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Development and evaluation of an indirect ELISA based on recombinant nonstructural protein 3A to detect antibodies to duck hepatitis A virus type 1

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ABSTRACT

To develop an indirect enzyme-linked immunosorbent assay(I-ELISA) method based on 3A protein of duck hepatitis A virus type 1(DHAV-1) for detection of DHAV-1 antibody, the recombinant protein 3A of DHAV-1 was expressed in *E.coli* and detected by Western blotting with DHAV-1 infected duck serum. A 3A-ELISA method using the expressed 3A protein as coating antigen for the detection of antibodies to DHAV-1 was developed. The optimal antigen, serum and enzyme-labeled antibody dilutions were 1:200(6.185 μg/ml), 1:20 and 1:2000, respectively. The optimal blocking buffer was 5% BSA. The cutoff value was determined to be 0.274, and the analytical sensitivity was 1:1280. There was no cross reaction between DHAV-1 infected duck serum and other common pathogenic duck serum, indicating that I-ELISA could be used to detect DHAV-1 infected duck serum. The coefficients of variation(CVs) were lower than 10%. The concordance between the I-ELISA based on the 3A subunit of DHAV-1 and that based on the whole DHAV-1 particle was 92.7%. Taken together, the 3A-ELISA method is a highly sensitive and specific test that could be used for screening for DHAV-1 infection and monitoring DHAV-1 antibody.

1. Introduction

Duck viral hepatitis(DVH) is a highly fatal, rapidly infectious disease in young ducklings, characterized by swelling livers mottled with haemorrhages, mainly caused by duck hepatitis A virus(genus Avihepatovirus, family Picornavirus, DHAV) infection (Ou et al., 2017b; Xie et al., 2018a, b). The timely and accurate detection is vital for disease control. Neutralization test is the most classical DHAV detection method but time-consuming and laborious (Li et al., 2017). Other assays, such as immune colloidal gold technique, RT-PCR and real-time fluorescence quantitative RT-PCR, are highly sensitive and specific but expensive and require special instruments (Hu et al., 2016; Wen et al., 2014). Clinically, a common method, such as indirect enzyme-linked immunosorbent assay(I-ELISA), is to identify viral infections by serotyping the proteins and antibodies produced during viral infection (Mao et al., 2016; Qi et al., 2015; Quanyun and Li, 1997; Shen et al., 2015; Yang et al., 2014). It is accurate, efficient, simple and easy to operate. To date, I-ELISA methods based on picornavirus non-structural proteins(NSPs) serve as potential markers of infection and present a reliable differential diagnostic method of distinguishing infected from

Abbreviations: DHAV-1, Duck hepatitis A virus type 1; DHAV-2, Duck hepatitis A virus type 2; DHAV-3, Duck hepatitis A virus type 3; DVH, Duck viral hepatitis; I-ELISA, Indirect enzyme-linked immunosorbent assay; Ni-NTA kit, Ni-NTA Sefinose TM Resin Kit; CVs, Coefficients of variation; NSPs, Non-structural proteins; SPs, Structural proteins; S.E, Salmonella enterica; R.A, Riemerella anatipestifer; *E. coli*, *Escherichia coli*; DSHSV, Duck swollen head septicemia virus; AIV, Avian influenza virus; DPV, Duck plague virus; SD, Standard deviation; RT-PCR, Reverse transcription-polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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inactivated vaccinated animals (Biswal et al., 2016; Fu et al., 2014, 2017; Hosamani et al., 2015; Mahajan et al., 2015; Yakovleva et al., 2006).

DHAV can be divided into three genotypes according to the evolutionary tree: Duck hepatitis A virus type 1(DHAV-1), Duck hepatitis A virus type 2(DHAV-2) and Duck hepatitis A virus type 3(DHAV-3), DHAV-1 and DHAV-3 are prevalent in mainland China, and DHAV-1 is the most widely distributed and hardest (Mao et al., 2017; Ou et al., 2017a; Wen et al., 2017). The genome of the DHAV-1 is a single strand positive-stranded RNA and contains an open frame that synthesizes an inactive precursor protein. After multistage cleavage, it finally forms structural proteins(SPs) P1 region(capsid proteins VP0, VP1 and VP3) (Wen et al., 2015) and non-structural proteins P2(2A, 2B and 2C proteins) (Cao et al., 2016; Yang et al., 2017) and P3 regions(3A, 3B, 3C and 3D proteins) (Cao et al., 2013; Sun et al., 2016, 2017; Zhang et al., 2017). Among them, 3A protein is the cleavage product of precursor protein 3AB, and 3A and 3B often exist in the form of two dipolymers (Gao et al., 2015). The C-terminal of 3A protein has a strong hydrophobic structure formed by six alpha-helical structures, and some hydrophobic regions formed by irregular curling (Gonzálezmagaldi et al., 2014; Jackson, 2014). These highly conserved hydrophobic structures can anchor viral RNA replication complexes to membrane and play an important role in viral replication and proliferation (Biswal et al., 2016).

In this study, we attempted to clone, express and purify the recombinant protein 3A in *Escherichia Coli*(*E. coli*). Coated with the purified recombinant 3A, an I-ELISA was established to provide a rapid and efficient detection method for DHAV antibody and pathogen epidemic trend, and provide a basis for differentiating DHAV infected ducks from inactivated vaccine immunized ducks.

2. Materials and methods

2.1. Viral strain and serum samples

DHAV-1 strain H was kept in our lab and its complete genome is available in GenBank(JQ301467.1). Positive serum samples were collected from breeding Peking ducks(Anas platyrhynchos domesticus) that were artificially infected with DHAV-1, and negative serum samples were obtained from healthy ducks which had never been infected with any pathogens. All of serum samples were collected from the jugular vein and preserved in a sterile manner at -80℃ in our lab until use. Anti-sera against several other common duck-sensitive antigens including DHAV-3, salmonella enterica(S.E), riemerella anatipestifer (R.A), *E. coli*, duck swollen head septicemia virus(DSHSV), avian influenza virus(AIV) and duck plague virus(DPV) were stored in our lab and were used to confirm the specificity of the new indirect ELISAs.

2.2. Cloning and expression of the recombinant protein

Primers specific for the 3A-encoding gene were designed using Primer premier 5.0 based on conserved nucleotide sequences from previously reported DHAV-1. The forward primer was 3AF: 5′−CCGG AATTCTCTAAGGTGAGGCGTTTCTCT-3′(the EcoR I site is underlined) and the reverse primer was 3AR: 5′−CCCAAGCTTCCGATTCCGCTCC AGAAAACC-3′(the Hind III site is underlined). Viral RNA was extracted using Trizol reagent(Takara) according the manufacturers instructions and was reversed-transcribed into cDNA(Takara). The 3A-encoding gene was amplified from the total genomic cDNA by PCR using primers 3AF and 3AR. The amplified product was separated on a 2% agarose gel and a 207-bp fragment was excised and purified with a gel extraction kit(OMEGA). The 207-bp PCR product was cloned into the EcoR I and Hind III sites of pET-32a(+). The correct orientation of the insert was confirmed by nucleotide sequencing. The plasmid was the transformed into *E. coli* BL21(DE3). Positive clones were selected for large-scale production and purification. The total amount of protein was quantified by using the BCA protein assay(Thermo Fisher Scientific). The 3A recombinant protein was purified by the Ni-NTA kit, detected by SDS-PAGE, and then analysed by Western blotting with DHAV-1 infected duck serum.

2.3. Standardization of 3A-DHAV-1-ELISA

A checkerboard titration in 96-well ELISA microplates was implemented to optimize the coating concentrations of antigen and dilutions of serum according to an essentially classical indirect ELISA protocol (Mao et al., 2016). The purified 3A protein was serially diluted by two-fold from 1:50 to 1:6400(24.74 μg/ml to 0.1933 μg/ml). The wells were blocked with blocking buffers(5% FBS, 10% FBS, 1% gelatin, 5% gelatin, 1% skim milk, 5% skim milk, 1% BSA and 5% BSA) at 37℃ for 30 min, 60 min, 90 min and 120 min. The positive and negative serum samples were diluted from 1:5-1:60. IgG detection was performed by using a conjugate of HRP-labeled goat anti-duck IgG that was optimized with two-fold serial dilutions(1:500 to 1:4000). All of the samples were tested in triplicate and measured with a microplate spectrophotometer (Model 680, Bio-Rad) at double wavelengths of 450 nm and 630 nm (OD_{450}-OD_{630}). A reaction with a corresponding positive value(P) of approximately 1.0, a negative value(N) of below 0.4, and the maximum difference(P/N) in optical densities that was no less than 2.1, was considered optimal (Crowther, 2009; Wu et al., 2011).

2.4. Validation of the assay parameters

The cutoff value was taken as the mean OD values($\bar{x}$) plus 3 fold standard deviation(SD) obtained from 48 DHAV-1 negative serum samples. A sample with an OD value above the cutoff value was considered positive (Jia et al., 2009; Upadhyay et al., 2009).

Analytical sensitivity that indicates the lower detection limit was estimated by end-point titration and defined as the maximum dilution of the sample detected just above the critical value. 8 DHAV-1 positive sera were diluted from 1:40 to 1:5120 with three repetitions for each dilution (Yang et al., 2014). DHAV-1 negative samples served as controls.

The antigen specificity was evaluated by an antigenic cross-reactivity test and blocking test (Liu et al., 2014). Confirmed antisera to DHAV-1, DHAV-3, S.E, R.A, *E. coli*, DSHSV, AIV and DPV were used to evaluate antigenic cross-reactivity test. Each antiserum was tested three times and DHAV-1 negative samples served as controls. A blocking test was conducted to further evaluate the specificity. Antisera to DHAV-1 mingled with DHAV-1 and DPV antigen separately at 1:10 ratio(v/v). The mixtures were incubated at 37℃ for 1 h, and diluted to the optimal concentration used as primary antibodies. Each sample was tested three times and DHAV-1 positive serum without antigen addition served as the positive control.

The repeatability and reproducibility of the method were determined as follows. Antigens from the same and different batches were used to test DHAV-1 positive(n = 3) and negative(n = 3) samples with four repetitions. The intra-assay and inter-assay CVs were calculated according to the formula: CVs = SD/$\bar{x}$.

2.5. Comparison of 3A-DHAV-1-ELISA and DHAV-1-ELISA for DHAV-1 antibody detection

DHAV-1-ELISA method was conducted as previously described (Quanyun and Li, 1997; Zhao et al., 1991) with some modifications. Serum samples in clinical specimens were tested by 3A-DHAV-1-ELISA and DHAV-1-ELISA separately to evaluate the similarities between the two assays. The similarity rate was calculated as follows: the sum of true positive and true negative samples divided by the total number of samples.

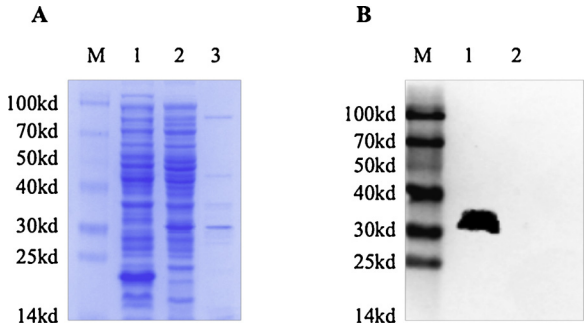

Fig. 1. Identification of the 3A protein from the pET-32a-3A plasmid by SDS-PAGE(A). M, molecular weight marker; lane 1, represented *E. coli* BL21(pET-32a(+)); lane 2, represented *E. coli* BL21 pET-32a-3A; lane 3, purification of 3A. Detection of the recombinant 3A protein by Western blotting(B). M, molecular weight marker; lane 1, *E. coli* expressing pET-32a-3A; lane 2, *E. coli* expressing the pET-32a(+) vector.

3. Results

3.1. Cloning, expression and identification of 3A-encoding gene

Recombinant plasmids of the correct size were identified by PCR and sequencing. The pET-32a-3A expression plasmid was over-expressed successfully in *E. coli*. Protein bands were visualised subsequently by coomassie brilliant blue staining. Analysis of extracts of pET-32a-3A-transformed *E. coli* by SDS-PAGE revealed the 3A recombinant protein with an approximate molecular mass of 31 kDa(including pET-32a vector(12×His tag) with a little more than 21.5 kDa) (Fig. 1A), which was consistent with the expected size of the 3A fusion protein. The recombinant protein was then analysed by Western blotting with DHAV-1 infected duck serum, which showed that the sera reacted specifically against the 3A fusion protein that had an approximate molecular mass of 31 kDa that was consistent with the predicting recombinant 3A protein molecular weight (Fig. 1B). This result indicated that 3A was a protein with good antigenicity and reactivity. No specific protein was detected in lysates derived from pET-32a(+)-transformed *E. coli* cells.

3.2. Standardization of the 3A-DHAV-1-ELISA procedure

The optimal concentrations of 3A protein as the coating antigen was 6.185 μg/ml, and the optimal dilution for serum and goat anti-duck IgG conjugate were 1:20 (Table 1) and 1:2000 (Table 2), respectively. The optimal coating condition was 4°C overnight, and the optimal blocking condition was 5% BSA 90 min(data not shown).

3.3. Selection of the optimal blocking buffer

In a comparison of the combinations of sample diluent and blocking agents, 5% BSA was the most efficient blocking agent for 3A detection with the lowest background(data not shown).

Table 1
Determination of optimal antigen coating concentration and serum dilutions.

Serum dilutions	Antigen dilutions							
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
1:5	2.945	3.099	3.506	3.171	2.615	2.508	2.347	2.581
1:10	3.257	3.430	3.382	3.265	2.780	2.095	2.475	3.086
1:20	3.263	3.484	**3.540**	3.085	2.371	2.016	2.199	2.061
1:40	2.873	3.171	3.010	3.036	2.213	1.913	2.468	1.872
1:80	3.184	2.703	3.394	2.853	2.215	2.067	2.222	2.011
1:160	2.895	2.640	3.271	1.692	1.642	1.777	1.504	1.655

Bold: P/N values corresponding to the optimal dilutions.

Table 2
Optimization of goat anti-duck IgG conjugate dilutions.

Dilutions	P	N	P/N
1:500	1.419	0.638	2.223
1:1000	1.296	0.470	2.757
1:2000	1.010	0.291	**3.474**
1:4000	0.686	0.198	3.466

P: average OD values of positive serum samples; N: average OD values of negative serum samples; P/N: average OD values of positive serum samples/ average OD values of negative serum samples; bold: P/N values corresponding to the optimal dilutions.

3.4. Validation of the method parameters

A batch of DHAV-1 antibody-negative serum samples was tested under the determined optimal conditions. The OD values ranged from 0.08 to 0.254, with a mean OD value of 0.157, and a SD of 0.0389. The cutoff value was 0.274 (mean ± 3SD).

Sensitivity, the end point of titration, was the maximum dilution of positive serum. The results showed that the analytical sensitivity of 3A-DHAV-1-ELISA method was 1:1280 (Fig. 2).

The results of the specificity evaluation showed that the OD values of positive reactions of DHAV-1- and DHAV-3-positive serum were above 0.274, while the OD values of negative reactions were below the cutoff value (Fig. 3). It showed that there was no cross reaction between DHAV-1 and other common pathogenic duck serum, and 3A-DHAV-1-ELISA method could be used to detect the positive serum of DHAV-1 and DHAV-3.

The repeatability of the assay was evaluated by determining the average intra-assay and inter-assay CVs. Serum samples with diverse OD values were tested. The intra-assay CVs ranged from 1.57% to 5.29%, with a mean of 3.79%, while the inter-assay CVs ranged from 1.25% to 7.73%, with a mean of 5.09% (Fig. 4). The CVs of all samples was no more than 10%, indicating that the method had good stability and reproducibility.

3.5. Comparison of efficacies of 3A-DHAV-1-ELISA and DHAV-1-ELISA for detecting DHAV-1 antibody

Diverse serum samples were tested by 3A-DHAV-1-ELISA and DHAV-1-ELISA for comparison (Table 3). The results showed that the positive rates for the two methods were 52% and 56%, respectively. Six samples that tested negative using DHAV-1-ELISA were found to be positive by 3A-DHAV-1-ELISA, while one sample that tested positive

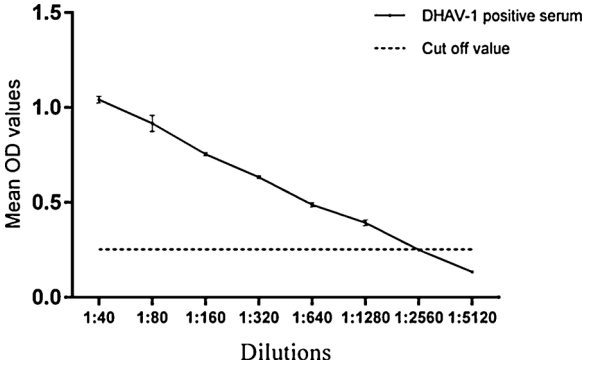

Fig. 2. The analytical sensitivity of the 3A-DHAV-1-ELISA method. DHAV-1 anti-serum was serially diluted by two-fold(from 1:40 to 1:5120) and was detected using the 3A-DHAV-1-ELISA method. The detected maximum dilution was 1:1280 according to the cutoff value of 0.274.

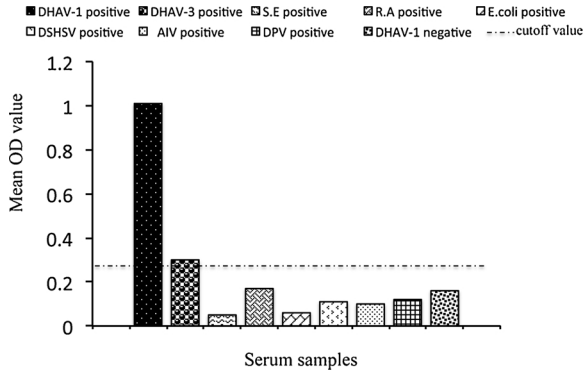

Fig. 3. Antigenic cross-reactivity analysis of the 3A-DHAV-1-ELISA method. Antiserum to all of common duck pathogens had the OD values of below the cutoff value except the DHAV-1 and DHAV-3 antisera.

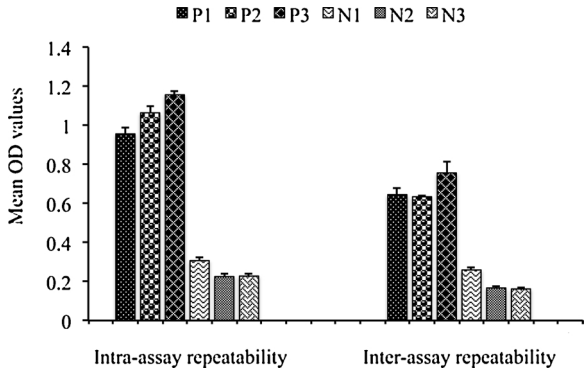

Fig. 4. Repeatability evaluation of the 3A-DHAV-1-ELISA method. P1-P3, positive serum samples 1-3; N1-N3, negative serum samples 1-3. Antigens from the same and different batches were used to evaluate intra-assay and inter-assay repeatability, and the CVs of all samples were below 10%.

Table 3
The coincidence rate between 3A-DHAV-1-ELISA and DHAV-1-ELISA.

DHAV-1-ELISA	3A-DHAV-1-ELISA		
	Positive	Negative	Total
Positive	**48**	1	49
Negative	6	**41**	47
Total	54	42	96

Bold: The coincidence number of two detection methods.

was found to be negative by the new method. Using the DHAV-1-ELISA method as a reference, the diagnostic sensitivity and specificity of the new method were 88.9% and 97.6%. The concordance between the two methods was 92.7%, indicating that the efficacy of 3A-DHAV-1-ELISA for antibody detection was almost equal to that of DHAV-1-ELISA.

4. Discussion

The pathogenic characteristics of DVH make it an intractable disease that must be identified and prevented in a timely manner (Ou et al., 2017c, 2018; Song et al., 2014). Clinically, naturally infected animal serum contains SPs and NSPs antibodies, while inactivated vaccine and subunit vaccine immunized animal serum only had SPs antibodies (Kraan et al., 2017; Oem et al., 2005; Talazadeh et al., 2013). The method based on NSPs antibody detection to distinguish vaccine-immunized ducks from infected ducks has been applied to a variety of infectious diseases (Cong et al., 2013; Mcelroy et al., 2009; Wang et al., 2018). I-ELISA methods based on picornavirus 3A, 3B, 3D, 3AB, 3ABC NSPs serve as potential markers of animal infection (Biswal et al., 2016; Fu et al., 2014, 2017; Hosamani et al., 2015; Mahajan et al., 2015; Yakovleva et al., 2006). However, the establishment of I-ELISA based on DHAV NSPs has not been reported, there has no ways of differentiating DHAV infected and vaccinated ducks as well.

In this study, an I-ELISA was developed and evaluated using prokaryotically expressed recombinant 3A NSP. Additionally, the performance of 3A-DHAV-1-ELISA was compared with that of widely used DHAV-1-ELISA. Full-length DHAV-1 3A gene with 279 bp was cloned into pET-32a(+) prokaryotic expression vector with 12×His tagged protein, but the expression was very low. The hydrophilicity analysis showed that the C-terminal region of 3A gene had transmembrane regions that might hinder the protein expression. Strauss et al. (2003) detected symmetrical dimers in 3A gene via Nuclear magnetic resonance technology, and each monomer contained an alpha helix that blocked the binding of His tag to Ni^{2+} column and increased the difficulty of protein purification. Therefore, this study removed the transmembrane domain and truncated the 3A gene. In a Western blotting assay, the purified recombinant protein of 31 kDa had specific immune response to DHAV-1 infected duck serum, demonstrating its suitability as an antigen for detection of infection specific antibodies in an immunoassay.

In theory, DHAV-1-ELISA that can identify all the virus antigen sites is more practical than 3A-DHAV-1-ELISA that just identify specific 3A antigen site. The analytical sensitivity of the 3A-DHAV-1-ELISA method was 1:1280(between 2^{10} and 2^{11}), which was slightly less than 2^{11} of DHAV-1-ELISA (Quanyun and Li, 1997; Zhao et al., 1991). There was only one dilution gradient difference. Dueing to the purification of DHAV particles was costly and difficult, the new established method was more worthy relying on its convenience.

The antigenic cross-reactivity test results indicated that the 3A-DHAV-1-ELISA method failed to detect antisera to other common duck-sensitive antigens but was capable of detecting antisera to both DHAV-1 and DHAV-3. It reported that VP1-DHAV-1-ELISA and VP3-DHAV-1-ELISA can both detect DHAV-1 and DHAV-3 (Liu et al., 2010; Shen et al., 2015). BLAST analysis showed that the 3A gene homology of DHAV-1 and DHAV-3 was 75%, VP1 gene homology was 72.81%, VP3 gene homology was 80%. High homology of DHAV genotypes was the potential reason for the slight cross-reaction of 3A-DHAV-1-ELISA. The method had good repeatability because both the intra-assay and inter-assay CVs were lower than 10%. Taken together, these results ascertain the sensitivity, specificity and stability of 3A-DHAV-1-ELISA and utility of that way to detect both DHAV-1 and DHAV-3 antibodies.

To evaluate the efficacy of the 3A-DHAV-1-ELISA method, it is critical to compare it with the standard method (Shen et al., 2015). The classical neutralization test is time-consuming and laborious, and it is not suitable for massive serological tests, nor suitable for the detection of viral particles used in this study that have not yet adapted to cell lines (Hu et al., 2016; Li et al., 2017; Shen et al., 2015). DHAV-1-ELISA method is sensitive, specific, convenient, efficient and widely used in clinical particles, and it has a high coincidence rate with neutralization test (Quanyun and Li, 1997; Zhao et al., 1991). Thus, this study used modified and improved DHAV-1-ELISA method as a benchmark to evaluate the newly established 3A-DHAV-1-ELISA method. When detecting identical clinical DHAV-1 infected samples, the coincidence rate of the two methods was 92.7%. The result showed that 3A NSP had good immunogenicity. Compared with other widely studied I-ELISA methods based on subunit of DHAV-1 particles, such as VP1-DHAV-1-ELISA and VP3-DHAV-1-ELISA methods, 3A-DHAV-1-ELISA had stronger specificity(97.6%) but lower sensitivity(88.9%). Massive conserved regions of 3A NSP may explicate this situation. The more conservative regions, the stronger the specificity. Overall, the 3A-DHAV-1-ELISA method can be used as a serological method for DHAV antibodies detection.

In developing countries, DVH control and eradication mainly rely on attenuated and inactivated vaccination. Whereas, distinguishing

infected from vaccinated flocks becomes an enormous challenge. The major problem is that the current detection methods are based on viral particles or SPs, which cannot detect or distinguish immune animals. Hence, the key to diagnose and distinguish inactivated from infected is NSPs antibody detection. Among picornaviruses, Hosamani et al. (2015) established an I-ELISA method based on picornavirus FMDV 3ABC for detecting NSPs antibodies, which has been widely used to distinguish infection in a vaccinated population. In addition, I-ELISA based on 2C, 3A, 3B, 3AB and 3D NSPs all can achieve above effect (Biswal et al., 2016; Fu et al., 2014, 2017; Mahajan et al., 2015; Mohapatra et al., 2014; Oem et al., 2005; Yakovleva et al., 2006). The concordances among these methods with standard method were at least 88.51% (Biswal et al., 2016; Fu et al., 2014, 2017; Mahajan et al., 2015; Mohapatra et al., 2014; Oem et al., 2005; Yakovleva et al., 2006). It indicates that these methods could be used as indexes to detect and distinguish naturally infected and inactivated animals.

5. Conclusion

In conclusion, we have developed a novel I-ELISA method based on DHAV-1 NSP 3A for detection DHAV antibody sensitively, specifically and rapidly. It has been demonstrated to be almost equally capable of sero-surveillance compared with DHAV-1-ELISA. It provides a novel supplement for monitoring viral infection and epidemic trend, and provides a theoretical basis for differentiating suspected DHAV-infected from inactivated vaccine-immunized flocks.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Zhang carried out the cloning of 3A and western gel analysis, developed the ELISA. Zhou performed data analysis and drafted manuscript. Zhou, Zhang, Cheng and Wang contributed to the analysis of the experimental data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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