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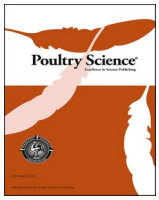

Full-Length Article

A highly sensitive one-step nanobody-based immunoassay to specifically detect antibodies against fowl adenovirus serotype 4

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ABSTRACT

Hepatitis-hydropericardium syndrome, caused by fowl adenovirus serotype 4 (FAdV-4), has resulted in significant economic damage to the poultry industry. To monitor viral exposure and vaccine efficacy, some traditional antibody-based immunoassays have been developed for detecting anti-FAdV-4 antibodies. However, these assays have some drawbacks including multi-step operations and higher production cost. Recently, nanobodies are regarded as a promising tool for developing immunoassays. In the study, 23 nanobodies against FAdV-4 were screened and expressed with horseradish peroxidase (HRP) in the HEK293T cells. Then, the FAdV-4-Nb28-HRP fusion protein was selected for developing competitive enzyme-linked immunoassays (cELISA) to detect anti-FAdV-4 antibodies in the chicken sera. The optimal concentrations and dilutions for the coating antigen, fusion protein and testing sera were determined to be 400 ng/well, 1:80 and 1:20, respectively. After the coated plates were vacuumized and stored, the operation of cELISA to detect clinical chicken sera was only one-step and the full time was 75 min. The cELISA also exhibited high sensitivity, specificity, reproducibility and good agreement with the commercial ELISA kit. When the sequential sera from the challenged chickens were tested, the cELISA showed superior sensitivity compared with the commercial ELISA kit. Moreover, epitope mapping revealed that the nanobody specifically recognized the sites GLN235 ASN236 SER238 of the fiber-1 protein, highly conserved among different FAdV-4 isolates and different from the FAdV-1 and -8. The results indicated that cELISA can specifically detect anti-FAdV-4 antibodies. Collectively, the developed one-step nanobody-based cELISA is an ideal method for epidemiological investigation and vaccine immune evaluation of FAdV-4.

Introduction

Fowl adenovirus (FAdV) is a non-enveloped virus with double-stranded DNA, affiliating to the family *Adenoviridae* and the genus *Aviadenovirus* (Benkö and Harrach, 2003) and is divided into three groups according to the genetic structure and sequence length. Group I (FAdV-I), isolated from chickens, turkeys, geese and quail with a common group-specific antigen, is further classified into five genotypes A-E with 12 serotypes (FAdV-1 to −7, −8a, −8b, −9, −10, −11) based on restriction enzyme digestion patterns and serum cross-neutralization tests (Hess, 2000; Zhang et al., 2024; Zsák and Kisary, 1984). Group II (FAdV-II) comprises the hemorrhagic enteritis virus (HEV) in turkeys and the marble spleen disease virus in pheasants. Group III (FAdV-III) is mainly linked to egg drop syndrome virus. At present, hepatitis-hydropericardium syndrome (HHS), caused by FAdV-4, has led to serious economic damage in the global poultry industry (Xie, et al., 2022). Since the first outbreak of HHS in China in 2015, HHS has been frequently identified in China with a mortality rate range of 20–80 % (Khawaja, et al., 1988; Pan, et al., 2017; Schachner, et al., 2018). In order to reduce the economic burden caused by HHS, the immunization with an inactivated vaccine is one of the most important methods for the prevention and control of FAdV-4 infection (Pouladi, et al., 2024). Serological surveillance is critical for assessing viral exposure and evaluating vaccine effectiveness in a flock, playing a key role in disease control (Zi, et al., 2018). In recent years, some methods for the detection of anti-FAdV antibodies have been developed for large-scale serological investigations and vaccine evaluation. Enzyme-linked immunosorbent assay (ELISA) is a more promising candidate for large-scale serological

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Table 1
Sequences of the primers used in this study.

Primers	sequences(5′−3′)	Usage
CALL001	GTCCTGGCTGCTCTTCTACAAGG	Overlap-VHH
CALL002	GGTACGTGCTGTTGAACTGTTCC	
VHH-FOR (*Pst* I)	CAGGTGCAGCTGCAGGAGTCTGGGGGAGR	
VHH-REV (*Not* I)	CTAGTGCGGCCGCTGAGGAGACGGTGACCTGGGT	
FAdV-4-hexon-HVR-F	CGCGGATCCTATTTTCACATCGCGGGC	pET28a-FAdV-4-hexon-HVR
FAdV-4-hexon-HVR-R	GCGAAGCTTGACGCGCTTGTTCATGTA	
FAdV-4-penton-F	CGCGGATCCATGTGGGGGTTGCAGCCGC	pET28a-FAdV-4-penton
FAdV-4-penton-R	CCCAAGCTTCTGCAAGGTCGCGGAACT	
FAdV-4-fiber1-F	CGCGGATCCATGTCGGCCCTAATCGCC	pET28a-FAdV-4-fiber1
FAdV-4-fiber1-R	CCCAAGCTTGGGGCTCGGAGCATGGTT	
FAdV-4-fiber2-F	CGCGGATCCATGCTCCGAGCCCCTAAA	pET28a-FAdV-4-fiber2
FAdV-4-fiber2-R	CCCAAGCTTGGGACGGAGGCCGCTGGA	
FAdV-4-fiber1-R-648	CGGAAGCTTGACTTCGTAGGTTGCAAAGGG	Truncated fragments from pET28a-FAdV-4-fiber1
FAdV-4-fiber1-F-649	CGCGAATTCACGCCCGTATTGGGAATATCGCAG	
FAdV-4-fiber1-R-972	CGGAAGCTTGAGATAGCCCACGTCGCTCTGGTT	
FAdV-4-fiber1-R-810	CGGAAGCTTTTCTCCGCTCGCCCCCGT	
FAdV-4-fiber1-F-811	CGCGAATTCAACAGTCTGACCAGCGGA	
FAdV-4-fiber1-F-325	CGCGGATCCATGGATCGAGCGGTCAGTCTG	
FAdV-4-fiber1-M235-238-R1	ATGTAGATGTAATAGCCTATCGCCCACGCCGCCAAGCCCTTGCTTTTACG	pET28a- FAdV-4-fiber1$^{M235\text{-}238}$
FAdV-4-fiber1-M235-238-F2	ATGGCTATTACACTACATAGG	
FAdV-4-fiber1-M263+265-R3	CTGTTTCTCCGCTCGCCCCCGCGAGCGCCTGGGCTAGCTCCGGGTGA	pET28a- FAdV-4-fiber1$^{M263+265}$
FAdV-4-fiber1-M263+265-F3	GGGGCGAGCGGAGAAAACAGTC	

[a]Restriction sites are underlined.

investigation because of its simple operation, high sensitivity and high throughput (Li, et al., 2017). At present, several in-house ELISAs based on FAdV-4 virions or its recombinant proteins have been established for detecting anti-FAdV-4 antibodies (Guo, et al., 2022; Pan, et al., 2020; Zi, et al., 2018).

The capsid of the FAdV-4 particle is composed of four key structural proteins including hexon, penton, fiber-1 and fiber-2 (Gelderblom and Maichle-Lauppe, 1982; Marek, et al., 2012). Fiber-1 protein, a major antigen on the surface of FAdV-4 particles contains important epitopes and is essential for facilitating viral infection and triggering immune responses in birds (Wang, et al., 2018; Zou, et al., 2021). Therefore, the fiber-1 protein is a good target for developing assays for the diagnosis of FAdV-4 infection and the evaluation of vaccine immune effect (Pandey, et al., 2021; Shao, et al., 2019a). For example, a sandwich ELISA using the monoclonal antibodies against fiber-1 protein as capture antibodies have been reported for detecting FAdV-4 and an indirect ELISA using fiber-1 protein as coating antigen has been reported for detecting anti-FAdV-4 antibodies (Feichtner, et al., 2018; Shao, et al., 2019a). However, these ELISAs were typically developed using conventional antibodies and required horseradish peroxidase (HRP)-labeled secondary antibodies, which resulted in the several-step operating procedures and higher production costs.

Nanobodies, derived from the variable domain of Camelidae heavy chain antibodies (VHH), have recently been applied in various immunoassays because of their small molecular weight, easy modification and production, and high thermal stability (Jin, et al., 2023; Muyldermans and Lauwereys, 1999; Zhu, et al., 2014). Therefore, the nanobody-based immunoassays revealed more sensitive, easier operation and lower production costs than the traditional antibody-based ones. Nanobodies can be fused with different reporters, which can be used as a probe to avoid the use of enzyme-labeled secondary antibodies and to develop one-step ELISA (Sun, et al., 2019; Yamagata and Sanes, 2018; Zhao, et al., 2022). However, until now, there are no studies on the use of nanobody-based immunoassays for the diagnosis of FAdV-4 infection. In the present study, the nanobodies against FAdV-4 were screened from an immunized Bactrian camel, and one nanobody was selected and fused with HRP. The nanobody-HRP fusion protein was subsequently employed as a probe to establish a one-step competitive ELISA (cELISA) for the detection of anti-FAdV-4 antibodies. Additionally, it was confirmed that the epitope recognized by the nanobody was highly conserved among various FAdV-4 isolates, indicating that the developed cELISA can specifically detect antibodies against different FAdV-4 isolates. Due to one-step operation and lower production cost, the cELISA is considered appropriate for large-scale monitoring antibody levels of FAdV-4 vaccine immune and epidemiological investigation of viral early infection in chicken flocks.

Materials and methods

Cells, viruses and serum samples

Human embryonic kidney cells (HEK293T) and Leghorn male hepatoma (LMH) chicken liver cells [American Type Culture Collection (ATCC); cat. no. CRL-2117] were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Thermo Fisher Scientific, Inc.) supplemented with 10 % fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37 °C and 5 % CO_2. The strains FAdV-1 (GenBank no. PP974337) and FAdV-4 (GenBank no. PP974336) were isolated from infected chickens in Shaanxi, China.

The 189 negative sera for anti-FAdV-4 antibodies from specific pathogen-free (SPF) chickens (Beijing Merial Vital Laboratory Animal Technology Co., Ltd.) and 47 positive sera from immunized chicken were analyzed to calculate the cut-off value for the developed cELISA. To determine the specificity, the 113 positive chicken sera for antibodies against other avian viruses including Newcastle disease virus (NDV; $n = 19$) confirmed by IDEXX NDV Antibody Test kit, avian hepatitis E virus (avian HEV) ($n = 22$) confirmed by Beijing WANTAI HEV antibody ELISA kit, influenza A virus subtype H9N2 ($n = 17$) confirmed by Shanghai Meiao Chicken H9 AIV Antibody ELISA Kit, FAdV-1 ($n = 25$), FAdV-8a ($n = 16$) and FAdV-8b ($n = 14$) that were prepared by challenge of chickens in our laboratory and determined through virus neutralization test (VNT) were detected using the developed cELISA. In addition, to evaluate the sensitivity and agreements of cELISA with the commercial FAdV Group I Antibody ELISA kit (BioChek BV), the 32 sera from SPF chickens challenged with FAdV-4 and 114 clinical sera were used. All animal experiments were performed according to the Guidance for Experimental Animal Welfare and Ethical Treatment by the Ministry of Science and Technology of China. The protocols were approved by the Committee on Ethical Use of Animals of Northwest A&F University.

Camel immunization and library construction

The FAdV-4 was cultured and titrated in LMH cells using DMEM/F12 supplemented with 10 % FBS. Cells and supernatant were harvested at 72 h after infection and subjected to three freeze-thaw cycles. Subsequently, the mixture was centrifuged at 10,000 x g for 30 min at 4 °C and layered onto a 15–45 % sucrose gradient. For purification, it was further centrifuged at 18,000 x g for 5 h at 4 °C, following a previously described method (Kuadkitkan, et al., 2021).

The 4-year-old male Bactrian camels were immunized five times with the purified FAdV-4 particles. The initial immunization used complete Freund's adjuvant (Sigma-Aldrich; Merck KGaA), while the subsequent four used incomplete Freund's adjuvant. Following five immunizations, 200 ml camel peripheral blood was collected to measure anti-FAdV-4 antibody titers using indirect ELISA. In brief, 96-well ELISA plates were coated with 400 ng FAdV-4 particles and incubated overnight at 4 °C. After washing with phosphate buffer saline (PBS; 0.1 M; pH 7.2) containing 0.05 % Tween 20, the plates were blocked with 2.5 % skim milk in PBS'T [PBS with 0.05 % Tween-20 (v/v)]. Diluted sera from the immunized camels were then used as primary antibodies. The rabbit anti-camel IgG was used as the secondary antibody (prepared in our lab) incubated 1h at 37 °C (Liu, et al., 2015), followed by use of the HRP-conjugated goat anti-rabbit IgG (1:5000, RS23910,Immunoway Biotechnology Company) incubated 1h at 37 °C.

Lymphocytes were isolated from 200 ml peripheral blood with Ficoll-Paque PLUS (Cytiva) and Leucosep™ tubes (Greiner Bio-One International GmbH) according to the manufacturer's protocol. The total RNA of peripheral blood lymphocytes (PBLs) was extracted using the RNeasy® Plus Mini kit (Qiagen AB). cDNA was obtained using the Transcript cDNA Synthesis Super Mix kit (TransGen Biotech Co., Ltd.) and used as a template to amplify the VHH genes (~400 bp) with nested PCR using primers (Table 1). After digestion with *Pst* I and *Not* I enzymes, the VHH genes were ligated into the pMECS vector with His and HA tags, and then the ligation products were electro-transformed into freshly prepared *Escherichia coli* (*E. coli*) TG1 competent cells to construct a phage display library. The bacteria were grown on solid Luria-Bertani (LB) medium plates supplemented with 100 μg/ml ampicillin and 20 % (w/v) glucose. The colonies were scraped from the plates and stored in LB medium containing 20 % glycerin at −80 °C. After gradient dilution, the library capacity was evaluated by counting colonies on the plates. The positive rate of the phage library was assessed by bacterial PCR using specific primers (Table 1).

Nanobody screening

The screening process was performed according to a previous study (Liu, et al., 2015). In brief, 100 μl phage library was rescued by 20 MOI M13K07 helper phage (New England BioLabs, Inc.). Microplate wells were coated with 40 μg FAdV-4 particles per well and incubated overnight at 4 °C. After blocking with 300 μl 2.5 % (w/v) non-fat powder milk in PBS'T, 100 μl rescued phage-displayed library at a concentration of 5×10^{10} pfu/ml was incubated with the coated plates for 2 h at room temperature. After washing the wells 10 times with PBS'T to remove unbound phages, the bound phages particles were eluted using 100 mM trimethylamine (100 μl/well; Sigma-Aldrich; Merck KGaA) for 10 min at 25 °C, and immediately neutralized with 1 M Tris-HCl (pH 7.4). Finally, the eluted phages were used to infect *E. coli* TG1 cells (Beyotime Institute of Biotechnology) for subsequent titration and amplification of the next round. Three rounds of screening were conducted and 96 clones were picked randomly from the third-round plates and cultured in 2X YT-AG medium [Tryptone (16 g L^{-1}), yeast extract (10 g L^{-1}) and NaCl (5 g L^{-1})]. The bacteria were grown overnight and collected for lysis. The periplasmic extracts containing the nanobodies from the lysis bacteria were incubated with the 96-well plates coated with purified FAdV-4 particles (400 ng/well). Then, the anti-HA monoclonal antibody (GenScript Biotech Corporation) and HRP-conjugated goat anti-mouse antibody (TransGen Biotech Corporation) were added in turn. After washing, 100 μl 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate solution was added per well. Finally, 50 μl 3M H_2SO_4 was added per well to stop the reaction, and the absorbance of each well was measured at 450 nm (OD_{450nm}). When the ratio of the OD_{450nm} value of clones to the negative is >3, the clone is positive. The positive clones were sequenced, and their sequences were classified based on their complementarity-determining region 3 (CDR3) amino acids (aa).

Expression of nanobody-HRP fusion proteins

For producing the nanobody-HRP fusion proteins, the genes encoding nanobodies were cloned into pCMV-N1-HRP vector including the secreting signal peptide, HA and His tags through the *Pst* I and *Not* I restriction (Sheng, et al., 2019). The ligation products were transformed into *E. coli* Trans5α cells (TransGen Biotech Corporation) and then sequenced. After that, the positive recombinant plasmids were transfected into HEK293T cells using polyetherimide agents (Polysciences Inc.) and the fusion proteins were secreted into the medium by a secreting signal peptide. The indirect immunofluorescence assay (IFA) was performed to determine the expression of fusion proteins in the cells. Briefly, the transfected cells were fixed with 4 % paraformaldehyde (Sigma Aldrich; Merck KGaA) and sequentially incubated with the anti-His monoclonal antibody (TransGen Biotech Corporation, Cat HT501; 1:400 dilution in PBS) and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., Cat 115-095-003; 1:500 dilution in PBS). Then, they were observed under a fluorescence microscope (Leica Biosystems; cat. no. AF6000).

Additionally, the titers of the fusion protein in the supernatant were determined by direct ELISA. Briefly, the plates were coated with purified FAdV-4 particles (400 ng/well) and incubated with different dilutions of the supernatant for 1 h at room temperature. After that, they were directly colored by adding TMB solution (100 μl/well). The OD_{450nm} values were read after the reaction was stopped with 3M H_2SO_4 (50 μl/well).

The fusion proteins in the supernatant with higher titer were selected to compare with the competitive ability by cELISA using the positive chicken sera for anti-FAdV-4 antibodies. The nanobody-HRP fusion protein with strongest competitive ability was selected as the probe to develop the cELISA for detection of anti-FAdV-4 antibodies in the chicken sera.

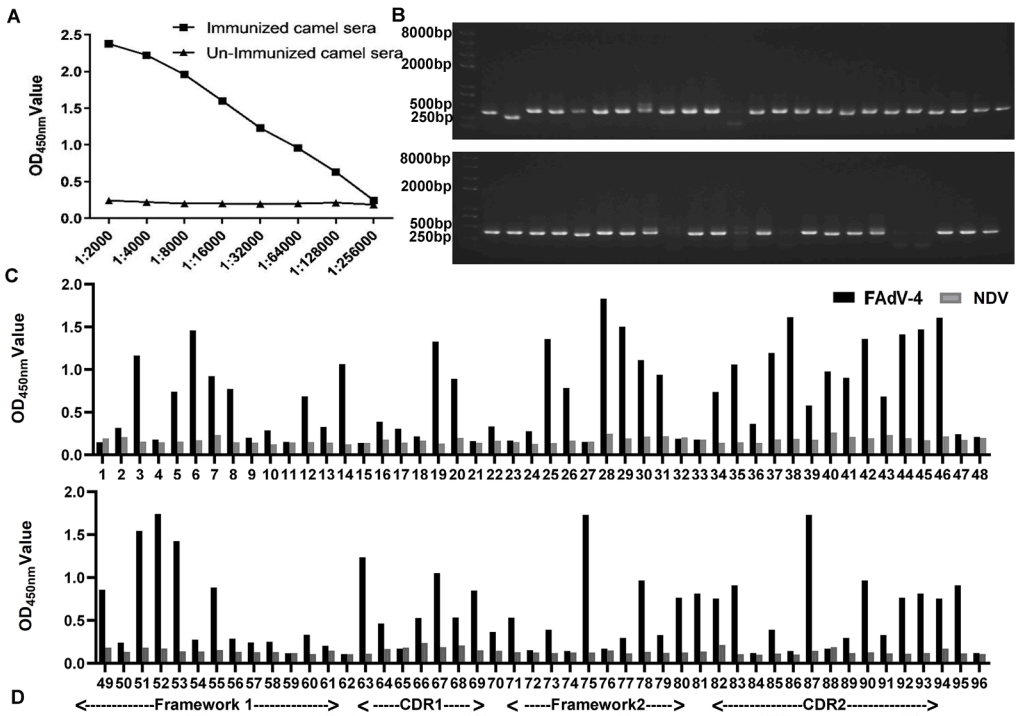
Development of cELISA to detect antibodies against FAdV-4

To optimize the amount of coated antigen and dilution of fusion protein for the cELISA, the purified FAdV-4 particles with different amounts (100, 200, 400 and 800 ng) per well and the nanobody-HRP fusion protein with different dilutions (1:20, 1:40, 1:80, 1:160 and 1:320) were used in the direct ELISA by a checkerboard titration. When the OD_{450nm} value of direct ELISA was 1.0, the optimal amount of coated antigen and dilution of fusion protein were used for the cELISA.

Each four positive and negative chicken sera for anti-FAdV-4 antibodies were diluted at different dilutions (1:20, 1:40, 1:80, 1:160 and 1:320) and detected with the developed cELISA to determine the optimal dilution of tested chicken sera. The optimal sera dilution was determined when the ratio of OD_{450nm} values of positive sera to negative sera (P/N) was the lowest.

The optimal incubation time between the mixture of the fusion protein and sera and the coated antigen was determined. The times of 30, 40, 50 and 60 min were used. After washed with PBS'T, TMB was added to the plates and incubated for 10 and 15 min, respectively. When the P/N value was the smallest, the incubation and color reaction times were the best.

Based on the aforementioned optimized conditions, the procedure of the developed cELISA was as follows: The 96-well ELISA plates were coated with the optimized amount of purified FAdV-4 particles



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	<----------Framework 1---------->	<-----CDR1----->	<-----Framework2----->	<----------CDR2---------->	
	10 20	30	40	50 60	
Nb6	ESGGGSVQAGGSLRLSCAKS	GYKHSRYCMG	WFRQAPGKEREGVA	AILS-DGSTLYADSVKG	59
Nb14	ESGGGSVQAGGSLRLSCATS	GDTYERNCMA	WFRQAPGKEREGVA	IIARNDGTTFYTDSVKG	61
Nb19	ESGGGSVQAGGSLRLSCAAS	GYTYSTYCMG	WFRQAPGKEREGVA	TITS-DGTTIYADSVKG	60
Nb21	ESGGGSVQPGGSLRLSCVAS	GYTDDSACMS	WFRQAPGKERERVA	TIYTGDDTTYTVDSVKG	61
Nb25	ESGGGSVQAGGSLTLSCEAP	GYTYSCDCIV	WFRQAPGKEREWVA	GIYVGSANTDYADSVKG	61
Nb28	ESGGGSVQAGGSLRLSCA--	-ASGYRTCMA	WFRQAPGKEREGVA	SISTVDGSTYYADSMKG	58
Nb29	ESGGGSVQAGGSLRLSCAAS	GYTYSNNCMA	WFRQAPGKEREGVA	GIRRSGETIFYSDSVKG	61
Nb37	ESGGDSVQAGGSLRLSCVLF	GYTYTRNCMG	WFRQVPGEEREGVA	SIYTGGGNTYYGDSTKG	61
Nb38	ESGGGSVQAGGSLRLSCAAS	GYIDSSGYMA	WFRQAPGKEREGVA	AIDLDG-STSYSDSVKG	60
Nb39	ESGGGSVQAGGSLRLSCGRS	GYRYSRYCMG	WFRQAPGKEREGVA	FFES-DGNTRYADSVKG	60
Nb40	ESGGGSVQAGGSLRLSCTAS	GYIYSNNCMA	WFRQGPGKEREGVA	GIRRSGETIYYSDSVKG	61
Nb42	ESGGDSVQAGGSLRLTCAAS	GYTYSRYCMG	WFRQAPGKDREEVA	HIGS-DGSTIYTDSVKG	60
Nb44	ESGGGSVQPGGSLRLSCAGA	TYAVSGGYMA	WFRQVSGKEREGVA	AISPGGGRSSYSDSVQG	61
Nb45	ESGGGSVQAGGSLRLSCAAS	GYTYSSYCMG	WFRQAPGKEREGVA	TIDS-LGSTIYADSVKG	60
Nb51	ESGGGSVQAGGSLRLSCVAS	GYTYSSNCMG	WFRQAPGKEREGVA	GIRTSGVPIYYADSVKD	61
Nb63	ESGGGSVQPGGSLRLSCS--	-ASYRRNCMG	WFRQVPGKEREGVA	SIYTPGGSTWYADFVKG	58
Nb75	ESGGDSVQAGGSLRLSCAAS	GYTYSSYLLG	WFRQAPGKEREGVA	VILSRGGNTYYADSGKG	61
Nb77	ESGGDSVQAGGSLRLSCAAS	GYTYSNNCMA	WFRQAPGKEREGVA	GIRRSGDTIHYSDSVKG	61
Nb86	ESGGGSVQAGGSLRLSCA--	-ASGYRTCMA	WFRQAPGKEREGVA	SISTVGGSTYYADSVKG	58
Nb87	ESGGDSVQAGGSLRLSCVAS	RSTYSTYSMA	WFRQAPGKEREVVA	TIYRRGGGTYYADSVKG	61
Nb90	ESGGGLVQPGGSLTLSCEAS	GYTYSRDCMV	WFRQAPGKEREWVA	GIYVGSGSTDYADSVKG	61
Nb93	ESGGGSVQAGGSLRLSCAAS	GYTYNNYCMG	WFRQAPGKEREGVA	HIDS-VGSTTYADSVKG	60
Nb95	ESGGDSVQAGGSLRLSCVAS	RYTYSTYSMA	WFRQAPGKEREVVA	TIYRRGGGTYYADSVKG	61

	<----------Framework3---------->	<----------CDR3---------->	<--Framework4-->	
	70 80 90	100 110	120	
Nb6	RFTIFKDNAKNTLYLQMNAMKPEDTAMYYCAA	S-----SGEYCST----EYEYKY	WGQGTQVTVSS	116
Nb14	RFTISQNNAKNTVYLQMNSLNPEDTGMYYCAA	NAGWHGCPPLNPN------KYEY	WGQGTQVTVSS	121
Nb19	RFTISKDKAKKTLYLQMNNPKPEDTAMYYCAQ	T-----TGPYCSG----AHEYQY	WGQGTQVTVSS	117
Nb21	RFTISQDNAKNTVYLQMNSLKPEDSAIYYCAA	S--FG-RYYGCGS---QPGWWNF	WGQGTQVTVSS	121
Nb25	RFTISQDIAKNTVYLQMNSLKPEDTAMYYCAA	DRTCRTWPPIWV-------EYNY	WGQGTQVTVSS	120
Nb28	RFTISQDNAKNTVYLQINSLKPEDSAKYYCAA	R-SLS---YNCAVH--QASRYSH	WGQGTQVTVSS	118
Nb29	RFTISQDNAKRTVYLQMNSLKPEDTAMYYCAA	T-----RGHYCDV-----NAYND	WGQGTQVTVSS	117
Nb37	RFTISQDNAKNTVYLQMNSLKSEDTAMYYCAA	APAFSRRGQDCYGG--FATDFSY	WGQGTQVTVSS	125
Nb38	RFIISKDNAKNTLYLQMNSLKPEDTAMYYCAT	DR---FSGALYRNYVLLSSWGY	WGQGTQVTVSS	123
Nb39	RFTISTDNAKNTLYLQMNSLKPEDTAMYYCAA	T-----SGAYCA-----DYEYIY	WGQGTQVTVSS	116
Nb40	RFAIFQDNAKRTVYLQMNSLKPEDTAMYYCAA	T-----RGSYCDV-----SAYND	WGQGTQVTVSS	117
Nb42	RFTIWKDNAKNTLYLQMNSLKPVDTAMYYCAQ	T-----TGPYCSG----AHEYAH	WGQGTQVTVSS	117
Nb44	RFTIARDNAENTVTLHMNSLKPEDTAMYYCAA	SP---VSGDTL-N----PNVYRY	WGQGTQVTVSS	119
Nb45	RFTISKDNAKN-LYLQMHSLKPEDTAMYYCAQ	T-----TGPYCSG----AHEYKY	WGQGTQVTVSS	116
Nb51	RFIISQDNAKRTVYLQMNSLKPEDTAMYYCAA	T-----RGDFCDI-----NAYNY	WGQGTQVTVSS	117
Nb63	RFTISQDNAKNTVYLQIYSMKPEDTAMYYCAA	APAFSRRGQDCGGG--IAADFSD	WGQGTQVTVSS	122
Nb75	RFTISQDNAKNTLYLQMNSLKPEDTAMYYCAA	GKNVRRYPPKSD-------DFAY	WGQGTQVTVSS	120
Nb77	RFTISQDNAKRTVYLQMNSLKPEDTAMYYCAA	T-----RGTYCDV-----NAYND	WGQGTQVTVSS	117
Nb86	RFTISQDNAKNTVYLQMNSLKPEDSAKYYCAA	R-SLS---YACDVL--QASRYSH	WGQGTQVTVSS	118
Nb87	RFTISQDNAKNTLYLQMNRLKPEDTAMYYCAA	AEGLLDYPPTNAL------AFGY	WGQGTQVTVSS	121
Nb90	RFTISQDNAKNTVYLQMNSLKPEDTAMYYCAA	DRTCRSWPPIWV-------DYGY	WGQGTQVTVSS	120
Nb93	RFTISKDNAENTLYLQMNNLKPEDTAMYYCAA	Y-----QG-YASDYG-GCPEYNR	WGQGTQVTVSS	119
Nb95	RFTISQDNAKNTLYLQMNRLKPEDTAMYYCAA	AEGLLGYPPTNDL------AFGY	WGQGTQVTVSS	121

(caption on next page)

Table 2
Three-round panning of phage particles against the FAdV-4 particles.

Round of panning	FAdV-4 (μg/well)	Phage input (PFU/well)	Phage output (PFU/well)	N output (PFU/Well)	Enrichment
1st round	40	5×10^{10}	1.4×10^{5}	4×10^{2}	3.5×10^{2}
2nd round	20	5×10^{10}	9.2×10^{6}	1.8×10^{3}	5.1×10^{3}
3rd round	10	5×10^{10}	1.5×10^{7}	4×10^{2}	3.75×10^{5}

overnight at 4 °C. After blocking with blocking buffer (2.5 % skim milk in PBS'T; 300 μl/well) for 1 h at 37 °C, the plates were added with the mixture of the optimized nanobody-HRP fusion proteins and dilution of testing chicken sera and incubated with the optimized time at 37 °C. After washing again, TMB (100 μl/well) was added and incubated at optimized time at room temperature following arrest using 3M H_2SO_4 (50 μl/well), and the $OD_{450\ nm}$ values were read. The percent of inhibition (PI) value was calculated as: PI = 100 % x [1 − (OD_{450nm} value of tested sera/OD_{450nm} value of negative sera)].

Determination of the cut-off value of the developed cELISA

A total of 189 negative and 47 positive chicken sera for anti-FAdV-4 antibodies were used to calculate the cut-off values for the developed cELISA. SPSS (version 20; IBM Corp.) was used for ROC (Receiver Operating Characteristic) analysis. The PI values of the 189 negative and

Table 3
The optimal coated amounts of FAdV-4 particles and the optimal dilution of FAdV-4-Nb28-HRP fusion protein were determined.

Different amounts of FAdV-4 particles (ng/well)	Different dilutions of FAdV-4-Nb28-HRP fusion in the medium					
	1:10	1:20	1:40	1:80	1:160	1:320
100	0.486	0.457	0.380	0.316	0.299	0.273
200	0.896	0.797	0.754	0.679	0.487	0.428
400	1.487	1.208	1.181	1.001	0.921	0.743
800	1.899	1.711	1.671	1.650	1.415	1.241

Table 4
Determination of optimal dilution of tested chicken sera for cELISA.

No. sera		Dilutions of tested chicken sera					
		1:10	1:20	1:40	1:80	1:160	1:320
Sera 1	Positive	0.221	0.232	0.341	0.462	0.535	0.801
	Negative	0.916	0.964	0.902	0.909	0.909	0.883
	P/N	0.242	0.241	0.378	0.508	0.588	0.907
Sera 2	Positive	0.213	0.239	0.365	0.450	0.613	0.855
	Negative	0.919	0.971	0.895	0.872	0.881	0.866
	P/N	0.232	0.246	0.408	0.516	0.696	0.987
Sera 3	Positive	0.219	0.258	0.359	0.473	0.558	0.763
	Negative	0.936	0.968	0.815	0.798	0.789	0.774
	P/N	0.234	0.267	0.441	0.593	0.706	0.985
Sera 4	Positive	0.237	0.260	0.326	0.516	0.566	0.753
	Negative	0.925	0.991	0.839	0.930	0.846	0.630
	P/N	0.256	0.263	0.388	0.554	0.668	1.194

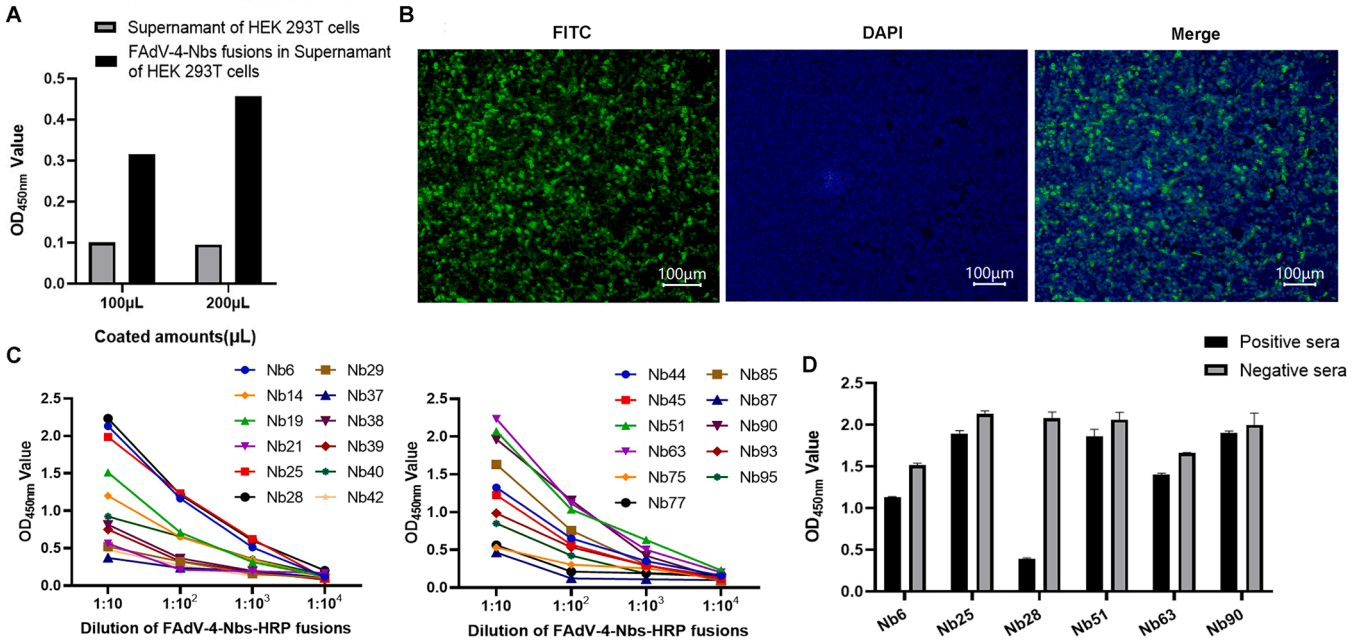

Fig. 2. Identification of anti-FAdV-4 nanobodies with HRP fusion protein secretion expressed in HEK293T cells. (A) Detection of the HRP activity in the FAdV-4-Nb-HRP fusion proteins secreted into the culture medium of HEK293T cells. (B) Detection of the FAdV-4-Nb-HRP fusion proteins expressed in HEK293T cells by IFA. A representative image of H9N2-Nb28-HRP is shown, and similar results were observed for the other 22 nanobodies-HRP against FAdV-4 in Fig. 2A and B. (C) Analysis of the binding of FAdV-4-Nb6-, -Nb14-, -Nb19-, -Nb21-, -Nb25-, -Nb28-, -Nb29-, -Nb37-, -Nb38-, -Nb39-, -Nb40-, -Nb42-, -Nb44-, -Nb45-, -Nb51-, -Nb63-, -Nb75-, -Nb77-, -Nb85-, -Nb87-, -Nb90-, -Nb93- and -Nb95-HRP fusions with FAdV-4 particles by direct ELISA. (D) cELISA comparative analysis of FAdV-4-Nb6-, -Nb25-, -Nb28-, -Nb51-, -Nb63- and -Nb90-HRP fusions blocking capability of the binding of FAdV-4 antibody-positive sera to FAdV-4 particles. FAdV, fowl adenovirus serotype 4; HRP, horseradish peroxidase; IFA, immunofluorescent assay; ELISA, enzyme-linked immunoassay; cELISA, competitive enzyme-linked immunoassay; H9N2, influenza A virus subtype H9N2.

Fig. 1. Screening of the nanobodies against the FAdV-4. (A) Titers of antibodies against FAdV-4 particles in the sera from the camel after the 5th immunization. (B) Identification of the positive rate for the VHH library by PCR. (C) Detection of the periplasmic extracts from 96 clones reacting with FAdV-4 particles by indirect ELISA. (D) Alignment of the amino acid sequences of the 23 screened nanobodies. Numbering and CDRs are in accordance with previous methods. FAdV, fowl adenovirus serotype 4; VHH, variable domain of Camelidae heavy chain antibodies; ELISA, enzyme-linked immunoassay; CDR, complementarity-determining region 3.

Table 5
Incubation time of the mixture containing chicken sera and fusions incubated with the antigen and optimal time for the colorimetric reaction after adding TMB.

Times of color reaction (min)		Incubation time of the mixture of chicken sera and FAdV-4-Nb28-HRP fusion with antigen (min)			
		30	40	50	60
10	Positive	0.138	0.133	0.136	0.193
	Negative	0.452	0.571	0.595	0.745
	P/N	0.305	0.2323	0.229	0.259
15	Positive	0.137	0.154	0.166	0.188
	Negative	0.563	0.612	0.745	0.945
	P/N	0.243	0.251	0.222	**0.198**

47 positive sera tested by cELISA were used to generate the ROC curve, with the X-axis representing 1-specificity and the Y-axis representing sensitivity. The area under the curve (AUC) was calculated, and the optimal cut-off value was determined using the maximum Youden's index, calculated as: Youden's index = sensitivity + specificity - 1.

Evaluation of specificity, sensitivity and reproducibility of cELISA

To evaluate the specificity, the 113 positive chicken sera for antibodies against other avian viruses including NDV (n = 19), HEV (n = 22), H9N2 (n = 17), FAdV-1 (n = 25), FAdV-8a (n = 16) and FAdV-8b (n = 14) were tested with the developed cELISA.

To determine sensitivity, the 32 sera were collected at different days post infection (0, 3, 5, 7, 11, 15, 20, 22 dpi) from four SPF chickens challenged with FAdV-4 and detected by cELISA. At the same time, the four positive sera were randomly selected for 2-fold serial dilution (from 1:20 to 1:5,120) and tested by cELISA to determine the minimum dilution of detection.

To evaluate reproducibility, each of the four positive and negative chicken sera were tested by cELISA to calculate the coefficient of variation (CV) of inter-assay (between plates) and the intra-assay (within a plate) variations. Inter-assay CV was determined by testing each sample on three different plates, while intra-assay CV was calculated from three replicates of each sample within a single plate.

Comparisons of cELISA using the commercial ELISA kit

A total of 114 chicken sera including 50 from vaccine immunized chickens and 64 from clinically suspected FAdV-4 infected chickens

Table 6
Area under of the curve Test Result Variable(s): PI.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.976	.015	.000	.947	1.000

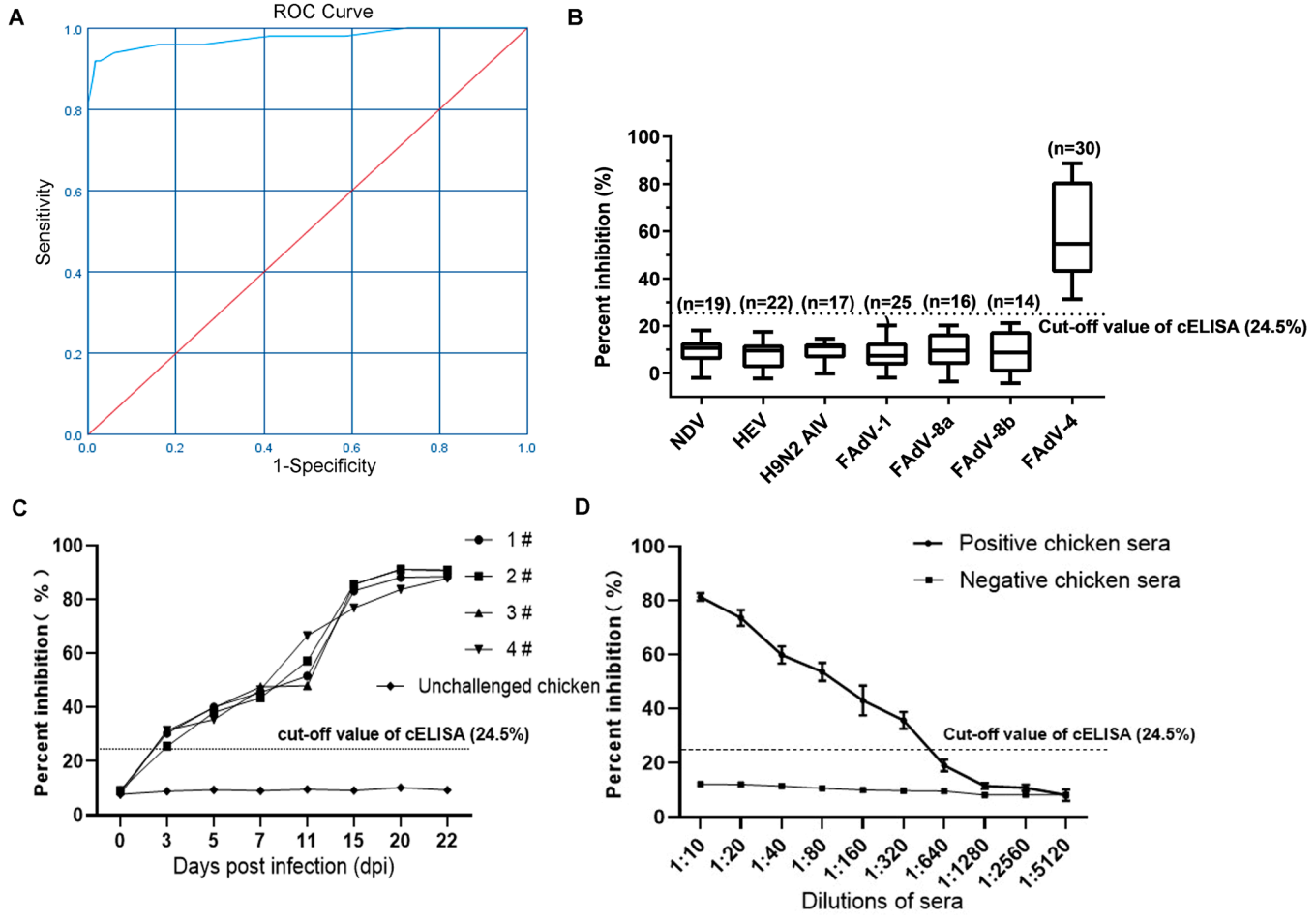

Fig. 3. Performance of the developed cELISA. (A) The ROC curve of the cELISA. (B) Specificity analysis of the developed cELISA by detection of NDV, avian HEV, H9N2, FAdV-1, −8a and −8b antibody-positive sera. (C) Detection of antibodies against FAdV-4 in the serial sera from the challenged SPF chickens with cELISA. (D) Determination of the maximum dilution of positive sera for anti-FAdV-4 antibodies. cELISA, competitive enzyme-linked immunoassay; NDV, Newcastle disease virus; avian HEV, avian hepatitis E virus; H9N2, influenza A virus subtype H9N2; FAdV, fowl adenovirus serotype 4; SPF, specific pathogen-free.

Table 7
Coordinates of the Curve Test Result Variable(s): PI.

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity	Youden's index
0.0000	1.000	1.000	0.000
0.1050	1.000	0.989	0.011
0.1150	1.000	0.979	0.021
0.1250	1.000	0.952	0.048
0.1350	1.000	0.936	0.064
0.1450	1.000	0.904	0.096
0.1550	0.980	0.888	0.092
0.1650	0.980	0.866	0.113
0.1750	0.980	0.818	0.161
0.1850	0.980	0.733	0.247
0.1950	0.980	0.583	0.397
0.2050	0.980	0.503	0.477
0.2150	0.980	0.412	0.568
0.2250	0.980	0.262	0.718
0.2350	0.980	0.160	0.819
0.2450	0.959	0.059	0.900
0.2550	0.918	0.032	0.886
0.2650	0.898	0.027	0.871
0.2750	0.898	0.021	0.877
0.2850	0.878	0.016	0.862
0.3050	0.837	0.005	0.831
0.3250	0.796	0.005	0.791
0.3350	0.755	0.005	0.750
0.3450	0.755	0.000	0.755
0.3600	0.735	0.000	0.735
0.3950	0.714	0.000	0.714
0.4250	0.673	0.000	0.673
0.4400	0.653	0.000	0.653
0.4550	0.633	0.000	0.633
0.4650	0.612	0.000	0.612
0.4850	0.592	0.000	0.592
0.5100	0.571	0.000	0.571
0.5250	0.531	0.000	0.531
0.5350	0.469	0.000	0.469
0.5500	0.429	0.000	0.429
0.5900	0.408	0.000	0.408
0.6250	0.388	0.000	0.388
0.6350	0.347	0.000	0.347
0.6500	0.265	0.000	0.265
0.6650	0.245	0.000	0.245
0.6900	0.224	0.000	0.224
0.7150	0.204	0.000	0.204
0.7250	0.184	0.000	0.184
0.7350	0.163	0.000	0.163
0.7650	0.143	0.000	0.143
0.8050	0.122	0.000	0.122
0.8250	0.102	0.000	0.102
0.8350	0.082	0.000	0.082
0.8550	0.061	0.000	0.061
0.8900	0.041	0.000	0.041
0.9150	0.020	0.000	0.020
1.0000	0.000	0.000	0.000

Table 8
Reproducibility of the cELISA determined by CV% value of intra and inter assay.

	CV% value range of 4 serum samples	Median value
Intra assay precision(CV%)*	1.65-5.34	2.31
Inter assay precision(CV %)**	2.22-4.26	3.34

CV, coefficient of variation.
* Intra-assay precision: Determined from 4 serum samples on the same plate for three repetitions (well-to-well).
** Inter-assay precision: Determined from three repetitions (plate-to-plate) of 4 serum samples on different plates.

were detected simultaneously by cELISA and a commercial FAdV-I antibody ELISA kit. The IFA was used to verify the inconsistent sera. The procedure of IFA was as follows: FAdV-4-infected LMH cells (0.1

Table 9
Comparisons of the developed cELISA with commercial ELISA kits by detecting immunized and clinical chicken serum samples.

Samples	Number	cELISA	Commercial ELISA kit		Agreement (%)	Kappa value
			+	-		
Sera of immunized chickens	47 (A)	+	47 (B)	0	100 %	1
	3 (C)	-	0	3 (D)		
Sera of clinical chickens	49 (A)	+	47 (B)	2	96.8 %	0.917
	15 (C)	-	0	15 (D)		

*Agreement (%) = $(B + D) / (A + C) \times 100\%$.
**Kappa value > 0.45.

MOI) were fixed with 4 % paraformaldehyde and were permeated with 0.25 % Triton X-100 (Sigma-Aldrich; Merck KGaA). Then, they were blocked with 1 % BSA. The inconsistent sera were diluted at 1:500 as the primary antibodies and were incubated for 1 h at 37 °C. After washing, the FITC-conjugated rabbit anti-chicken IgG (1:500; Immunoway Biotechnology Company) was used as the secondary antibody. The nuclei were stained with 4′, 6-diamidino-2-phenylindole. Finally, the stained cells were observed under a fluorescence microscope (Leica Biosystems; cat. no. AF6000). The coincidence rates were calculated using Microsoft Excel's CORREL function (Microsoft Corporation).

Identification of the epitope recognized by the nanobody

To determine the developed cELISA for specifically detecting antibodies against different FAdV-4 isolates, the epitope recognized by the nanobody was identified and aligned among different FAdV isolates. Firstly, the structural protein of FAdV-4 recognized by the nanobody was identified. Different proteins including hypervariable region (HVR) of hexon (Crawford-Miksza and Schnurr, 1996), fiber-1, fiber-2 and penton of FAdV-4 were expressed using the prokaryotic system. Briefly, the viral DNA was extracted using a DNA extraction kit (TransGen Biotech Corporation) following the manufacturer's instructions. The genes encoding hexon, penton, fiber-1 and −2 proteins were separately amplified using the respective primer pairs (Table 1) and inserted into the pET-28a vector (Novagen) containing His tag with the *BamH* I and *Hind* III restriction sites. After sequencing, the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells (TransGen Biotech Corporation) for expression. The four proteins were expressed through by adding 1 mM isopropyl β-D-1-thiogalactopyranoside into the positive bacteria for 8 h at 37 °C. After the bacterial cells were collected and sonicated, the cell lysate was purified by a Ni-NTA column (Cytiva). The purified recombinant proteins were analyzed by SDS-PAGE and western blotting with anti-His monoclonal antibody as the primary antibody and HRP-conjugated goat anti-mouse as the secondary antibody. Using these proteins as antigens, the viral protein recognized by the nanobody was determined by western blotting with the nanobody-HRP fusion protein as the primary antibody. After the viral protein was recognized by the nanobody, a series of truncated and overlapping fragments from the viral protein was designed and also expressed with the bacterial system. The expression and purification of these truncated fragments were same as the structural proteins of FAdV-4. Similarly, the expression and purification of these truncated fragments were analyzed with SDS-PAGE and western blotting. Subsequently, western blotting was also used to identify the antigenic region of the viral protein recognized by the nanobody.

To precisely fine the epitope recognized by the nanobody, the 3D structures of homology modeling for the viral protein and the nanobody were first predicted by the AlphaFold 3 server. Then, based on the aforementioned identification of the antigenic region, the key motifs of

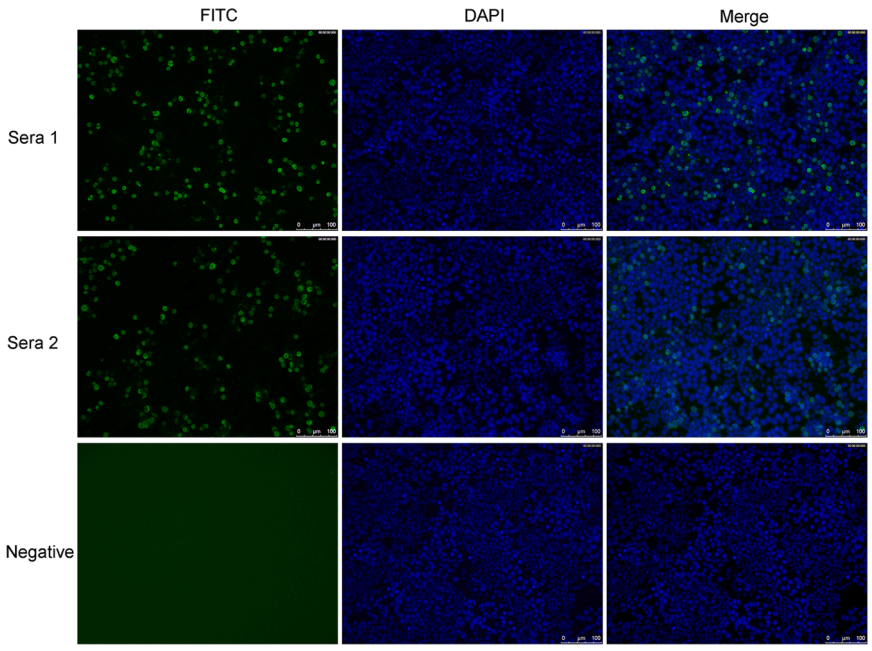

Fig. 4. Evaluation of the two inconsistent serum samples tested with developed cELISA and the commercial ELISA kit with IFA. ELISA, enzyme-linked immunoassay; cELISA, competitive enzyme-linked immunoassay; IFA, immunofluorescent assay.

the epitope for the nanobody binding to viral protein were accurately predicted using the AlphaFold 3 server. The key aa involved in the interaction were analyzed using PyMOL (version 4.6; pymol.org/2/support.html). Then, the mutated viral proteins were also expressed using the bacterial system. Using the mutated proteins as the antigen, western blotting was performed to confirm the predicted key motifs of the epitope. Notably, only the fiber proteins of the species FAdV-A and -C, including the serotypes FAdV-1 and FAdV-4/FAdV-10, respectively, are transcribed by two separate genes that differ in sequence and length and are known as fiber-1 and −2. Finally, the conservation and unique for serotype 4 of key sites recognized by nanobody among different isolates of serotype 4 and serotype 1 of FAdV-I were analyzed using the Clustal W module of Lasergene 7.1 (DNASTAR, Inc.).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0; Dotmatics). The Kappa index values were calculated using SPSS (version 20; IBM Corp.) ROC analysis was also analyzed using SPSS (version 26; IBM Corp.).

Results

Construction of the phage display VHH library and screening of specific nanobodies against FAdV-4

After the camels were immunized with the purified FAdV-4 particles five times, the indirect ELISA showed that the titers of the sera from the camels increased to 1:128, 000, indicating that the viral particles induce strong immune responses in the camels (Fig. 1A). Subsequently, PBLs were isolated from the immunized camels. Total RNA was extracted from the PBLs. The VHH genes with expected size of 400 bp were successfully amplified by reverse transcription and two rounds of PCR. After cloning of the VHH gene into the pMECS vector, a phage display VHH library consisting of $\sim 1.75 \times 10^7$ clones with a positive rate of 90 % was successfully constructed (Fig. 1B).

After three rounds of bio-panning, the phage particles against FAdV-4 were found to be significantly enriched (Table 2). Using the periplasmic extract from 96 clones as the primary antibody and NDV particles as the negative control, the indirect ELISA showed that 54 clones were positive and selected for sequencing (Fig. 1C). After sequencing, the alignment of aa revealed that a total of 23 nanobodies against FAdV-4 were obtained according to the CDR3 region of VHH genes, named Nb6, Nb14, Nb19, Nb21, Nb25, Nb28, Nb29, Nb37, Nb38, Nb39, Nb40, Nb42, Nb44, Nb45, Nb51, Nb63, Nb75, Nb77, Nb85, Nb87, Nb90, Nb93 and Nb95 (Fig. 1D).

Expression of nanobody-HRP fusion proteins against FAdV-4

The genes encoding 23 nanobodies were cloned into the pCMV-N1-HRP vector. After transfection of HEK293T cells with the positive recombinant plasmids, the cells and supernatant were collected after 72 h. The direct ELISA results showed that the 23 nanobody-HRP fusion proteins were successfully released into the cell supernatant and still exhibited the activity of the HRP biological enzyme, which could be directly developed with the substrate chromogenic TMB (Fig. 2A). The IFA results showed that the 23 nanobody-HRP fusion proteins were all successfully expressed in HEK293T cells (Fig. 2B).

Additionally, the direct ELISA using the purified FAdV-4 particles as the coating antigens showed that six of 23 fusion proteins exhibited strong specific binding to FAdV-4 with titers of 1:1,000 (Fig. 2C). They were named FAdV-4-Nb6-, -Nb25-, -Nb28-, -Nb51-, -Nb63- and -Nb90-HRP fusion proteins. Subsequently, using the six nanobodies-HRP fusion proteins as the probes, the cELISAs showed that the FAdV-4-Nb28-HRP fusion protein has the highest competitive ability (PI, 80 %; Fig. 2D), indicating that the fusion protein can be selected for developing the cELISA to detect anti-FAdV-4 antibodies.

Development of the cELISA using the Nb28-HRP fusion protein as a probe

When the coating amounts of FAdV-4 particles was 400 ng/well and the dilution of FADV-4-Nb28-HRP fusion protein was 1:80, the OD_{450nm} value of direct ELISA was ~1.0 (Table 3). The best dilution of chicken sera was 1:20 for the cELISA with the lowest P/N value (Table 4). Additionally, when the incubation time was 60 min and the color reaction time was 15 min, the P/N value was the smallest (Table 5).

For developing one-step cELISA, after the plates were coated with FAdV-4 particles (400 ng/well), blocked with blocking buffer and

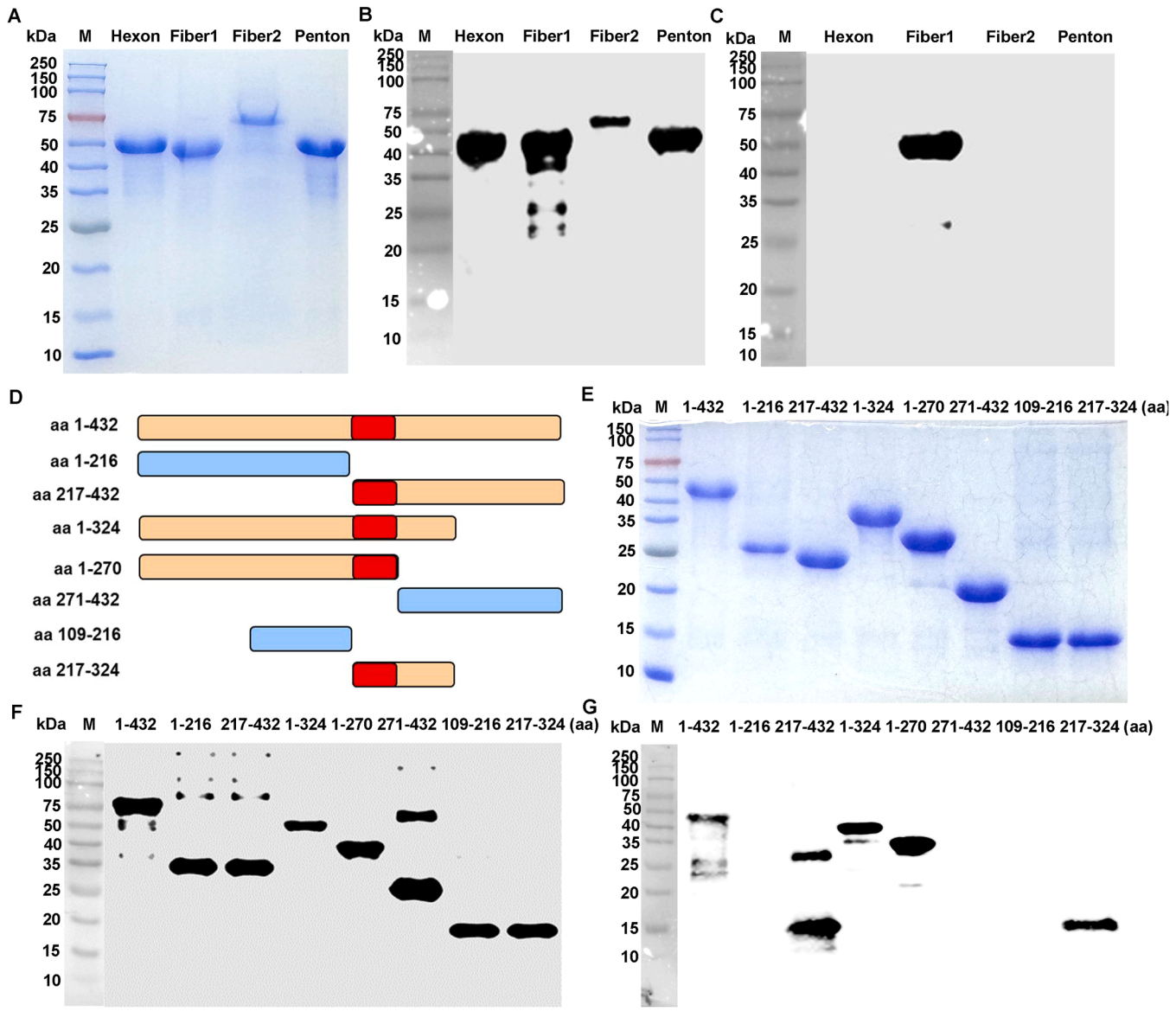

Fig. 5. Identification of key antigenic regions recognized by FAdV-4-Nb28-HRP fusion protein. (A) SDS-PAGE of purified hexon-HVR, fiber-1, fiber-2 and penton proteins expressed using the *E. coli* system. (B) Antigenic analysis of the recombinant protein with western blotting using the anti-His monoclonal antibody as the primary antibody. (C) Western blotting of viral structural proteins identified by FAdV-4-Nb28-HRP fusion protein. M, protein molecular markers; lane 1, hexon-HVR; lane 2, fiber-1; lane 3, fiber-2; lane 4, penton. (D) Schematic diagram of different FAdV-4-fiber1 truncated fragments. (E) SDS-PAGE of different fragments expressed using the *E. coli* system. (F) Western blotting of expression of different fragments using anti-His monoclonal antibody as the primary antibody. (G) Western blotting of key domains of fiber-1 proteins identified by FAdV-4-Nb28-HRP fusion protein. M, protein molecular markers; lane 1-8: 1-432, 1-216, 217-432, 1-324, 271-432, 109-216 and 217-324 aa, respectively. FAdV, fowl adenovirus serotype 4; HRP, horseradish peroxidase; HVR, hypervariable region; *E. coli, Escherichia coli*; aa, amino acids.

washed, they were dried in the fume hood, vacuumed and packed. For the clinical application, the packed plates were opened. Then, the testing chicken sera (1:20) were mixed with the FAdV-4-Nb28-HRP fusion protein (1:80) and added into the plates. After incubated for 60 min at 37 °C and washed, the TMB (100 μl/well) was added for color reaction at room temperature for 15 min. After the reaction was stopped with 3M H_2SO_4 (50 μl), the OD_{450nm} values were read.

Cut-off value of the cELISA

By testing 189 negative and 47 positive chicken sera using the developed cELISA, the ROC curve displays an AUC of 97.6 % (95 % CI, 0.94-1; $P < 0.001$; Fig. 3A; Table 6). A series of sensitivity and 1-specificity values were derived from the curve's coordinates (Table 7). The maximum Youden's index was 0.9, with 94.1 % specificity and 95.9 % sensitivity at a cut-off value of 24.5 % for the cELISA. The sera with a PI $\geq$ 24.5 % were regarded as positive, while those with a PI < 24.5 % were negative (Table 7).

Specificity, sensitivity and reproducibility of the cELISA

By testing 113 positive sera containing antibodies against other avian virus including NDV, HEV, H9N2, FAdV-1 (FAdV-A), FAdV-8a and FAdV-8b (FAdV-E) with the cELISA, the results showed that all sera had PI values <24.5 %, indicating that cELISA specifically detects antibodies against FAdV-4 (Fig. 3B).

When the sequential sera from the four chickens challenged with FAdV-4 were tested using cELISA, the results showed that all four chickens tested positive for anti-FAdV-4 antibodies at 3 dpi, and the sera at 22 dpi from the four chickens were all positive (Fig. 3C). Additionally,

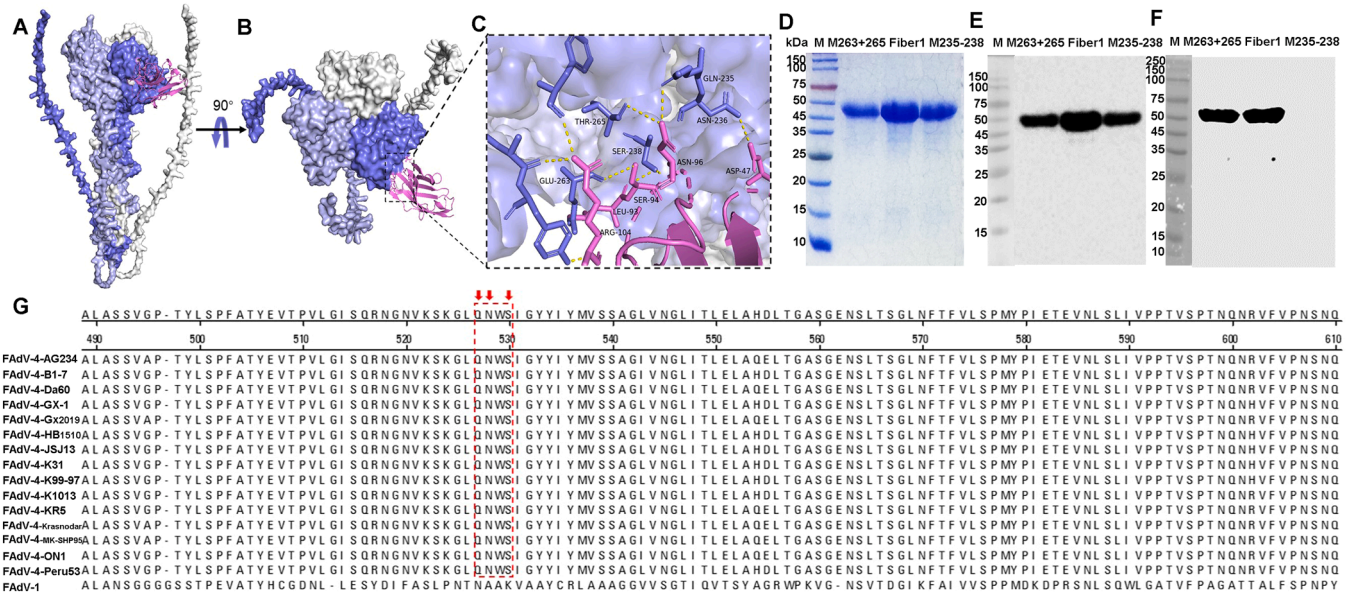

Fig. 6. Identification of the epitope recognized by FAdV-4-Nb28-HRP fusion protein. (A-B) The structure of complex between FAdV-4 fiber-1 protein and FAdV-4-Nb28 was predicted docking by AlphaFold3. Each protomer of the fiber-1 in surface form is indicated with different colors (dark blue, light blue and white) and Nb28 in cartoon form was colored in violet. (C) The rectangular region in the Fig. B has been enlarged to better observe the amino acids involved in the interaction between Nb28 and fiber-1. (D) SDS-PAGE of different mutated FAdV-4 fiber-1 proteins expressed using the *E. coli* system. (E) Western blotting of different mutated FAdV-4 fiber-1 protein using anti-His monoclonal antibody as the primary antibody. (F) Western blotting of different mutated or wild type fiber-1 proteins reaction with FAdV-4-Nb28-HRP. M, protein molecular markers; lane 1 to 3, fiber-1-M263+265, fiber-1 and fiber-1-M235-238, respectively. (G) Sequence alignments of the key motifs binding to FAdV-4-Nb28 of different FAdV-4 isolates and FAdV-1 fiber-1 genes. FAdV, fowl adenovirus serotype 4; HRP, horseradish peroxidas; *E. coli, Escherichia coli.*

different dilutions of the four positive chicken sera were also detected to determine the cELISA's limit detection (LOD). The results showed that all four sera were negative at the dilution of 1:640 and positive at 1:320 (Fig. 3D), indicating that the LOD of the cELISA was 1:320. These findings demonstrate that the cELISA exhibited high sensitivity.

Each of the four positive and negative chicken sera was tested using cELISA to evaluate the CV of inter-assay (between plates) and intra-assay (within a plate) variations. The results showed that the intra-assay CV range was 1.65–5.34 % with a median of 2.31 %, and the inter-assay CV range was 2.22–4.26 % with a median of 3.34 %. The median CV values were all <10 %, which indicated that the developed cELISA had excellent reproducibility (Table 8).

Agreement between the cELISA and commercial ELISA kit

The 114 sera containing 50 immunized and 64 clinical chicken sera were simultaneously tested with the developed cELISA and the commercial FAdV Group I Antibody ELISA kit. For the 50 immunized chicken sera, the agreement between the two methods was 100 %, with 47 identified as positive and three identified as negative for anti-FAdV-4 antibodies. For the 64 clinical chicken sera, the coincidence rate was 96.8 %, with a Kappa index of 0.917, indicating a strong agreement between the two assays (Kappa > 0.45; Table 9). Additionally, the two chicken sera with inconsistent results were further detected using IFA, which confirmed both as positive (Fig. 4). These results suggested that the developed cELISA exhibits a high coincidence rate with the commercial ELISA kit and may offer greater sensitivity.

Epitope recognized by the FAdV-4-Nb28-HRP fusion protein

SDS-PAGE showed that the hexon-HVR, fiber-1, fiber-2 and penton proteins were all successfully expressed with predicted sizes of 51, 45, 89 and 57 kDa by the *E. coil* system (Fig. 5A). Western blotting with anti-His monoclonal antibody as the primary antibody showed that the four proteins were expressed with His tags (Fig. 5B). Western blotting with the FAdV-4-Nb28-HRP fusion protein as the primary antibody demonstrated that the fusion protein specifically reacted with the fiber-1 protein of FAdV-4 (Fig. 5C), indicating that the epitope recognized by the fusion protein resides within the fiber-1 protein.

To further map the epitope recognized by the FAdV-4-Nb28-HRP fusion protein, different truncated and overlapping fragments of fiber-1 protein were designed (Fig. 5D). SDS-PAGE and western blotting confirmed the successful expression of these fragments at the expected sizes (Fig. 5E and F). Then, using the FAdV-4-Nb28-HRP fusion protein as the primary antibody, western blotting showed that the fusion protein can react with the fragments spanning 1–432, 217–432, 1–324, 1–270 and 217–324 aa, but not with 1–216, 109–216 and 271–432 aa indicating that the 217–270 aa region of the fiber-1 protein was the key domain recognized by the FAdV-4-Nb28-HRP fusion protein (Fig. 5D and G).

To finely define the key motifs of the epitope, the 3D structure of the C-terminal of fiber-1 and FAdV-4-Nb28 were separately predicted and docked by AlphaFold3. The docking model showed that Q235, N236, S238, E263, T265 formed hydrogen bonds with FAdV-4-Nb28 in the 217-270 aa region of fibre-1 protein, indicating that these sites may be the key sites involved in the interaction between fibre-1 protein and FAdV-4-Nb28 (Fig. 6A-C). Consistently, these binding sites are included in the truncated segments selected earlier (Fig. 5D). Furthermore, the sites Q235, N236, S238 and the sites E263 and T265 of the fiber-1 protein were mutated into methionine (M) and alanine (A) named FAdV-4-fiber1-M235-238 and FAdV-4-fiber1-M263+265, respectively, and expressed using the *E. coli* system to verify key sites for interaction. SDS-PAGE and western blotting with anti-His monoclonal antibody as the primary antibody confirmed the successful expression of the two fiber-1 mutants at the expected sizes 45 kDa (Fig. 6D and E). Subsequently, western blotting with FAdV-4-Nb28-HRP fusion protein as the primary antibody showed that the fusion protein did not react with FAdV-4-fiber1-M235-238, suggesting that the motifs Q235, N236, S238 of fiber-1 were recognized by FAdV-4-Nb28 with four hydrogen bonds involved in its interaction (Fig. 6C and F). Sequence alignments of the

motifs of FAdV-1, FAdV-4 fiber-1 genes showed that the epitope is unique and highly conserved to FAdV-4 (Fig. 6G), indicating that cELISA with FAdV-4-Nb28-HRP fusion protein as the probe can detect antibodies against different FAdV-4 isolates.

Discussion

At present, only one commercial ELISA kit (FAdV-I Antibody ELISA kit; BioChek BV) is universally used to detect antibodies against FAdV (Lu, et al., 2020a). Several in-house indirect ELISAs were established for the detection of antibodies against different serotypes of FAdV-I using inactivated FAdV-4 particles or the FAdV-1 penton protein as coating antigens, but none of them are specific for detecting anti-FAdV-4 antibodies (Pan, et al., 2020; Shao, et al., 2019b). While the most indirect ELISA based on viral particles or proteins is highly sensitive, it needs high purity of the coating antigens to reduce non-specific reaction. cELISA has the advantages of simple operation, short detection time, no need for HRP-labeled secondary antibodies of different animals and low purity requirement of coated antigen compared with indirect ELISA. However, there are few studies on the use of cELISA for specific detection of anti-FAdV-4 antibodies. In the current study, a nanobody-based one-step cELISA was developed for the detection of anti-FAdV-4 antibodies without using secondary antibody, which has a high consistency with the existing commercial indirect ELISA kit and is more sensitive at detecting the sequential sera from the challenged chickens with FAdV-4.

In view of the characteristics of poultry, the low-cost and high-efficiency detection technology for the evaluation of vaccine immunity and surveillance of disease has important practical utility. However, the traditional antibody-based ELISAs have high purity of antigen and enzyme-labelled secondary antibodies, which resulted in higher production cost (Gu, et al., 2022; Ji, et al., 2022). By contrast, nanobodies present an excellent alternative to overcome the limitations of traditional antibodies while delivering comparable performance (Vattekatte, et al., 2020). On one hand, nanobody can be easily generated in prokaryotic expression systems, which can reduce production costs (Ji, et al., 2022; Lu, et al., 2020b). On the other hand, nanobody can be expressed with HRP or other reporters as tags, which can avoid the use of enzyme-labelled secondary antibodies, and also reduce operating time and production costs (Sun, et al., 2019; Yamagata and Sanes, 2018; Zhao, et al., 2022). In the present study, the developed nanobody-based cELISA using nanobody-HRP fusion as the probe simplifies the operating procedure to one-step when the coated plates were vacuumized and stored. Meanwhile, the full detection time is only 75 min, which is shorter than that of the commercial ELISA kit. In this study, the cost of nanobody production obtained through transient transfection of HEK293T cells at laboratory (5 × T75 flasks, 100 mL culture medium, sufficient for 800 ELISA plates) was approximately 290 RMB per batch. The main cost drivers include plasmid preparation (50 RMB, 17.2 %), DMEM (21 RMB, 7.1 %), FBS (120 RMB, 41.3 %), transfection reagents (50 RMB, 17.2 %) and quality control (50 RMB, 17.2 %). The nanobody-HRP fusion protein was efficiently secreted into the culture supernatant using the HEK293T expression system, eliminating the need for labor-intensive purification steps and in vitro enzymatic conjugation. This approach significantly reduces costs while greatly simplifying the production process. Importantly, the HEK293T platform offers industrial-grade scalability, demonstrating compatibility with high-density bioreactor cultures and ensuring exceptional batch-to-batch consistency (inter-batch CV <10 %). These advantages—cost efficiency, standardized manufacturing, and reagent stability—position the nanobody-based cELISA as a strong candidate for industrial-scale production. Furthermore, the development of a stable Nb28-HRP expressing cell line could enable even more efficient large-scale production in the future.

Fiber-1 protein is one of the main structural proteins located on the surface of FAdV and can induce neutralization antibody responses (Watanabe et al., 2023). In the present study, it was determined that the epitope recognized by the FAdV-4-Nb28-HRP fusion protein was located in the fiber-1 protein, suggesting that the cELISA can be used to evaluate the vaccine immunization of FAdV-4 in chickens. Following the comparisons between the cELISA and commercial ELISA kit to detect 50 immunized chicken sera, it was shown that the coincidence rate was 100 %, indicating that the cELISA could be used to evaluate antibody level after vaccine immunization of FAdV-4. Moreover, some previous studies documented that the fiber-1 protein is essential for FAdV replication and assembly, and the Shaft and Knob domain of the protein directly mediates viral infection (Wang et al., 2020; Zou et al., 2021). Importantly, the fiber-1 protein is highly expressed in the early stage of FAdV-4 infection in chickens, making it a good target for early clinical diagnosis (Li et al., 2017; Pandey et al., 2021). Consistently, the results of the current study showed that anti-FAdV-4 antibodies can be detected at 3 dpi by the developed cELISA, whereas the commercial ELISA kit or some in-house indirect ELISAs detect early only at 7 dpi (Guo, et al., 2022), indicating that the assay has higher sensitivity. Further studies are needed to evaluate the sensitivity of the developed cELISA with sera from additional infected chickens, especially at different days post vaccination. Collectively, the practical feasibility of the cELISA is demonstrated through its cost-efficiency ratio, which includes lower costs, higher throughput, and improved operational efficiency. Additionally, it exhibits standardization potential (inter-plate CV < 10 % as shown in Table 8), meeting the World Organization for Animal Health (OIE) requirements for serological assays, and user-friendly protocols suitable for implementation in basic-tier laboratories. Crucially, the utility and feasibility of cELISA in real-world applications are further validated by its adaptability in detecting specifically anti-FAdV-4 antibodies across sequential sera from challenged chickens, sera of immunized flocks and clinical field sera. In the future, the performance of the cELISA will need to be validated in a wider range of clinical sera, particularly those from different regions, infection stages, and immune statuses.

Previously, the indirect ELISA using fiber proteins as coating antigens was often developed for specific detection of anti-FAdV antibodies (Lu, et al., 2020a). Fiber-1 and −2 proteins are only located and conserved in the FAdV-A (FAdV-1) and FAdV-4C (FAdV-4/10) particles (Hess, et al., 1995; Sohaimi and Bejo, 2021). Therefore, indirect ELISAs based on fiber proteins cannot distinguish anti-FAdV-1, −4 and −10 antibodies. In the present study, the alignment showed that the epitope recognized by the FAdV-4-Nb28-HRP fusion protein was highly conserved among different FAdV-4, but inconsistent with FAdV-1. Therefore, the developed cELISA can be used for specifically detect anti-FAdV-4 antibodies. Although FAdV-10 belongs to FAdV-C, it is rarely detected in the field and is considered a low pathogenicity FAdV (Shao, et al., 2019a). As a result, further investigation is required to assess whether the developed cELISA can detect antibodies against FAdV-10. In addition, previous epidemiological analysis has indicated that FAdV-8 is one of the major pathogenic FAdVs and is currently widespread in domestic flocks (Lu, et al., 2019). However, the results of the current study showed that cELISA cannot detect anti-FAdV-8 antibodies. Therefore, the developed cELISA can serve as a valuable tool to specifically and sensitively detect anti-FAdV-4 antibodies in the chicken sera.

In conclusion, the current study is the first to report a one-step cELISA based on nanobody-HRP fusion proteins to detect anti-FAdV-4 antibodies in vaccine-immunized and infected chickens. The cELISA with high specificity, sensitivity and repeatability, serves as a powerful tool for epidemiological studies, assessing the immune response to FAdV-4 vaccines and developing precise vaccination schedule, import quarantine and biosafety measures, and providing an alternative to VNT for areas without equipment.

CRediT authorship contribution statement

Pinpin Ji: Writing – review & editing, Writing – original draft,

Formal analysis, Data curation, Conceptualization. **Hao Zhang:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Xiri Yangzong:** Writing – original draft. **Ziling Li:** Writing – original draft. **Zhixiang Liu:** Writing – original draft. **Miao Dan:** Writing – original draft. **Xiaoxuan Li:** Writing – original draft. **Xuwen Sun:** Writing – original draft. **Qin Zhao:** Writing – review & editing, Supervision, Resources, Formal analysis, Data curation, Conceptualization. **Yani Sun:** Writing – review & editing, Supervision, Resources, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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