surveillance of ASFV in animals in endemic areas.

Currently, the only available antigen ELISA kit (INGEZIM PPA DAS; Ingenasa, Spain) for the detection of ASFV is manufactured by Ingenasa, and it is based on the use of traditional monoclonal antibodies against the major viral capsid protein p72, enabling the accurate analysis of acute infection in blood and tissue samples. The efficacy of the nanobody-based sandwich ELISA developed in this study was compared with that of the commercial kit. The coincidence rate of the two assays with 282 clinical samples was 93.62% (Table 2), suggesting high correlation and agreement between the nanobody- and monoclonal antibody-based assays. In addition, both assays were applied to the detection of virus in blood and tissue samples collected from a sick pig affected by ASFV and diluted to various concentrations. The detection limits of the nanobody-based assay were comparable to those of the monoclonal antibody in diluted spleen samples but were significantly higher in diluted blood, liver, kidney, and lung samples (Fig. 7), suggesting its higher sensitivity compared to the traditional method ($P < 0.05$). The superiority and favorable properties of nanobodies make the novel sandwich Nb-ELISA ideal for the diagnosis of ASFV infection performed in laboratories, especially in laboratories with limited resources. Moreover, the performance of this novel method was evaluated by testing genotype II strains of ASFV in this study; other ASFV genotype strains should be further tested and evaluated in the future.

In conclusion, a diverse phage display library consisting of specific VHs raised against p30, p72, and K205R of ASFV was successfully constructed. Using the phage display

technique, 19 nanobodies specifically binding to the ASFV-p30 protein were identified and isolated by three rounds of bio-panning. Subsequently, a sandwich Nb-ELISA with Nb17 and HPR-coupled Nb30 as capture and detector reagents, respectively, was developed for the detection of ASFV in blood and tissue samples. In comparison, high correlation and agreement were observed between the Nb-ELISA and the commercial kit and the WOAHP real-time PCR for the detection of ASFV in clinical samples, and the results showed no cross-reactivity with other porcine viruses. This nanobody-based immune assay offers an affordable and valuable tool for detection of ASFV from affected animals during an acute outbreak of disease.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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