Development of a novel competitive ELISA based on nanobody-horseradish peroxidase fusion protein for rapid detection of antibodies against avian hepatitis E virus

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ABSTRACT Avian hepatitis E virus (**avian HEV**) increases poultry mortality and decreases egg production, leading to huge economic losses worldwide. However, there is no effective serological test for avian HEV. Researchers previously created a testing platform using the nanobody (**Nb**)-horseradish peroxidase (**HRP**) fusion protein as an ultrasensitive probe to develop competitive ELISA (**cELISA**) to detect antibodies against different animal viruses. In this study, a rapid and reliable cELISA was developed to test for antibodies against avian HEV using the same platform. Six anti-avian HEV capsid protein nanobodies were selected from an immunized Bactrian camel using phage display technology. The avian HEV-Nb49-HRP fusion protein was expressed and used as a probe for developing a cELISA assay to test for avian HEV antibodies. The cut-off value of the developed cELISA was 22.0%. There was no cross-reaction with other anti-avian virus antibodies, suggesting that the cELISA had good specificity. The coefficients of variation were 0.91% to 4.21% (intra-assay) and 1.52% to 6.35% (inter-assay). Both cELISA and indirect ELISA showed a consistency of 86.7% (kappa = 0.738) for clinical chicken serum samples, and coincidence between cELISA and Western blot was 96.0% (kappa = 0.919). The epitope recognized by Nb49 was located in aa 593-604 of the avian HEV capsid protein, and the peptide (**TFPS**) in aa 601-604 was essential for binding. The novel cELISA is a saving cost, rapid, useful, and reliable assay for the serological investigation of avian HEV. More importantly, the peptide TFPS may be crucial to immunodominant antigen composition and protection.

Key words: nanobody-HRP fusion protein, HEK293T, competitive ELISA, avian HEV, antibody detection

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INTRODUCTION

The pathogenic avian hepatitis E virus (**HEV**) is a major cause of big liver and spleen disease and hepatitis-splenomegaly syndrome in chicken (Handlinger and Williams, 1988; Ritchie and Riddell, 1991). These diseases increase mortality (1%−4%), decrease egg production (10%−40%), and enlarge the liver and spleen in laying hens and broiler breeders aged 30 to 72 wk (Agunos et al., 2006; Morrow et al., 2008; Massi et al., 2010). The virus has also been found in healthy chickens, which indicates that avian HEV can cause subclinical infection (Huang et al., 2002). To date, 4 major avian HEV genotypes have been divided: genotype 1 avian HEV was documented in South Korea and Australia (Payne et al., 1999; Bilic et al., 2009; Kwon et al., 2012); genotype 2 was found in Korea, Central Europe and United States (Haqshenas et al., 2001; Moon et al., 2016); genotype 3 avian HEV has been reported in China and Europe (Morrow et al., 2008; Marek et al., 2010); and there have been genotype 4 epidemics in Taiwan region and Hungary (Banyai et al., 2012; Hsu and Tsai, 2014). Two novel avian HEV strains have recently been identified in chickens and in silkie fowls in China (Su et al., 2018; Liu et al., 2020).

Avian HEV is a quasi-enveloped, single-stranded positive sense RNA virus. It encodes 3 open-reading frames, ORF1, ORF2, and ORF3 (Huang et al., 2004). ORF1 encodes a non-structural protein, ORF2 encodes a viral capsid protein that contains the major antigenic epitopes, and ORF3 encodes a small multifunctional

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phosphoprotein (Haqshenas et al., 2002; Guo et al., 2007). At present, there is no widely accepted commercial detection kit available to detect the seroprevalence of avian HEV in chickens. Two indirect ELISA kits and a blocking ELISA kit with truncated ORF2 protein as the coating antigen have been developed to detect chicken IgG antibodies against avian HEV (Huang et al., 2002; Zhao et al., 2013). However, these methods have high requirements for the purity and nonspecific binding of the coating antigen and can be time-consuming and complicated to perform (Liu et al., 2014).

Nanobodies (single-domain antibodies) derived from camel, alpaca, and shark heavy chain-only antibodies have recently become widely used in diagnosis and therapy (De Meyer et al., 2014). Nanobodies (15 kDa) are single-domain and strictly monomeric antibody fragments that can be easily cloned and are selectable from naive or immune VHH libraries using phage display technology (Holliger and Hudson, 2005). They are highly stable, easy to produce, and inclined to combine with concave-shaped epitopes (Desmyter et al., 2001). Nanobodies, compared with conventional antibodies, are a promising technology for diagnosing and treating.

In this study, nanobodies against the truncated avian HEV ORF2 protein were selected from a Bactrian camel immunized with the recombinant truncated ORF2 protein using phage display technology. We developed a nanobody-horseradish peroxidase (**HRP**) fusion protein-based competitive ELISA (**cELISA**) from this platform (Sheng et al., 2019) for convenient and faster testing anti-avian HEV antibodies.

MATERIALS AND METHODS

Antigen, Vectors, and Cells

The truncated ORF2 protein (aa 339-606, Ca268 protein) of avian HEV strain isolated from China (CaHEV, GenBank No. GU954430) was used as the antigen for Bactrian camel immunization and as the coated antigen in the cELISA assay (Zhao et al., 2013).

The pCMV-N1-HRP vector was used to construct the expression platform for nanobody-HRP fusion, and the nanobody-HRP fusion protein was expressed in HEK293T cells (Sheng et al., 2019).

Serum Samples

A total of 288 specific pathogen-free (**SPF**) chicken serum samples were used to determine the cELISA cutoff value. A total of 180 sequential chicken serum samples that had been collected from 20 infected chickens at 0, 7, 14, 21, 28, 35, 42, 49, and 56 d post-inoculation (**dpi**) in a previous study (Liu et al., 2014) were used to validate the cELISA assay. One hundred twenty clinical positive serum samples (kept in our laboratory) raised were investigated against other avian viruses were used to determine if the developed cELISA assay had cross-reacted with other chicken virus antibodies; the samples included, the Newcastle disease virus (n = 30), fowl adenovirus (n = 30), avian influenza virus (n = 30), and infectious bursal disease virus (**IBDV**) (n = 30).

In addition, serum samples were collected from 300 clinically healthy chickens (Hy-Line Variety Brown laying hens) of different ages (25−37 wk old) in 4 flocks in Shaanxi province and were used to evaluate the consistency of the cELISA assays with other testing methods.

Selection of Specific Nanobody Against Ca268 Protein

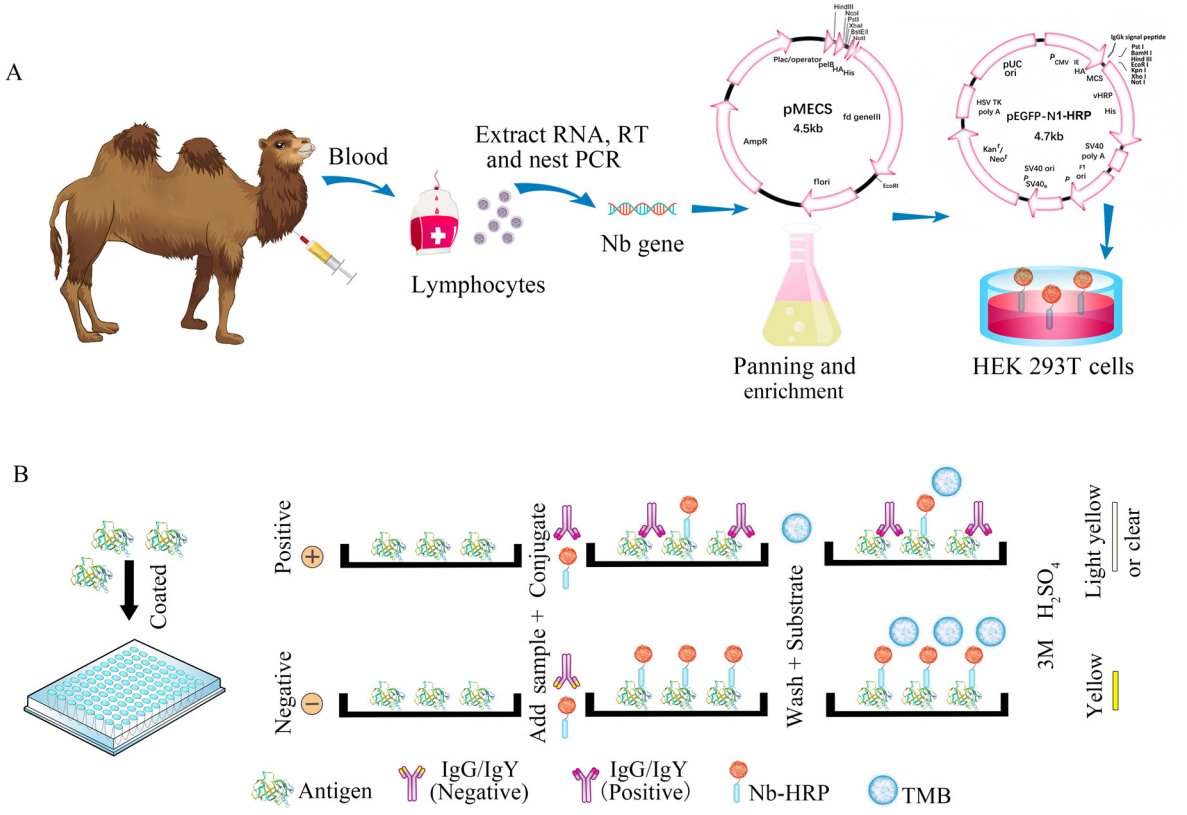
The specific nanobody against the Ca268 protein was selected according to the process described by Sheng et al. (2019). Briefly, a male Bactrian camel (4-yr-old) was immunized with 2 mg Ca268 protein by subcutaneous injection. Complete Freund's adjuvant was used for the first immunization and an equal volume of incomplete Freund's adjuvant was used for the 3 subsequent immunizations, which were carried out at 2-wk intervals. After the last immunization, peripheral blood lymphocytes were isolated from anticoagulated collected blood and total RNA was extracted. The nanobody library was then constructed by transforming recombinant phagemids into TG1 cells. Finally, the specific nanobodies against Ca268 protein were selected using phage display technology. After classified, the best nanobody was selected as the candidate reagent for developing the proposed cELISA assay (Scheme 1A).

Production of Nanobody-HRP Fusion Protein in HEK293T

The platform for nanobody-HRP fusion protein was expressed in HEK293T cells according to the method described by Sheng et al. (2019). Briefly, the candidate nanobody gene was inserted into the pCMV-N1-HRP vector, and the recombinant plasmid was then transfected into the HEK293T cells using Lipo 8000 transfection reagent. After 48 h, the cell culture medium was collected and filtered using a 0.45 μm filter (Scheme 1A).

Development of cELISA Using Nanobody-HRP Fusion Protein as Reagent

First, the concentration of the coated antigen and the dilution of the nanobody-HRP fusion were optimized by a checkerboard titration using direct ELISA. Different concentrations of Ca268 proteins were coated (0.5, 1, 2, and 4 μg/mL), and the dilution ratios of nanobody-HRP fusions were from $1:2^4$ to $1:2^{11}$. The final conditions were selected that produced an OD_{450nm} value of approximately 1.0. Subsequently, the optimal dilution of chicken serum was also determined. Four separate anti-aHEV antibody positive and antibody negative chicken serum samples were diluted at 1:5, 1:10, 1:20, and 1:40 for cELISA assay, and the optimal dilution selected was that which produced the least OD_{450nm} ratio between the positive and negative sera (**P**/**N**).



Scheme 1. Graphic abstract. (A) Diagram for the acquisition of the VHH library and the expression of nanobody-HRP fusion proteins. (B) Design of the developed cELISA. Abbreviations: cELISA, competitive ELISA; HRP, horseradish peroxidase.

Finally, the reaction times between testing serum and nanobody-HRP fusion with antigen and the colorimetric reaction were further optimized. The incubation times of the mixtures were set to 15, 30, 45, and 60 min. After tetramethylbenzidine (**TMB**) was added, the colorimetric reaction times were set to 10 and 15 min, and the reaction that produced the smallest P/N ratio was selected as optimal.

After optimization, the optimum concentration of Ca268 protein was coated on the plates overnight at 4° C. The plates were washed 3 times with PBS'T and blocked with blocking buffer for 1 h at room temperature (**RT**). The washing operation was performed again, and 100 μL of the optimal testing mixture was added to the wells, which were then incubated at RT for the optimal time. After another 3 times washing, 100 μL TMB was added, and the plates were incubated for the optimal time at RT. In the final step, the colorimetric reaction was stopped using 3 M H_2SO_4 (50 μL/well), and the OD_{450nm} values were read by an automatic ELISA microplate reader (Scheme 1B).

Validation of cELISA

The percent competitive inhibition (**PI**) was calculated using the following formula: PI (%) = [1 − (OD_{450nm} value of testing serum sample / OD_{450nm} value of negative serum sample)] × 100%. The 288 negative serum samples from SPF chickens were tested using the developed cELISA assay, and the cut-off value was determined by the mean PI of 288 negative serum samples plus 3 standard deviations (**SD**) to ensure 99% confidence for the negative serum samples in this range.

To determine whether the development of cELISA had cross-reactivity with other positive serums against the chicken virus, including Newcastle disease virus, fowl adenovirus, avian influenza virus, and IBDV, 120 sera samples were assayed. To determine the sensitivity of cELISA, 180 sequential chicken serum samples from 20 infected chickens were tested with cELISA and indirect ELISA (**iELISA**). The iELISA assay was conducted as described by Zhao et al. (2013). Besides, 5 positive sera twice diluted from 1:10 to 1:1280 were also detected with the cELISA to determine the lowest detection limit.

Three positive and 3 negative serum samples were selected to evaluate the repeatability of cELISA. Each sample was added to 3 different plates at different times to calculate the inter-assay coefficient of variance (**CV**), and 3 replicates within each plate were used to determine the intra-assay CV.

Comparisons of cELISA With iELISA and With Western Blot

To date, there is no available commercial kit specifically for detecting avian HEV antibodies, and iELISA

and Western blotting are generally used for detection. A total of 300 clinical serum samples were collected from healthy chickens of different ages in 4 flocks in Shaanxi province. The samples were tested using the developed cELISA, iELISA, and Western blotting, respectively. The iELISA and Western blot assays were carried out according to the procedures described by Zhao et al. (2013).

Determination of Epitopes Recognized by Nanobody

Currently, 5 antigen regions (I−V) in Ca268 protein have been predicted using the Welling method (Haqshenas et al., 2002; Dong et al., 2011). In order to identify the epitope recognized by a nanobody, the truncated protein ap237 (aa 313-549) preserved in our laboratory was first tested with nanobody-HRP fusions by Western blot. The Ca268 protein was then continuously truncated and identified. The truncated proteins were prokaryotically expressed and renatured in the same way as was the Ca268 protein. Two overlapping short peptides were synthesized for verification using direct ELISA. In future research, sequence analysis and amino acid mutation will be used to identify whether the epitopes of other avian HEV strains will also be recognized by the nanobody.

Statistical Analysis

Kappa values were calculated to determine the agreement between cELISA and iELISA and between cELISA and Western blot using IBM SPSS Statistics 20.

RESULTS

Construction of a Phage Display VHH Library

The titer of antibodies against Ca268 protein in camel sera from the last immunization was evaluated using iELISA and reached $1{:}10^7$ (Figure 1A). A VHH library was successfully constructed, based on previously described methods, with 6.3×10^8 individual colonies. Subsequently, 98% (47/48) of the VHH gene fragments were inserted into the phage display vector (Figure S1), and after sequencing, the library showed wide diversity. The library was rescued with the M13KO7 helper phage, and the titer of the recombinant phage library reached 4.5×10^{12} pfu/mL.

Screening and Sequencing of Nanobodies Against Ca268 Protein

The phage particles (carrying specific VHHs) against Ca268 protein were strongly enriched after 3 rounds of panning (Table 1). Periplasmic extracts were expressed from 96 individual colonies and screened for binding to Ca268 protein by iELISA, out of which 80 fragments were identified for specific binding to the Ca268 protein (Figure S2). Sequence analysis showed that 6 nanobodies (Nb12, Nb13, Nb20, Nb49, Nb58, and Nb76) were identified according to the amino acid sequences of the CDR3 region, and the conserved residues from the 6 nanobodies (at positions 37, 44, 45, and 47) were determined to be hydrophilic amino acids (Figure 1B). These nanobodies reacted only with Ca268 protein and not with IBDV-VP2 protein (as a control protein), as shown by iELISA (Figure 1C). Nb49 showed the strongest binding ability with Ca268 (Figure 1D), so it was chosen and expressed to create a cELISA with nanobody-HRP fusions as a probe.

Expression of CaHEV-Nb49-HRP Fusion Protein in HEK293T Cells

After the CaHEV-Nb49 VHH gene was inserted into the pCMV-N1-HRP vector, the recombinant plasmid was transfected into the HEK293T cells. The CaHEV-Nb49-HRP fusion protein was successfully expressed in the HEK293T cells using anti-HA mAb for detection by IFA (Figure 2A), and the titer of this protein in the medium exceeded 1:1000 (Figure 2B).

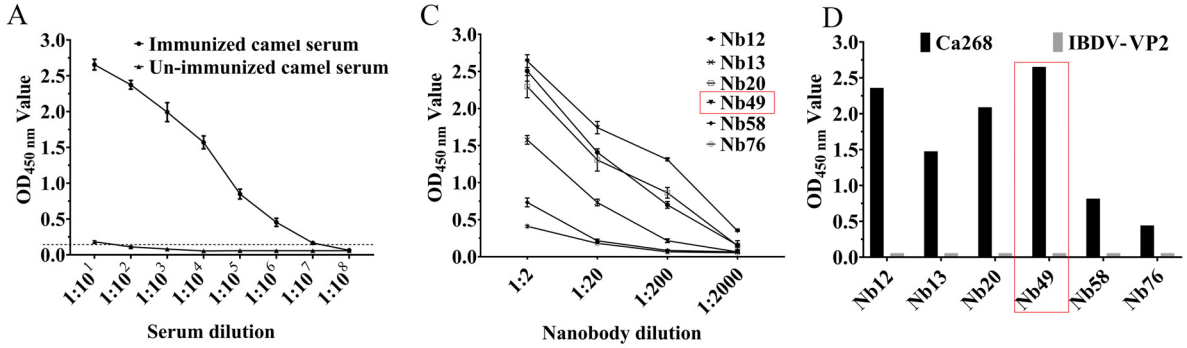
cELISA Using CaHEV-Nb49-HRP Fusions as Reagents

The checkerboard titration assay showed that the optimal coating concentration of Ca268 protein was 1 μg/mL, and the dilution of CaHEV-Nb49-HRP fusions was $1{:}2^8$ (Table 2). The optimal dilution of chicken sera was 1:5 in cELISA (Table 3). A checkerboard assay was subsequently used to determine the optimal incubation times for mixtures of chicken sera and CaHEV-Nb49-HRP fusions, and colorimetric reaction showed that the P/N ratio was least when the incubation time was 45 min, and the reaction time was 15 min (Table 4).

After optimization, the cELISA assays were carried out as follows. First, 1 μg/mL of Ca268 protein was coated on the 96-well ELISA plates overnight at 4℃. After washing 3 times with PBS'T, the plates were blocked with blocking buffer (200 μL) at RT for 1 h. The washing operation was performed again, and the 100 μL of testing mixture consisting of 20 μL chicken serum test sample and 80 μL CaHEV-Nb49-HRP fusion was added and incubated at RT for 45 min. After washing 3 times, 100 μL TMB was added and the plates were incubated in the dark at RT for 15 min. In the final step, the colorimetric reaction was stopped using 3 M H_2SO_4 (50 μL/well) and the OD_{450nm} values were read by an automatic ELISA microplate reader.

Cut-off Values for the cELISA

The 288 SPF chicken serum samples were assayed by cELISA to determine the cut-off values. The results showed that the average PI (X) value was 4.0%, with SD of 6.0%. Thus, the cut-off value for the developed cELISA was 22.0% (4.0% + 3 × 6.0%). When the PI



B

	Framework1			CDR1	Framework2			CDR2		
	10		20	30	40			50		60
CaHEV-Nb12	QVQLQESGGGSVQAGGSLRLSCAAS			GYIYSTNYMG	WFRQAPGKEREGVA			AIYTGDGSTLYADSVKG		
CaHEV-Nb13	QVQLQESGGGSVQAGGSLRLSCAYS			GYDFCNYDIS	WYRQGPGKEREFVS			GLEG-DGRISYADAVKG		
CaHEV-Nb20	QVQLQESGGGLVQPGGSLRLSCAAS			GYTFSSGCMG	WIRQAPGKEPEGVA			AVDI-DGTTSFADSVKG		
CaHEV-Nb49	QVQLQESGGDSVQAGGSLRVSCAAA			GFTYSGDCMA	WFRQAPGKEREGVA			TIAR-DYSTNYADSVKG		
CaHEV-Nb58	QVQLQESGGGSVQAGGSLRLSCAAS			GLIYHNNYWG	WFRQNPGKEREGVA			SLYTGDYSTFYGDSVKG		
CaHEV-Nb76	QVQLQESGGGSVQAGGSLRLSCAAS			GLIYSNNYWG	WFRQVPGKEREGVA			GYYTGNGATYYDDSVKG		

	Framework3				CDR3			Framework4		
	70		80	90	100		110	120		
CaHEV-Nb12	RFTISQDNAKNTVYLQMNSLKPEDTAMYYCAS				GDPAMYSDYALRGDGYNY			WGQGTQVTVSS		
CaHEV-Nb13	RFTISKDHAKNTAYLQMNSLKPEDTARYYCKI				EGG-ASLCYNGRG-----			WGQGTQVTVSS		
CaHEV-Nb20	RFTISKDNAKNTLYLQMNSLKPEDTALYYCAA				DTP--ERCASDYDVPFAY			SGQGTQVTVSS		
CaHEV-Nb49	RFTISKDDAKSTLYLQMNSLKPEDTAMYYCAA				RPWAGYRCDFAGG-EYNY			WGQGTQVTVSS		
CaHEV-Nb58	RFTISRDSAKNTLYLHMNSLRPEDTAMYYCAA				GGRNGGSSSLETS-TYAY			WGQGTQVTVSS		
CaHEV-Nb76	RFTISRDSANNMLYLQMQRLKPEDTAMYYCAA				GRLR--APWYSSD-EYPY			WGQGTQVTVSS		

Figure 1. Screening nanobodies against Ca268 protein. (A) Titers of antibodies against Ca268 protein in immunized camel serum. (B) Alignment of amino acid sequence of 6 screened nanobodies. The residues are labeled by red triangles. (C) Specific reactions of the 6 screened nanobodies with Ca268 protein. IBDV-VP2 protein was used as a His-tag control protein. (D) Titration of the 6 screened nanobodies for binding ability with Ca268 protein. Abbreviation: IBDV, infectious bursal disease virus.

Table 1. Enrichment of nanobodies against the Ca268 protein from the phages during 3 rounds panning.

Round of panning	Phage input (PFU/well)	Phage output/P (PFU/ well)	PBS output/N (PFU/well)	Recovery rate (P/input)	Enrichment (P/N)
1st round	5×10^{10}	1.7×10^{3}	1.7×10^{1}	3.4×10^{-8}	1.0×10^{2}
2nd round	5×10^{10}	1.6×10^{7}	1.16×10^{5}	3.2×10^{-4}	1.38×10^{2}
3rd round	5×10^{10}	1.5×10^{7}	7.1×10^{3}	3.0×10^{-2}	2.1×10^{3}

Abbreviation: P/N, positive and negative; PBS, phosphate buffer saline; PFU, plaque forming unit.

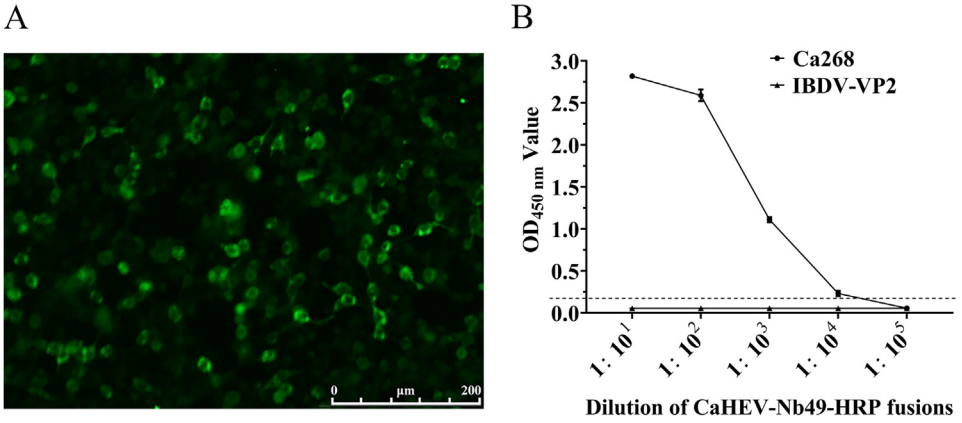


Figure 2. Identification of CaHEV-Nb49-HRP fusion protein expression. (A) Analysis of CaHEV-Nb49-HRP expressed in HEK293T cells by IFA. (B) Detection of CaHEV-Nb49-HRP reaction with Ca268 protein using ELISA. Abbreviation: HRP, horseradish peroxidase.

Table 2. Determination of the optimal coating concentration of Ca268 protein and the optimal dilution of CaHEV-Nb49-HRP fusions by direct ELISA.

Different amounts of the Ca268 protein (μg/mL)	Different dilutions of CaHEV-Nb49-HRP fusions in the medium							
	$1:2^4$	$1:2^5$	$1:2^6$	$1:2^7$	$1:2^8$	$1:2^9$	$1:2^{10}$	$1:2^{11}$
0.5	1.508	1.257	0.957	0.679	0.232	0.172	0.084	0.063
1	2.153	1.921	1.675	1.380	0.988	0.438	0.258	0.215
2	2.470	2.296	2.013	1.839	1.424	0.962	0.454	0.343
4	3.040	2.859	2.686	2.470	1.912	1.550	1.132	0.650

Abbreviation: HRP, horseradish peroxidase.

The optimal concentration of Ca268 protein and dilution of CaHEV-Nb49-HRP were chosen when the OD_{450nm} value of the direct ELISA was approximately 1.0.

Table 3. Determination of the optimal dilution of tested chicken serum for cELISA.

No. serum	Sera type	1:5	1:10	1:20	1:40
1	Positive	0.654	0.733	0.890	1.051
	Negative	1.029	0.996	1.009	1.084
	P/N	0.636	0.736	0.882	0.970
2	Positive	0.551	0.703	0.931	1.018
	Negative	1.046	1.020	0.971	1.042
	P/N	0.527	0.689	0.959	0.977
3	Positive	0.113	0.292	0.494	0.685
	Negative	0.975	0.985	0.950	1.008
	P/N	0.116	0.296	0.520	0.680
4	Positive	0.183	0.339	0.566	0.737
	Negative	0.996	1.004	1.038	1.148
	P/N	0.184	0.338	0.545	0.642

Abbreviations: cELISA, competitive ELISA, P/N, positive and negative.

Four positive and negative chicken serums were separately used for cELISA testing. The dilutions of serum were 1:5, 1:10, 1:20 and 1:40. The best dilution was chosen when the OD_{450nm} value of positive to negative (P/N) sera was smallest.

value of the tested chicken serum was ≥22.0%, it was considered to be positive, otherwise it was considered to be negative.

Sensitivity, Specificity, and Reproducibility of cELISA

A total of 180 sequential sera were assayed to determine the sensitivity of the developed cELISA. Both cELISA and iELISA showed that all chickens had seroconverted at 14 dpi and remained positive until 28 dpi (Figure 3A). For the different dilution of the 5 positive chicken sera, all samples at the dilution of 1:320 were negative using cELISA, whereas only 1 sample was positive when diluted 1:160 (Figure 3B). Therefore, for most positive chicken serum samples, the largest dilution was 1:80 for detecting anti-avian HEV antibodies. To determine the specificity of cELISA, the positive serum against other chicken viruses was negative for detection (PI values from 0.12% to 18.91%, Figure 3C). The reproducibility results showed that the intra-assay CV of the PI ranged from 0.91% to 4.21% (median 1.63%), and the inter-assay CV ranged from 1.52% to 6.35% (median 3.95%)

Agreement of cELISA With iELISA and With Western Blot

To determine if the developed cELISA can be used to test clinical samples, 300 clinical serum samples from chickens in large-scale farms in Shaanxi provinces were assayed by the developed cELISA, iELISA, and Western blot. The results of cELISA and iELISA coincided for 260 of the 300 serum samples, an agreement rate of 86.7%, with kappa = 0.738 (Table 5). The results of cELISA and Western blot agreed in 288 of the 300 serum samples, an agreement rate of 96%, with kappa = 0.919 (Table 5). There were no significant differences between cELISA and either iELISA or the Western blot; all kappa values were >0.4.

Epitopes Recognized by Nanobody

Six antigen regions (I–VI) were identified on the avian HEV capsid protein (Figure 4A) (Haqshenas et al., 2002; Dong et al., 2011; Wang et al., 2014). No reactivity was observed for the truncated

Table 4. Optimized incubation time of the mixtures and the optimal time for colorimetric reaction after adding TMB by a checkerboard assay.

Times (min) of color reaction	Sera type	Incubation times (min) of chicken sera and CaHEV-Nb49-HRP fusions			
		15	30	45	60
10	Positive	0.337	0.126	0.129	0.130
	Negative	0.512	0.602	0.621	0.675
	P/N	0.658	0.209	0.208	0.193
15	Positive	0.234	0.209	0.161	0.159
	Negative	0.582	0.733	0.985	0.988
	P/N	0.402	0.285	0.163	0.161

Abbreviations: HRP, horseradish peroxidase; P/N, positive and negative; TMB, tetramethylbenzidine.

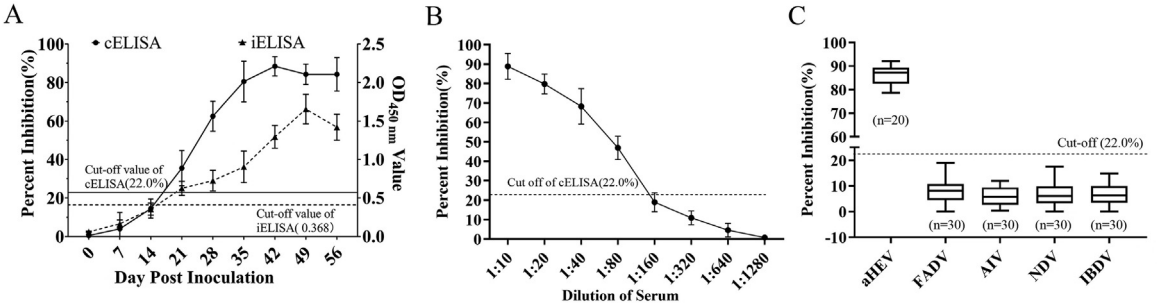


Figure 3. Sensitivity and specificity of cELISA using CaHEV-Nb49-HRP as a probe. (A) Detection of antibodies against avian HEV in sera using cELISA and iELISA. (B) Determination of the largest dilution of positive chicken sera for anti-avian HEV antibodies. (C) Analysis of the cELISA testing the antibodies against other chicken disease viruses, including NDV, FADV, AIV, and IBDV. Abbreviations: AIV, avian influenza virus; cELISA, competitive ELISA; FADV, fowl adenovirus; HEV, hepatitis E virus; HRP, horseradish peroxidase; IBDV, infectious bursal disease virus; iELISA, indirect ELISA; NDV, Newcastle disease virus.

protein ap237 (aa 313-549) binding to Nb49-HRP fusion protein using Western blotting (Figure 4B). Three truncated overlapping fragments (aa 339-583, aa 339-600, and aa 339-604) were therefore designed using Lasergene Protein software (Figure 4A). The Western blotting results showed that Nb49-HRP fusion protein reacted with Ca268 protein fragments spanning aa 339-604, and 339-606, but not aa 339-583 or 339-600 (Figure 4B), which suggests that the epitope recognized by Nb49-HRP fusion protein was located in the C-terminus of Ca268 protein. Two peptides (aa 593-604 and aa 589-600) were then synthesized based on the already identified antigenic domains (Figure 4A). Peptide aa 593-604 showed higher reactivity than peptide aa 589-600 in binding to the Nb49-HRP fusion protein (Figure 4C). In all avian HEV sequences of different genotypes, only one amino acid had been mutated (R or K in aa 600) in aa 593-604 (Figure 4D). The mutation Ca268 protein (R or K in aa 600) was expressed, and the Western blot showed that the Nb49-HRP fusion protein reacted with it (Figure 4E). These results suggest that Nb49 combines with all known HEV ORF2 proteins of different genotypes.

DISCUSSION

Current serological tests (including iELISA, bELISA, and Western blot) are mainly diagnostic methods of avian HEV infection (Huang et al., 2002; Zhao et al., 2013; Liu et al., 2014). However, these methods have high requirements for the purity and non-specific binding of the coating antigen, and the process is time-consuming, complicated and inconvenient for large-scale serological investigations (Liu et al., 2014). In contrast with conventional polyclonal and monoclonal antibodies, nanobodies have higher specificity, higher affinity, and wider antigen-binding sites and have been used to develop diagnostic technologies for animal diseases (De Meyer et al., 2014). These characteristics enable them to overcome aforementioned limitations and be used as probes for ELISA development. A nanobody-HRP fusion technique was used for the first time to create a cELISA assay to test for antibodies against avian HEV. No secondary antibody is required for detection in this method, which therefore greatly saves cost and time.

In the previous study, 5 antigen regions (I−V) in Ca268 protein were predicted using the Welling method (Haqshenas et al., 2002; Dong et al., 2011). In this study, the recognized epitope (aa 593-604), and particularly the peptide TFPS (aa 601-604, outside region IV) was essential for Nb49 binding (Figure 4B and Figure 4C). Nb49, used as a probe in the developed cELISA, showed a good response with clinical chicken serum. Studies have shown that region IV (aa 583-600) was not an immunodominant antigen and also had no protective function against avian HEV infection, but that Ca268 protein (aa 339-606) provided effective immune protection (Guo et al., 2007; Zhao et al., 2012). We therefore hypothesized that peptide TFPS is an essential component for the antigenic region IV, and it may also have an important protective function. Further experiments will be conducted to verify our hypothesis.

At present, 4 major avian HEV genotypes have been divided, and 2 novel avian HEV strains have recently been separately identified in chickens and silkie fowl in China (Su et al., 2018; Liu et al., 2020). In this study, the epitope recognized by Nb49 was located in aa 593-604 of CaHEV ORF2 (genotype 3). Compared

Table 5. Comparisons of the developed cELISA with iELISA and with Western blot by testing clinical chicken serum.

		iELISA				Western blot			
Samples number	cELISA	+	−	Agreement (%)[a]	Kappa value	+	−	Agreement (%)[a]	Kappa value
124	+	124 (A)	0 (B)	86.7	0.738	124 (A)	0 (B)	96.0	0.919
176	−	40 (C)	136 (D)			12 (C)	164 (D)		

Abbreviations: cELISA, competitive ELISA; iELISA, indirect ELISA.
[a]agreement (%) = (A+D)/300 × 100.

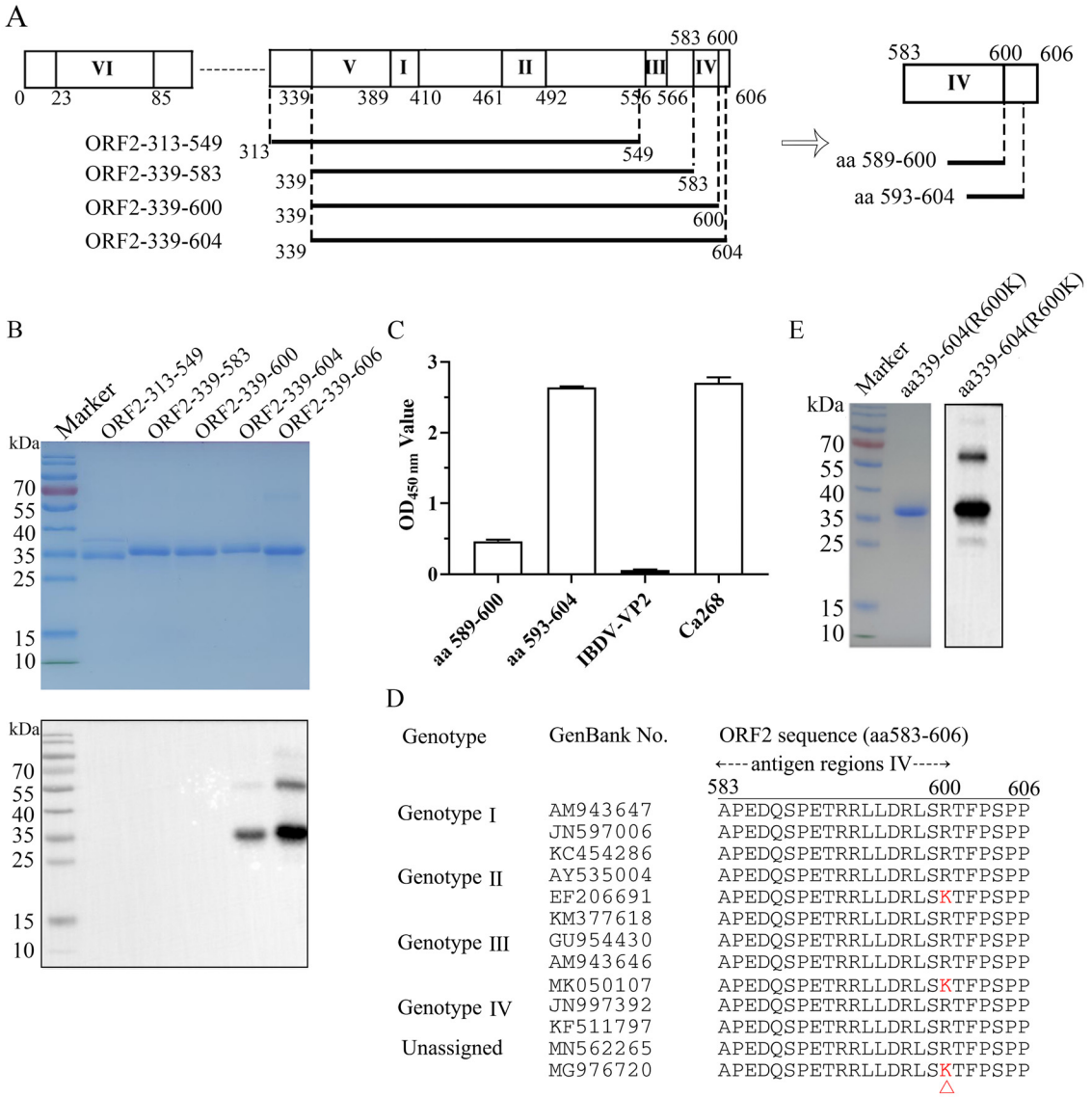


Figure 4. Determination of epitopes by nanobody. (A) The linear locations of various truncated fragments, showing 6 antigen regions (I−VI) and synthetic peptide fragments. (B) SDS-PAGE analysis of expressed truncated fragments and Western blotting of CaHEV-Nb49-HRP fusions binding to these fragments. (C) ELISA analysis showing binding of CaHEV-Nb49-HRP fusions to peptides, Ca268 protein (positive control) and IBDV-VP2 protein (negative control). (D) Amino acid alignments of capsid proteins from 13 avian HEV isolates using Lasergene 7.1 (DNASTAR, Inc.). The mutation (R or K) at position 600 is indicated by the red triangle. (E) SDS-PAGE analysis of expressed truncated fragment (aa 339-604, R600K) and Western blotting of CaHEV-Nb49-HRP fusions binding to this fragment. Abbreviations: HRP, horseradish peroxidase; IBDV, infectious bursal disease virus; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

with all avian HEV sequences of different genotypes, only one amino acid has mutated (R or K in aa 600), in aa 593-604 (Figure 4D). Western blotting showed that Nb49-HRP fusion protein reacted with the mutated Ca268 protein (Figure 4E), which suggests that cELISA can be used to detect antibodies against all aHEV genotypes.

There is no gold standard assay for avian HEV antibody detection. Conventionally, iELISA and Western blot methods can be used to detect antibodies against avian HEV. We compared cELISA against iELISA and Western blot; our results showed high reproducibility and no significant differences (Kappa = 0.738 and Kappa = 0.919), which suggests that the cELISA can replace iELISA and Western blot in detecting anti-avian HEV antibodies in poultry.

In summary, a nanobody Nb49-based cELISA was developed that can be used to test for antibodies against all known genotypes of avian HEV. This assay is time-saving, convenient, reproducible, highly specific, and sensitive. Therefore, this method is ideal for large-scale serological testing for avian HEV infections in poultry. In addition, a vital antigenic region in avian HEV capsid protein was identified that may provide an important site for future immunodominant antigen and protective function research.

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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102326.

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