Virology 528 (2019) 101–109

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Identification and characterization of a novel nanobody against duck hepatitis A virus type 1

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ARTICLE INFO

Keywords:
DHAV-1
VP1
Phage display
Nanobody
Epitope

ABSTRACT

Duck virus hepatitis (DVH) caused by duck hepatitis A virus type 1 (DHAV-1) is an acute and highly contagious disease affecting young ducklings. The VP1 protein is one of the major structural proteins of DHAV-1 carries critical epitopes responsible for the induction of neutralizing antibodies. In this study, we have successfully constructed an immune phage display VHHs library against DHAV-1 with the size of 6×10^6 colonies. A nanobody (Nb) against VP1 protein of DHAV-1, named Nb25, was identified from the immunized phage display library. Nb25 could react with the conserved linear B-cell epitope of ^{174}PAPTST^{179} in DHAV-1 VP1, even though Nb25 showed no neutralizing activity to DHAV-1. To the best of our knowledge, this is the first report about preparation of anti-DHAV-1 Nbs and identification of the specific conserved linear B-cell epitope of DHAV-1 with Nb, which will facilitate the serologic diagnosis of DHAV-1 infection.

1. Introduction

Duck viral hepatitis (DVH) is an acute, contagious, rapidly spreading viral infection of young ducklings that is characterized primarily by ecchymotic hemorrhage and liver necrosis (Chen et al., 2013). DVH is mainly caused by duck hepatitis A virus (DHAV), which is the only member of a novel genus *Avihepatovirus* in the family *Picornaviridae* (Li et al., 2013; Wang et al., 2018). By phylogenetic analyses and neutralization tests, DHAV strains are categorized into three different serotypes: the traditional serotype 1 (DHAV-1) (Ding and Zhang, 2007; Tseng et al., 2007), a serotype only reported in Taiwan (DHAV-2) (Tseng and Tsai, 2007) and the serotype that was isolated in South Korea and China (DHAV-3) (Fu et al., 2008; Kim et al., 2007; Xu et al., 2012). DHAV-1 is the most widespread and virulent serotype that has the most significant impact on the worldwide poultry industry (Erfan et al., 2015; Soliman et al., 2015).

DHAV-1 is a small nonenveloped virus with an approximately 7.8 kb single-stranded, positive-sense RNA genome, which contains a single open reading frame (ORF) encoding a large polyprotein, flanked by the 5′ and 3′ untranslated regions (UTRs) (Ding and Zhang, 2007; Tseng et al., 2007). The polyprotein is cleaved into three mature structural proteins (VP0, VP1, and VP3) and nine nonstructural proteins (2A1, 2A2, 2A3, 2B, 2C, 3A, 3B, 3C, and 3D) (Tseng and Tsai, 2007). Among these proteins, the principal antigenic determinant VP1 plays an essential role in pathogenicity, evolution, and virulence (Jin et al., 2008; Li et al., 2013; Zhang et al., 2015).

The *Camelidae* family produces a particular class of immunoglobulins, called heavy-chain antibodies (HCAbs) with lack of light chain naturally, and their antigen-binding site consists of a single domain, known as Nanobodies (Nbs) or variable domain of the heavy chain of the heavy-chain antibodies (VHHs), which are considered as the smallest available intact antigen-binding fragments (Hamers-Casterman et al., 1993; Muyldermans, 2001; Muyldermans et al., 2009). For possessing many attractive features, such as low immunogenicity, strong stability, good solubility, ease of genetic manipulation and easy expression, Nbs has been widely used in biomedicine and immunoassay field (Chakravarty et al., 2014; Liu et al., 2017).

To date, the application of Nbs in the diagnosis and treatment of DHAV has not been studied. In this study, an immune library of 6×10^6 individual Nb clones was constructed, a DHAV-1 VP1-specific Nb was isolated via phage display and characterized by ELISA, and its conserved linear B-cell epitope was identified by dot blot. These results may facilitate future investigations on the function of VP1 protein and the development of immunoassays for the diagnosis of DHAV-1

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https://doi.org/10.1016/j.virol.2018.12.013
Received 29 August 2018; Received in revised form 17 December 2018; Accepted 18 December 2018
Available online 24 December 2018
0042-6822/

infection.

2. Results

2.1. Construction of VHH library

Camel immunization with live vaccine of DHAV-1 was assessed via ELISA. The specific antibody titers rapidly raised after the second injection. Then, RT-PCR was performed to obtain the coding genes of variable domains of the heavy-chain antibodies from PBMCs. The PCR fragments were cloned into phagemid vector pCANTAB 5E and transformed into *Escherichia coli* (*E. coli*) TG1 cells. The final library was calculated to contain about 6×10^6 independent clones. A total of 96 individual colonies were randomly chosen for colony PCR and an amplicon of expected size (around 750 bp) was obtained from 93 of the randomly picked clones. The PCR amplicons of forty positive clones were sequenced, and each clone contained a distinct VHH sequence respectively, which confirmed the heterogeneity of the individual clones from the library.

2.2. Phage bio-panning

The DHAV-1 VP1 gene was expressed as a His_6-fusion protein and a GST-fusion protein, respectively. As shown by western blot, both fusion proteins could react with anti-DHAV-1 mAb 4F8 (Zhang et al., 2015) (Fig. 1A).

The VHH phage library was subjected to bio-panning against captured DHAV-1 VP1-His protein. After three rounds of panning, the ratio of the numbers of positive/negative colonies increased from 6.9 to 1349 (Table 1). Polyclonal phage ELISA of specific phage particles from each round of bio-panning also showed that the phage particles binding to DHAV-1 VP1-His were strongly enriched after three rounds of bio-panning (Fig. 1B). These results indicates that the phage particles expressing VP1-specific Nbs effectively enriched.

2.3. Selection and expression of VP1 specific Nbs

The periplasmic extracts from 48 selected clones were tested by an ELISA with DHAV-1 VP1-His protein as coating antigen. The result showed that the vast majority of extracts had a signal strength, which was at least three-fold higher than the signal of control wells containing the same extract but without coated VP1-His (Fig. 2A). Sequence analysis found a unique clone presented in this set of antibody fragments, named Nb25, which contained the major hallmark amino acids of Nb (Fig. 2B). The coding gene of Nb25 was recloned into expression vector, the purified Nb25 was confirmed by Western blot (Fig. 2C).

Table 1
Enrichment of phage particles carrying DHAV-1 VP1-specific Nbs.

Round of panning	Input phage (PFU/ml)	Output phage (PFU/ml)		P/N
		Positive	Negative	
1st round	5×10^{10}	3.30×10^5	4.8×10^4	6.9
2nd round	5×10^{10}	4.60×10^6	3.6×10^4	128
3rd round	5×10^{10}	5.80×10^7	4.3×10^4	1349

PFU: plaque forming unit.

2.4. The reactivity and specificity of Nb25

Using DHAV-1 VP1-GST as coating antigen, indirect ELISA was used to detect the reactivity and specificity of Nb25. The Nb25 was found to have an obvious reaction with VP1-GST protein when approximately 250 μg/ml, and when the Nb25 concentration increased, the reaction was more obvious (Fig. 3A). According to the result of specificity assay, Nb25 exhibited a very strong signal with DHAV-1 VP1-GST ($OD_{450} > 1.50$) and purified DHAV-1 particles ($OD_{450} > 0.8$), while other non-VP1 proteins showed low background-signals ($OD_{450} < 0.20$) (Fig. 3B). IFA result showed that Nb25 had intracellular binding activity with DHAV-1, whereas did not reacted with DHAV-3 and the mock control (Fig. 3C). Those results showed that Nb25 has good binding activity and specificity with DHAV-1 VP1 protein.

Neutralization assay results demonstrated that Nb25 failed to neutralize DHAV-1, the results showed that the identified Nb25 could only be used for the application of DHAV-1 detection rather than as neutralizing antibody against DHAV-1.

2.5. Identification of the liner epitope in DHAV-1 VP1 to Nb25

Totally five overlapping GST-fused fragments (VP1-1, VP1-2, VP1-3, VP1-4 and VP1-5) spanning the DHAV-1 VP1 protein were expressed and conducted to dot blot analysis. As demonstrated by dot blot assay, VP1-4 and VP1-5 showed reactivity with Nb25 (Fig. 4A). So the liner epitope recognized by Nb25 was preliminary speculated as

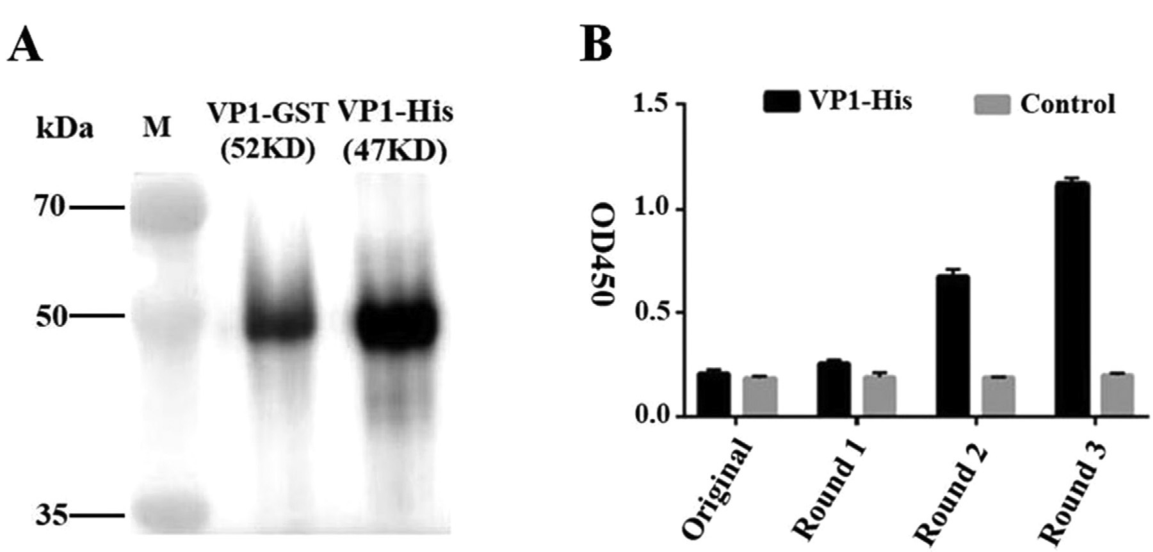

Fig. 1. Identification of DHAV-1 VP1 protein and the enrichment degree of phage particles expressing VP1-specific Nbs. (A) Analysis of the expressed fusion VP1 proteins by western blot using mAb 4F8. (B) Polyclonal phage ELISA of specific phage particles from each round of bio-panning. The coated antigen was DHAV-1 VP1-His and the negative controls was SMP. The OD_{450} values were the means ± SDs from triplicate measurements.

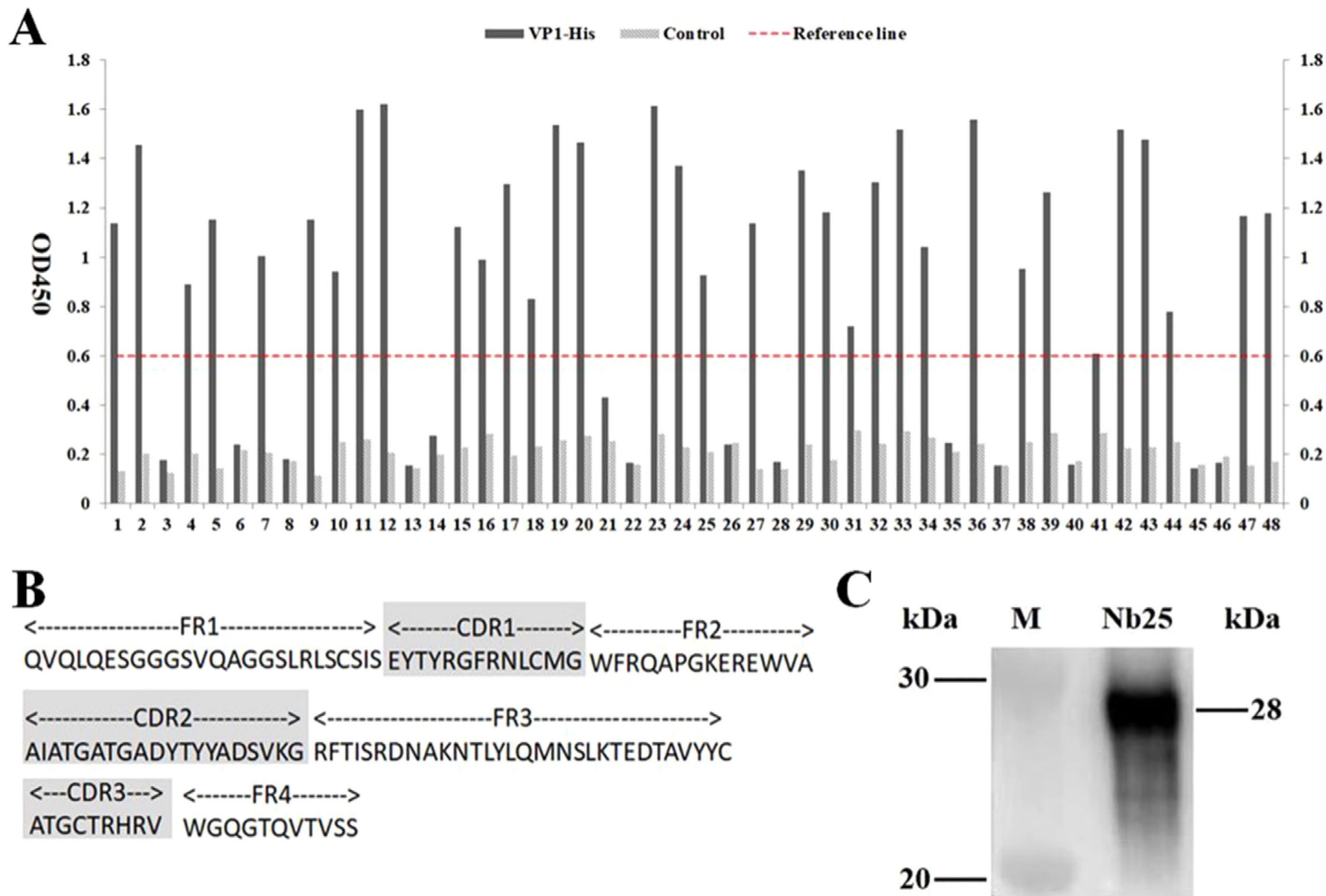

Fig. 2. Identification of the isolated Nbs of DHAV-1 VP1 protein. (A) Periplasmic extract ELISA of obtained VP1-His soluble Nb by osmotic shock. The coated antigen was DHAV-1 VP1-His and the negative controls was SMP. The ratio higher than 3 was considered as positive. The given OD_{450} values were the means ± SDs from triplicate measurements. (B) Amino acid sequence of Nb25. (C) Analysis of the expressed fusion Nb25 by western blot. Mrker, protein molecular weight marker.

^{172}PLPAPTSTTS^{181} in the DHAV-1 VP1 protein.

Subsequently, based on the peptide ^{172}PLPAPTSTTS^{181}, a panel of six shortened peptides were generated by deleting amino acids individually, at either the amino or the carboxy terminus in sequence. According to dot blot hybridisation results, the peptides ^{173}LPAPTSTTS^{181}, ^{174}PAPTSTTS^{181}, ^{172}PLPAPTSTT^{180} and ^{172}PLPAPTST^{179} were recognized by Nb25, whereas ^{175}APTSTTS^{181} and ^{172}PLPAPTS^{178} showed no reactivity with Nb25 (Fig. 4B). These results showed that the liner epitope recognized by Nb25 was ^{174}PAPTST^{179} in the DHAV-1 VP1 protein.

To verify our presumption described above and define the epitope precisely, these truncated peptides ^{174}PAPTST^{179}, ^{174}PAPTS^{178} and ^{175}APTST^{179} were expressed. Dot blot analysis showed that the fused 6-aa peptide ^{174}PAPTST^{179} can be bound specifically to Nb25, whereas the other two truncated peptides showed no reactivity with Nb25 (Fig. 4C). Based on the dot blot analysis results, the accurate position of the epitope recognized by the Nb25 was deduced at ^{174}PAPTST^{179} in the DHAV-1 VP1 protein (Fig. 5).

2.6. Homology analysis of the liner epitope to Nb25 in different serotypes DHAVs

The homology alignment results showed that the ^{174}PAPTST^{179} was a conserved motif in the VP1 protein of DHAV-1 (Fig. S1). Based on the result of homology analysis (Fig. 6A), four mutant peptides were expressed with a GST-Tag which were used to identify the specificity of the liner epitope recognized by Nb25 among three serotypes of DHAV. Dot blot analysis showed that Nb25 reacted with ^{174}PAPTST^{179} in DHAV-1 VP1, while not with ^{174}KDVKNN^{179} in DHAV-2 VP1, ^{174}KDVTNN^{179} in DHAV-2 VP1, ^{174}PASTYT^{179} in DHAV-3 VP1 and ^{174}PAPTYT^{179} in DHAV-3 VP1 (Fig. 6B), which indicated that Nb25 was a specific Nb for DHAV-1.

3. Discussion

DHAV-1 is of great concern for the duck industry due to the potentially high mortality when an outbreak occurs. Mortality in the field often exceeds 50% and even reaches 95% (Li et al., 2017). In recent year, mixed infection of DHAV-1 and DHAV-3 is becoming more and more common (Chen et al., 2013; Gan et al., 2014; Soliman et al., 2015). On the other hand, some DHAV-1 strains exhibit genetic and serological diversity from prototype strains (Wen et al., 2018). Based on molecular epidemiology investigation of DHAV-1 in recent years, we urgently need to establish stable, low cost and highly specific detection methods of DHAV-1. A variety of methods for the molecular detection of DHAV-1 infection have been reported in numerous studies (Chen et al., 2013; Mao et al., 2016; Shen et al., 2015). Antibody-mediated immunoassays is the most specific and precise assay for detecting DHAV-1 (Zhang et al., 2014). However, traditional monoclonal antibodies used in virus detection need more support costs and they are difficult for massive production. In order to eliminating the drawbacks of conventional antibodies, we aimed to develop nanobodies against DHAV-1.

In the current study, a VHH of DHAV-1 named Nb25 was

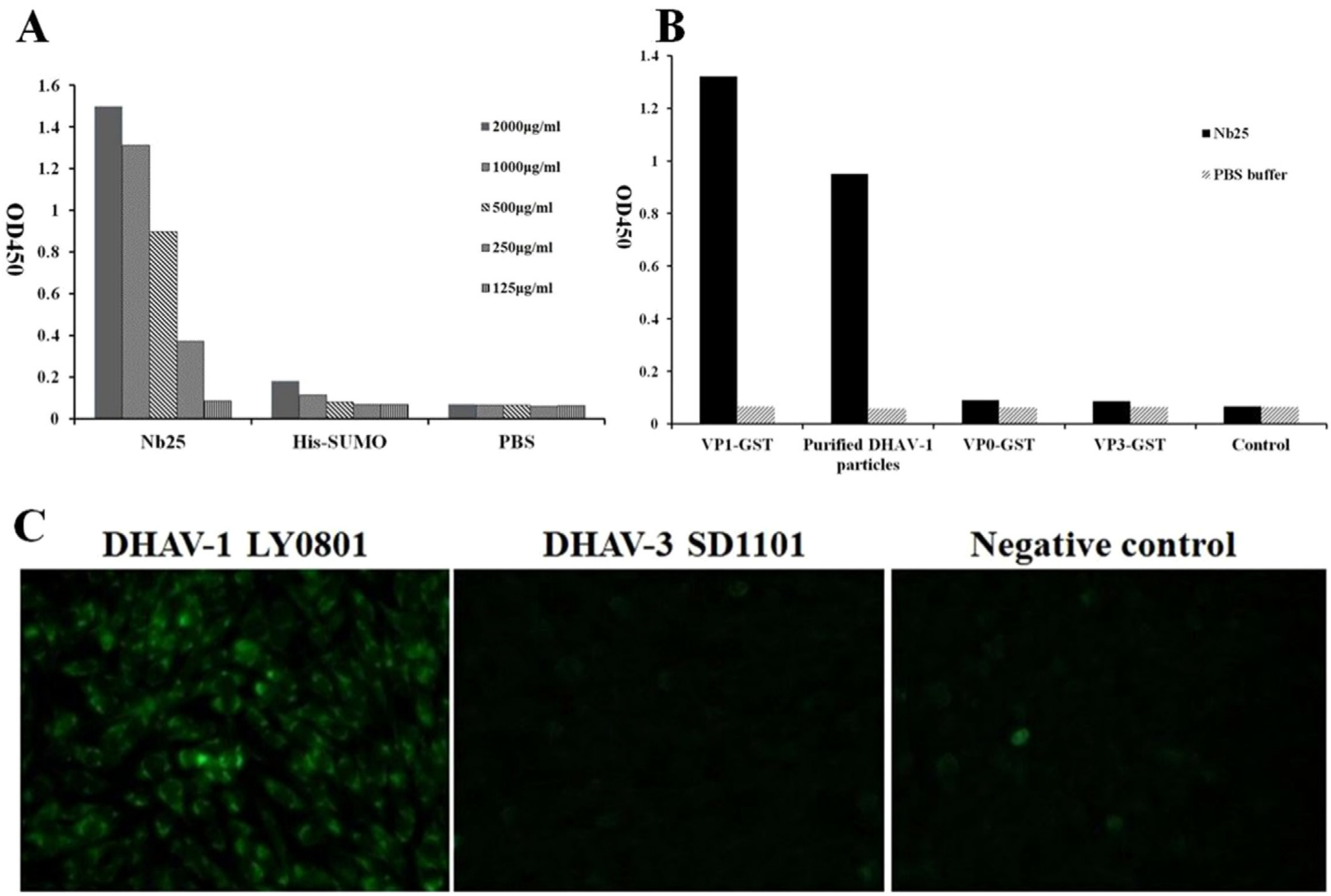

Fig. 3. Reactivity and specificity of the Nb25 against DHAV-1. (A) Reactivity of the Nb25, His-SUMO tag protein and PBS buffer were negative control. The OD_{450} values were the means ± SDs from triplicate measurements. (B) Specificity of the Nb25, SMP was used as control. The OD_{450} values were the means ± SDs from triplicate measurements. (C) DHAV-1 LY0801 strain and DHAV-3 SD1101 strain infected BHK-21 cells detected with Nb25 by IFA, and uninfected BHK-21 cells as negative control.

successfully selected and characterized, and the conserved accurate linear B-cell epitope to Nb25 was identified. To our best knowledge, this is the first report to select specific Nb against DHAV-1 and identify the accurate epitope to the specific Nb.

Owing to their reproducibility, stability, and cost-effective production, the recombinant VHHs are becoming a salient option as immunoassay reagents (Hassanzadeh-Ghassabeh et al., 2013). For instance, a streptavidin-biotin-based directional double Nanobodies sandwich ELISA for H5N1 detection showed superiority over the commonly undirectional ELISA protocol (Zhu et al., 2014). A nanobody-based competitive dot ELISA had high sensitivity and specificity in visual screening of Ochratoxin A in cereals (Sun et al., 2017). Thus, the identified specific nanobody of DHAV-1 in this study could serve as a potential new diagnostic reagent for DHAV-1.

Antibodies are key reagents to investigate molecular target at the cellular level. Conventional antibodies are used to stain antigens in fixed cells, whereas their use in living cells is limited owing to the inefficient folding and assembly applications of their variable heavy and light chains. As a new class of molecular tracers, Nbs had been used as nanoprobe or biosensor to dynamically trace FMDV Aisa 1 and HIV-1 in fixed cells or in living cells (Helma et al., 2012; Yin et al., 2013). To date, it remained impossible to directly and dynamically visualize DHAV-1 in living cells. In this study, neutralization assay results demonstrated that Nb25 had no neutralizing activity on DHAV-1. The results of specific assay and IFA indicated the feasibility of detection intracellular DHAV-1 antigen with Nb25. Hence, Nb25 could be used as nanoprobe to dynamically trace DHAV-1 morphogenesis in living cells.

In the genome of DHAV-1, mutations of nucleotide and amino acid are mainly contained in VP1 protein (Jin et al., 2008), There are three hypervariable regions (HVRs) at the C-terminus (158–160, 180–193 and 205–219) and other variable points in VP1 protein (Gao et al., 2012). The variability of VP1 may lead to the antibodies to VP1 with failure to recognize different DHAV-1 strains. Fortunately, the identified epitope ^{174}PAPTST^{179} by Nb25 was highly conserved in different DHAV-1 strains (Fig. S1). Moreover, we found that Nb25 can only identify DHAV-1 specifically and effectively (Fig. 6). So, Nb25 was a specific antibody for differential diagnosis of the mixed infection of DHAV-1 and DHAV-3 in ducklings. In other study, with a neutralizing monoclonal antibody 2D10, a conserved B-cell epitope ^{173}LPAPTS^{178} in the VP1 protein of DHAV-1 had been identified (Wu et al., 2015). Interestingly, although there was only one amino acid position shift in the two conserved epitopes, ^{173}LPAPTS^{178} was a neutralizing epitope while ^{174}PAPTST^{179} was a non-neutralizing epitope.

In conclusion, DHAV-1 VP1 specific nanobody Nb25 was isolated from a camel VHH library and the conserved linear epitope of DHAV-1 VP1 recognized by Nb25 was identified for the first time. Nb25 was a highly specific, high-yield and stable antibody to DHAV-1, which should be made a lower cost and high sensitive diagnosis kits to DHAV-1. For without neutralizing activity, Nb25 could be used as nanoprobe to dynamically trace DHAV-1 morphogenesis in living cells.

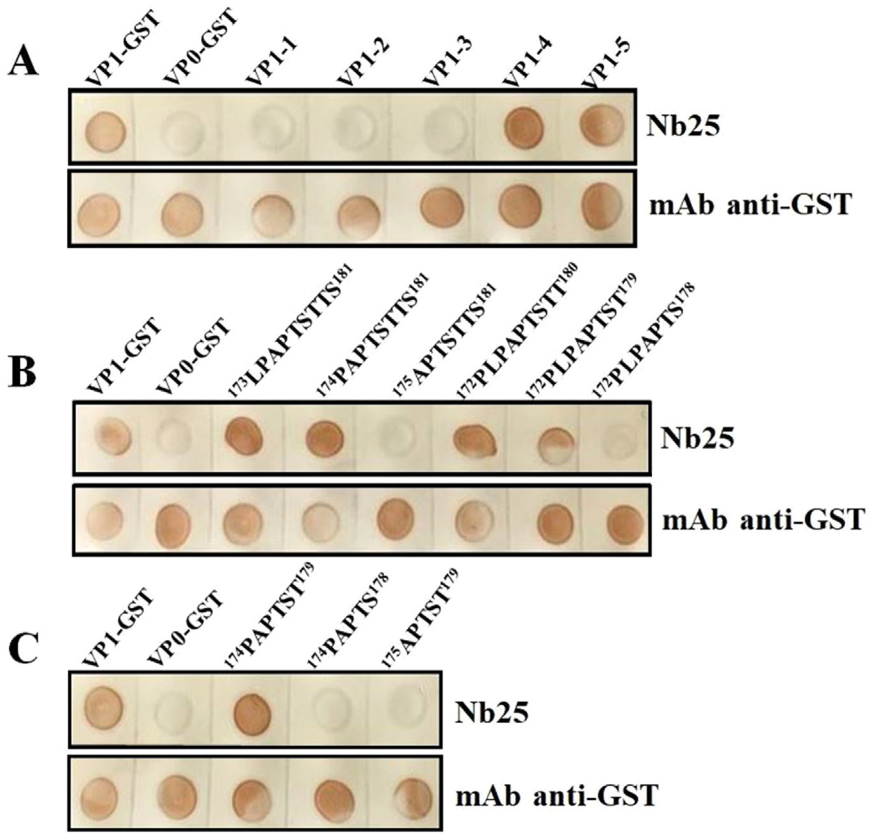

Fig. 4. Analysis of the expressed various DHAV-1 VP1 fusion proteins and truncated versions of the peptide ^{172}PLPAPTSTTS^{181} in *E. coli* Rosetta (DE3) cells by dot blot assay using Nb25 and mAb anti-GST.

4. Materials and methods

4.1. Bactrian camel immunization and library construction

A male Bactrian camel was immunized subcutaneously six times at biweekly intervals with live vaccine of DHAV-1 (NJTB bio-industry, NJ, China) as previously described (Vincke et al., 2012). The animal experiments were carried out in accordance with the guidelines issued by Shandong Agricultural University Animal Care and Use Committee (Approval no.: SDAUA-2016-032). Four days after the last

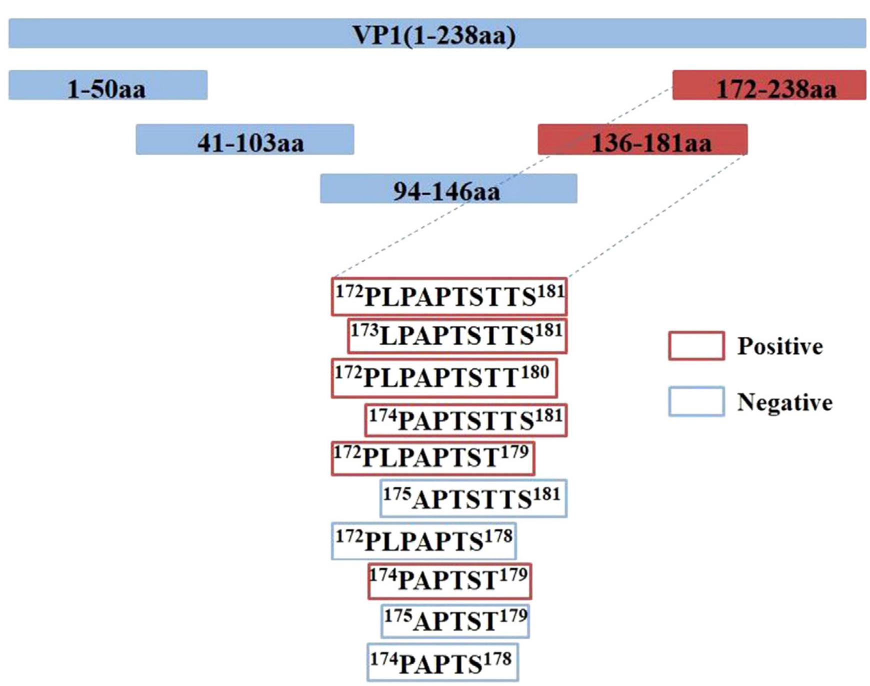

Fig. 5. The accurate position of the epitope recognized by the Nb25 was deduced at ^{174}PAPTST^{179} in the DHAV-1 VP1 protein.

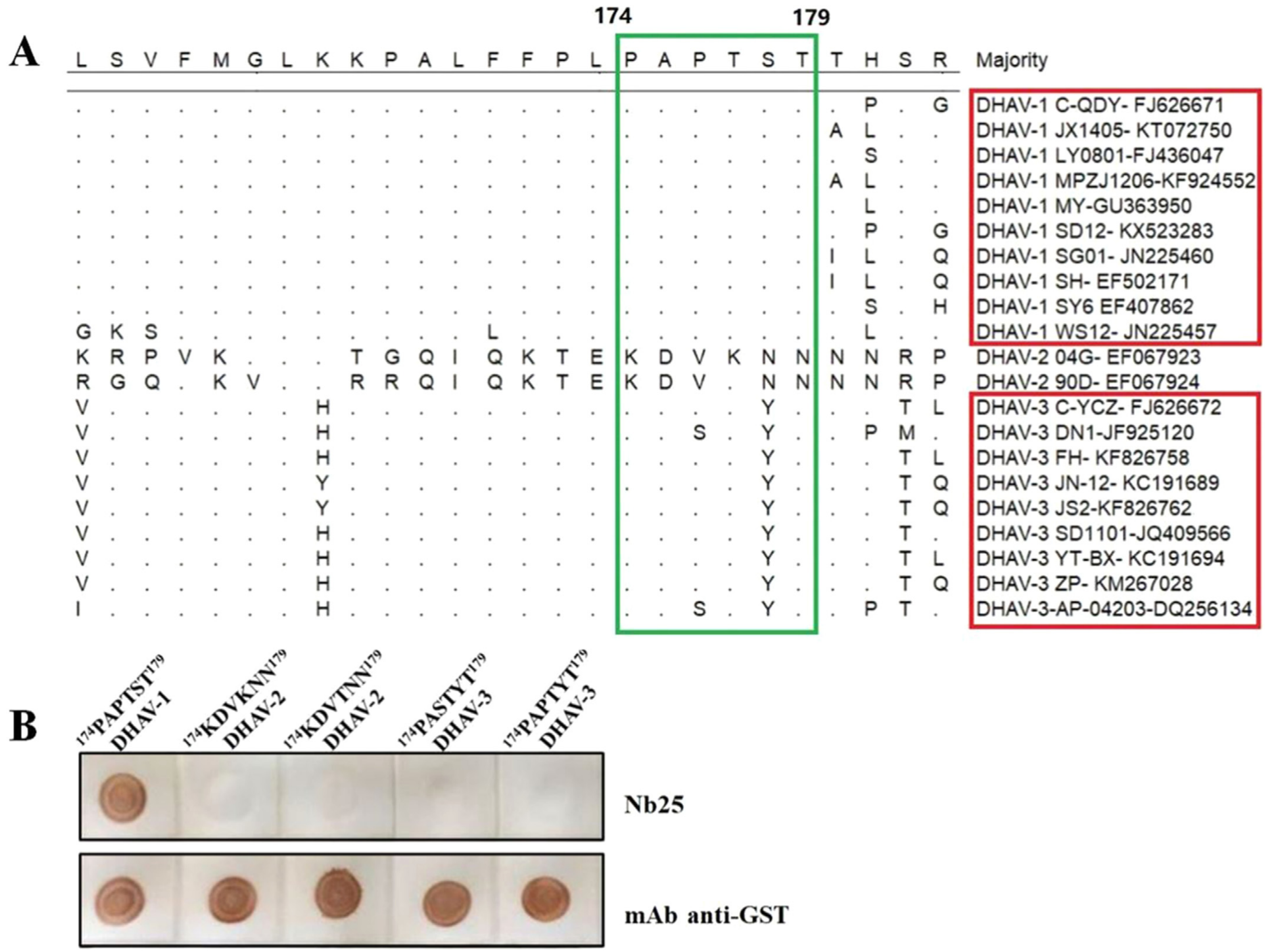

Fig. 6. Homology analysis and verification of the liner epitope to Nb25 in different serotypes DHAVs. (A) Sequence alignment of three serotypes of DHAV strains around the epitope-coding region of the VP1 protein. Major sequences are shown at the top; dots indicate identical amino acids. The identified epitope is in box. (B) Verification of the linear epitope to Nb25 among three serotypes of DHAV by dot blot.

immunization, peripheral blood mononuclear cells (PBMCs) were isolated from a 250 ml blood sample. Total RNA was extracted and VHH genes were cloned by nested PCR as described previously (Vincke et al., 2012). The final PCR products were cloned into the phagemid vector pCANTAB 5E (GEHealthcare Life Science, Pittsburgh, USA) and transformed into freshly prepared electro-competent *E. coli* TG1 cells. Cells were plated on LB agar plates supplemented with ampicillin and glucose. After culturing overnight at 37 °C, colonies were scraped from the plates and stored at − 80 °C in LB supplemented with 20% glycerol.

4.2. Expression and purification of DHAV-1 VP1 recombinant protein

A DHAV-1 DNA-launched infectious clone constructed in our previous work (Chen et al., 2017) was used as templates to amplify VP1 gene. Two restriction sites of *EcoR* I and *Xho* I were designed in primers to help to insert VP1 into pET-32a (Novagen, Darmstadt, Germany) and pGEX-6p-1 (GEHealthcare Life Science, Pittsburgh, USA) prokaryotic expression vector, respectively (Table 2). The protein was expressed in *E. coli* Rosetta (DE3) cells (Transgen Biotech, Beijing, China) after 3 h induction with 1 mM IPTG at 37 °C. The recombinant proteins VP1-His and VP1-GST were purified by eluting from SDS-PAGE as described by Zhang et al. previously (Zhang et al., 2012).

Table 2
The primers used in the present study for producing major structure proteins of DHAV-1.

Primers	Sequence (5′–3′)[a]	Usage
VP1-F	GGGGAATTCGGTGATTCTAACCAGT	pET32a-VP1
VP1-R	CGGCTCGAGTTCAATTTCCAAAT	pGEX-6p-VP1
VP0-F	GGGGAATTCATGGATACTCTTACTAAAAAC	pGEX-6p-VP0
VP0-R	TATCTCGAGCTGATTGTCAAATGGT	
VP3-F	GGGGGATCCGGAAAGAGAAAACCACGC	pGEX-6p-VP3
VP3-R	GGGCTCGAGCTGATTATTGGTTGCCATC	

[a] Restriction sites are underlined.

4.3. Bio-panning

For bio-panning, 100 µl VP1-His protein solution (100 µg/ml, 80 µg/ml and 40 µg/ml in PBS buffer for the first, second and third round, respectively) was added to a well of a microtiter plate. A well without VP1-His protein was performed as negative control. Residual protein binding sites were blocked with 4% skimmed-milk proteins (BD, USA). Phage particles of 10^{10} were added to wells and incubated for 2 h at room temperature. After removing non-adsorbed phages, VP1-His protein bound phage particles were eluted with 100 mM triethylamine (pH 11.0) for 5 min. Next, the solution was transferred to a fresh tube and neutralized with 1 M Tris-HCl (pH 7.4). The eluted phages were used to infect exponentially growing *E. coli* TG1 cells to amplify target phages. Then, the amplified phages were taken to the next round of panning (Lee et al., 2007).

A total of 3 rounds of panning were conducted in the experiment. To assess the phage enrichment of every round, a polyclonal phage ELISA was performed on each phage sample. Briefly, 100 µl (8 µg/ml) of DHAV-1 VP1-His protein was coated in per well of 96-well microplate and incubated overnight at 4 °C. After blocking residual protein binding sites with 4% skimmed-milk proteins (SMP), 10^{10} phage particles from each round of the panning were added to the wells and incubated for 1 h at room temperature. After washing three times with PBS containing 0.01% Tween-20 (PBST), 1/4000 HRP conjugated anti-M13 antibody (GEHealthcare Life Science, Pittsburgh, USA) and TMB substrate (Transgen Biotech, Beijing, China) were added to detect antigen-bound phages in the wells.

4.4. Selection and identification of VP1-specific Nbs

According to the results of polyclonal phages ELISA, the enriched phage particles were employed for randomly picking 48 individual colonies, and the expression of VHHs with an E-Tag was induced using 1 mM IPTG in Terrific Broth supplemented with ampicillin. The recombinant VHHs-E-Tag protein was extracted from the periplasm by osmotic shock (Skerra and Pluckthun, 1988) and tested for its capacity to recognize VP1-His using an ELISA with an anti-E-Tag antibody (Genscript, Piscataway, NJ, USA). Finally, all VHH genes from the positive clones were sequenced.

4.5. Expression and purification of specific Nbs

The VHH genes of the selected clones were amplified using primers as following: (VHH-F: 5′-GGG gga tcc CAG GTC CAA CTG CAG GAG TCT-3′) and (VHH-R: 5′-GCC aag ctt TGA GGA GAC GGT GAC CTG GG-3′). Two restriction enzymes sites *BamH I* and *Hind III* designed in the primers, were used to sub-clone VHH genes into the expression vector pET28a, which was beforehand added N-terminal fusion of His_6 tag and SUMO tag (prepared and stored in our lab). The recombinant plasmid pET28a-VHH was transformed into *E. coli* Rosetta (DE3) cells to express protein by the induction of IPTG. Recombinant proteins of VHHs were purified on Ni-NTA resins (Genscript, Piscataway, NJ, USA). Samples were concentrated and purified using 15 ml 10 kDa centrifugal filter columns (Millipore, MA, USA). SDS-PAGE was conducted to test the purity of the protein. To demonstrate the identity of the protein, a Western blot test with mouse anti-His antibody (GEHealthcare Life Science, Pittsburgh, USA) was conducted.

4.6. The reactivity of isolated Nbs against VP1

An ELISA method was developed to assess the reactivity of Nb. The wells were coated with 100 µl (8 µg/ml) DHAV-1 VP1-GST protein and incubated overnight at 4 °C. After blocking the residual protein binding sites with 4% skimmed-milk proteins (SMP), the Nbs (100 µl), which is diluting in 4% SMP by serial 2-fold, were added into the wells. PBS buffer and His-SUMO tag protein (expression and purification by using the same method as Nbs) as negative control. After washing three times with PBST, the wells were added with 100ul mouse anti-His antibody (primary antibody). Subsequently, goat anti-mouse IgG conjugated with HRP (KPL, MD, USA), which as secondary antibody, was filled in the wells. The reaction was completed with TMB as the substrate and the absorption values were measured at 450 nm.

4.7. The specificity of isolated Nbs against VP1

To determine binding specificity of the Nbs towards other major structural proteins, an ELISA test was performed using VP0-GST and VP3-GST of DHAV-1. VP0-GST and VP3-GST were prior expressed and purified by the method resembling to VP1-GST (primers are listed in Table 2). Those proteins were coated onto microtiter ELISA plates at 8 µg/ml overnight. The next ELISA assay procedure was previously described in the reactivity of isolated Nbs against VP1, expect the working concentration of the selected Nbs was 1000 µg/ml.

4.8. Indirect immunofluorescent assay (IFA)

To verify the specific intracellular binding activity between selected VHHs and DHAV-1 virus, BHK-21 cells were infected with DHAV-1 LY0801 strain (Genbank no. FJ436047, median embryo lethal dose, $ELD_{50} = 10^{-4.7}$, 0.1 ml) and DHAV-3 SD1101 strain (Genbank no. JQ409566, median embryo lethal dose, $ELD_{50} = 10^{-4.9}$, 0.1 ml) when reaching around 70% confluence in a 6-well plate. After incubation for 2 h at 37 °C, the cells were washed by PBS buffer for three times and fresh DMEM was added to each well. At 60 h postinfection (hpi), DHVA-1 and DHAV-3 infected or negative control cells were fixed with ice-chilled acetone-methanol mix (1:1 v/v) for 15 min at room temperature. After washing, the cells were incubated with the identified Nb25 for 2 h at 37 °C. FITC-labeled mouse anti-His antibody (Bioss, Beijing, China) was used as secondary antibody (1:50) and the cells were incubated at 37 °C for 1 h in the dark. The stained cells were analyzed by fluorescence microscopy ((Leica AF6000, Wetzlar, Germany).

4.9. Neutralization capacity of isolated Nbs against VP1

Nb25 was quantified as 2.536 mg/ml. With 2, 4, 8, 16 and 32 dilution in normal saline, the neutralizing activities of Nb25 against DHAV-1 LY0801 strain (FJ436047, median embryo lethal dose, $ELD_{50} = 10^{-4.7}$, 0.1 ml) was determined using quantitative virus (100 ELD_{50}, 0.1 ml).

4.10. Epitope mapping

To identify the linear epitope on VP1 protein, GST-tagged fragments of DHAV-1 VP1 were expressed in *E. coli* Rosetta (DE3) cells. Five overlapping fragments of the DHAV-1 VP1 gene were amplified with specific primers (Table 3) for construction of the recombinant plasmids VP1-1, VP1-2, VP1-3, VP1-4 and VP1-5. The PCR products were subcloned into the pGEX-6p-1 expression vector with the help of *EcoR I* and *Xho I* restriction sites. The recombinant plasmids were transformed into *E. coli* Rosetta (DE3) cells to express recombinant proteins. SDS-PAGE was used to assess the crude *E. coli* lysates, besides, crude *E. coli* lysates were purified for dot blot analysis. Approximately 2 µg of each purified protein diluted with PBS buffer was spotted onto the nitrocellulose membrane (Millipore, MA, USA) at the center of the grid. After blocking the membranes by incubating with 4% SMP, the membrane was incubated at 37 °C for 2 h with Nb25, respectively. After washing, the membranes were incubated with mouse anti-His antibody or mouse anti-GST antibody (GEHealthcare Life Science, Pittsburgh, USA).

Table 3
Primers for identification of the linear epitope in DHAV-1 VP1.

Primers	Sequence (5′–3′)[a]	AA position in VP1
VP1-1F	GGCGAATTCGGTGATTCTAACCAG	1–50
VP1-1R	GCCTCGAGAGTTGAAGTGTGTT	
VP1-2F	GGCGAATTCTTAGTTAGGACTGTAC	41–103
VP1-2R	GCCTCGAGCTCTGAAGTGAGG	
VP1-3F	GGCGAATTCTCCTATTCTATGGATG	94–146
VP1-3R	GCCTCGAGAAAAGTTGCCTC	
VP1-4F	GGCGAATTCCGACCAATTCCTGGC	136–181
VP1-4R	GCCTCGAGTGATGATGTTGTGGAAG	
VP1-5F	GCGAATTCCCACTCCCTGCTCC	172–238
VP1-5F	GCCTCGAGTTCAATTTCCAAATTG	

[a] Restriction sites are underlined.

Table 4
The primers used in the present study for producing epitope peptides.

Primers	Oligonucleotide sequence (5′→3′)	Coding motif
173–181-F	aattc CTCCCTGCTCCCACTTCCACAACATCAtaac	^{173}LPAPTSTTS^{181}
173–181-R	tcgagtta TGATGTTGTGGAAGTGGGAGCAGGGAGg	
174–181-F	aattcCCTGCTCCCACTTCCACAACATCAtaac	^{174}PAPTSTTS^{181}
174–181-R	tcgagttaTGATGTTGTGGAAGTGGGAGCAGGg	
175–181-F	aattcGCTCCCACTTCCACAACATCAtaac	^{175}APTSTTS^{181}
175–181-R	tcgagttaTGATGTTGTGGAAGTGGGAGCg	
172–180-F	aattc CCACTCCCTGCTCCCACTTCCACAACAtaac	^{172}PLPAPTSTT^{180}
172–180-R	tcgagtta TGTTGTGGAAGTGGGAGCAGGGAGTGGg	
172–179-F	aattcCCACTCCCTGCTCCCACTTCCACAtaac	^{172}PLPAPTST^{179}
172–179-R	tcgagttaTGTGGAAGTGGGAGCAGGGAGTGGg	
172–178-F	aattcCCACTCCCTGCTCCCACTTCCtaac	^{172}PLPAPTS^{178}
172–178-R	tcgagttaGGAAGTGGGAGCAGGGAGTGGg	
174–179-F	aattcCCTGCTCCCACTTCCACAtaac	^{174}PAPTST^{179}
174–179-R	tcgagttaTGTGGAAGTGGGAGCAGGg	
174–178-F	aattcCCTGCTCCCACTTCCtaac	^{174}PAPTS^{178}
174–178-R	tcgagttaGGAAGTGGGAGCAGGg	
175–179-F	aattcGCTCCCACTTCCACAtaac	^{175}APTST^{179}
175–179-R	tcgagttaTGTGGAAGTGGGAGCg	
DHAV-2-F1	aattcAAGGACGTAAAGAACAACtaac	^{174}KDVKNN^{179}
DHAV-2-R1	tcgagttaGTTGTTCTTTACGTCCTTg	
DHAV-2-F2	aattcAAGGACGTAACTAACAACtaac	^{174}KDVTNN^{179}
DHAV-2-R2	tcgagttaGTTGTTAGTTACGTCCTTg	
DHAV-3-F1	aattcCCTGCACCAACTTACACAtaac	^{174}PAPTYT^{179}
DHAV-3-R1	tcgagttaTGTGTAAGTTGGTGCAGGg	
DHAV-3-F2	aattcCCTGCATCGACTTACACAtaac	^{174}PASTYT^{179}
DHAV-3-R2	tcgagttaTGTGTAAGTCGATGCAGGg	

Note: Introduced bases (to form termination codon and overhanging ends of EcoR I and Xho I after annealing of the two complementary oligonucleotides) are shown in lowercase letters.

Afterwards, the membranes incubated with goat anti-mouse IgG conjugated with HRP at 37 °C for 1 h. Then, the substrate of DAB (TianGen, Beijing, China) was used to visualize the reaction.

Based on the result of dot blot test, a series of complementary oligonucleotides coding for the truncated versions of the peptide ^{172}PLPAPTSTTS^{181} were synthesized, annealed, and cloned into the *EcoR I* and *Xho I* restriction sites of the prokaryotic expression vector pGEX-6p-1 for further determination of epitope position (Table 4). The recombinant proteins were detected by dot blot assay as described previously.

4.11. Homology analysis

To analyze the conservation of the identified epitope among different DHAV strains, the epitope sequence and flanking sequences of the VP1 protein were compared with those of 82 DHAV-1 strains, nine DHAV-3 strains, and two DHAV-2 strain by using the DNASTAR Lasergene program (DNASTAR Inc. Madison, WI, USA).

Based on the results of homology analysis, a series of complementary oligonucleotides encoding for the corresponding epitopes of DHAV-2 and DHAV-3 were synthesized, annealed, and cloned into the *EcoR I* and *Xho I* restriction sites of the prokaryotic expression vector pGEX-6p-1 for further determinate the specific of the epitope recogenized by Nb25 of DHAV-1 (Table 4). The recombinant proteins were detected by dot blot assay as described previously.

Acknowledgements

This work was funded by grants from the National Key Research and Development Program of China (2016YFD0500800, 2017YFD0500800), National Natural Science Foundation of China (31772754), Funds of Shandong "Double Tops" Program, China (SYL2017YSTD11), Shandong Modern Agricultural Technology & Industry System, China (SDAIT-11-15) and Agricultural Science and Technology Innovation Project of Shandong Academy of Agricultural Sciences, China (CXGC2016B14).

Competing interests statement

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.12.013.

References

Chakravarty, R., Goel, S., Cai, W., 2014. Nanobody: the "magic bullet" for molecular imaging? Theranostics 4, 386–398.
Chen, J., Zhang, R., Lin, S., Li, P., Lan, J., Xie, Z., Wang, Y., Jiang, S., 2017. Construction and characterization of an improved DNA-launched infectious clone of duck hepatitis a virus type 1. Virol. J. 14, 212.
Chen, L.L., Xu, Q., Zhang, R.H., Yang, L., Li, J.X., Xie, Z.J., Zhu, Y.L., Jiang, S.J., Si, X.K., 2013. Improved duplex RT-PCR assay for differential diagnosis of mixed infection of duck hepatitis A virus type 1 and type 3 in ducklings. J. Virol. Methods 192, 12–17.
Ding, C., Zhang, D., 2007. Molecular analysis of duck hepatitis virus type 1. Virology 361, 9–17.
Erfan, A.M., Selim, A.A., Moursi, M.K., Nasef, S.A., Abdelwhab, E.M., 2015. Epidemiology and molecular characterisation of duck hepatitis A virus from different duck breeds in Egypt. Vet. Microbiol. 177, 347–352.
Fu, Y., Pan, M., Wang, X., Xu, Y., Yang, H., Zhang, D., 2008. Molecular detection and typing of duck hepatitis A virus directly from clinical specimens. Vet. Microbiol. 131, 247–257.
Gan, Y.J., Huang, C.H., Wang, X.Z., Chen, S.H., Li, J.X., 2014. Epidemiological investigation of duck hepatitis A virus (DHAV) isolated from Sichuan Basin by RT-PCR disclose the existence of mixed infection and the feasibility of DHAV – a evolved from C80 strain. Pak. Vet. J. 34, 356–360.
Gao, J., Chen, J., Si, X., Xie, Z., Zhu, Y., Zhang, X., Wang, S., Jiang, S., 2012. Genetic variation of the VP1 gene of the virulent duck hepatitis A virus type 1 (DHAV-1) isolates in Shandong province of China. Virol. Sin. 27, 248–253.
Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R., 1993. Naturally occurring antibodies devoid of light chains. Nature 363, 446–448.
Hassanzadeh-Ghassabeh, G., Devoogdt, N., De Pauw, P., Vincke, C., Muyldermans, S., 2013. Nanobodies and their potential applications. Nanomedicine 8, 1013–1026.
Helma, J., Schmidthals, K., Lux, V., Nuske, S., Scholz, A.M., Krausslich, H.G., Rothbauer, U., Leonhardt, H., 2012. Direct and dynamic detection of HIV-1 in living cells. PLoS One 7, e50026.
Jin, X., Zhang, W., Zhang, W., Gu, C., Cheng, G., Hu, X., 2008. Identification and molecular analysis of the highly pathogenic duck hepatitis virus type 1 in Hubei province of China. Res. Vet. Sci. 85, 595–598.
Kim, M.C., Kwon, Y.K., Joh, S.J., Kim, S.J., Tolf, C., Kim, J.H., Sung, H.W., Lindberg, A.M., Kwon, J.H., 2007. Recent Korean isolates of duck hepatitis virus reveal the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains. Arch. Virol. 152, 2059–2072.
Lee, C.M., Iorno, N., Sierro, F., Christ, D., 2007. Selection of human antibody fragments by phage display. Nat. Protoc. 2, 3001–3008.
Li, C., Chen, Z., Meng, C., Li, L., Liu, G., 2013. High yield expression of duck hepatitis A virus VP1 protein in Escherichia coli, and production and characterization of polyclonal antibody. J. Virol. Methods 191, 69–75.
Li, X., Zhao, R., Lin, W., Li, C., Zhang, T., Meng, F., Liu, M., Zhang, Y., 2017. Evidence of VP1 of duck hepatitis A type 1 virus as a target of neutralizing antibodies and involving receptor-binding activity. Virus Res. 227, 240–244.
Liu, X., Tang, Z., Duan, Z., He, Z., Shu, M., Wang, X., Gee, S.J., Hammock, B.D., Xu, Y., 2017. Nanobody-based enzyme immunoassay for ochratoxin A in cereal with high resistance to matrix interference. Talanta 164, 154–158.
Mao, S., Ou, X., Zhu, D., Chen, S., Ma, G., Wang, M., Jia, R., Liu, M., Sun, K., Yang, Q., Wu, Y., Chen, X., Cheng, A., 2016. Development and evaluation of indirect ELISAs for the detection of IgG, IgM and IgA1 against duck hepatitis A virus 1. J. Virol. Methods 237, 79–85.
Muyldermans, S., 2001. Single domain camel antibodies: current status. J. Biotechnol. 74, 277–302.
Muyldermans, S., Baral, T.N., Retamozzo, V.C., De Baetselier, P., De Genst, E., Kinne, J., Leonhardt, H., Magez, S., Nguyen, V.K., Revets, H., Rothbauer, U., Stijlemans, B., Tillib, S., Wernery, U., Wyns, L., Hassanzadeh-Ghassabeh, G., Saerens, D., 2009. Camelid immunoglobulins and nanobody technology. Vet. Immunol. Immunopathol. 128, 178–183.
Shen, Y., Cheng, A., Wang, M., Chen, S., Jia, R., Zhu, D., Liu, M., Sun, K., Yang, Q., Chen, X., 2015. Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies. J. Virol. Methods 225, 30–34.
Skerra, A., Pluckthun, A., 1988. Assembly of a functional immunoglobulin Fv fragment in

Escherichia coli. Science 240, 1038–1041.
Soliman, M., Alfajaro, M.M., Lee, M.H., Jeong, Y.J., Kim, D.S., Son, K.Y., Kwon, J., Choi, J.S., Lim, J.S., Choi, J.S., Lee, T.U., Cho, K.O., Kang, M.I., 2015. The prevalence of duck hepatitis A virus types 1 and 3 on Korean duck farms. Arch. Virol. 160, 493–498.
Sun, Z., Duan, Z., Liu, X., Deng, X., Tang, Z., 2017. Development of a nanobody-based competitive dot ELISA for visual screening of ochratoxin A in cereals. Food Anal. Methods 10, 3558–3564.
Tseng, C.H., Knowles, N.J., Tsai, H.J., 2007. Molecular analysis of duck hepatitis virus type 1 indicates that it should be assigned to a new genus. Virus Res. 123, 190–203.
Tseng, C.H., Tsai, H.J., 2007. Molecular characterization of a new serotype of duck hepatitis virus. Virus Res. 126, 19–31.
Vincke, C., Gutierrez, C., Wernery, U., Devoogdt, N., Hassanzadeh-Ghassabeh, G., Muyldermans, S., 2012. Generation of single domain antibody fragments derived from camelids and generation of manifold constructs. Methods Mol. Biol. 907, 145–176.
Wang, A., Gu, L., Wu, S., Zhu, S., 2018. Duck hepatitis A virus structural proteins expressed in insect cells self-assemble into virus-like particles with strong immunogenicity in ducklings. Vet. Microbiol. 215, 23–28.
Wen, X., Zhu, D., Cheng, A., Wang, M., Chen, S., Jia, R., Liu, M., Sun, K., Zhao, X., Yang, Q., Wu, Y., Chen, X., 2018. Molecular epidemiology of duck hepatitis a virus types 1 and 3 in China, 2010–2015. Transbound. Emerg. Dis. 65, 10–15.
Wu, X., Li, X., Zhang, Q., Wulin, S., Bai, X., Zhang, T., Wang, Y., Liu, M., Zhang, Y., 2015. PLoS One 10, e0118041.
Xu, Q., Zhang, R., Chen, L., Yang, L., Li, J., Dou, P., Wang, H., Xie, Z., Wang, Y., Jiang, S., 2012. Complete genome sequence of a duck hepatitis a virus type 3 identified in eastern China. J. Virol. 86, 13848.
Yin, S., Yang, S., Shang, Y., Sun, S., Zhou, G., Jin, Y., Tian, H., Wu, J., Liu, X., 2013. Characterization of Asia 1 sdAb from camels bactrianus (C. bactrianus) and conjugation with quantum dots for imaging FMDV in BHK-21 cells. PLoS One 8, e63500.
Zhang, R., Zhou, G., Xin, Y., Chen, J., Lin, S., Tian, Y., Xie, Z., Jiang, S., 2015. Identification of a conserved neutralizing linear B-cell epitope in the VP1 proteins of duck hepatitis A virus type 1 and 3. Vet. Microbiol. 180, 196–204.
Zhang, T., Li, X., Wu, X., Shaozhou, W., Bai, X., Liu, S., Liu, M., Zhang, Y., 2014. Characterization of monoclonal antibodies against duck hepatitis type 1 virus VP1 protein. J. Virol. Methods 208, 166–170.
Zhang, Z., Chen, J., Shi, H., Chen, X., Shi, D., Feng, L., Yang, B., 2012. Identification of a conserved linear B-cell epitope in the M protein of porcine epidemic diarrhea virus. Virol. J. 9, 225.
Zhu, M., Gong, X., Hu, Y., Ou, W., Wan, Y., 2014. Streptavidin-biotin-based directional double nanobody sandwich ELISA for clinical rapid and sensitive detection of influenza H5N1. J. Transl. Med. 12, 352.