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

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ABSTRACT

An indirect enzyme-linked immunosorbent assay (I-ELISA) based on the recombinant VP3 protein of duck hepatitis A virus type 1 (DHAV-1) was developed and evaluated in this study. The optimal antigen, serum and enzyme-labeled antibody dilutions were 1:160 (0.94 μg), 1:160 and 1:2000, respectively. The optimal blocking buffer was 1% gelatin. The cutoff value was determined to be 0.332, and the analytical sensitivity was 1:1280 ($OD_{450-630} = 0.37$). The results of the specificity evaluation showed that no cross-reactivity existed between DHAV-1 antiserum and other common duck-sensitive pathogens, except for duck hepatitis A virus type 3 (DHAV-3), suggesting that this could be a common approach for the simultaneous detection of DHAV-1 and DHAV-3 antibodies. The coefficients of variation (CVs) for all of the tested samples were lower than 10%. The concordance between the I-ELISA based on the VP3 subunit of DHAV-1 and that based on the whole DHAV-1 particle was 96%. These results indicate that the VP3-based I-ELISA method has high sensitivity, specificity, and repeatability and is as effective as the DHAV-1-based I-ELISA method for sero-surveillance. Thus, it may be a convenient and novel method for DHAV antibody detection and epidemiological surveillance of DHAV prevalence.

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1. Introduction

Duck viral hepatitis (DVH) is a highly fatal and rapidly spreading contagious disease of ducklings that is mainly caused by duck hepatitis A virus (DHAV). It belongs to the genus *Avihepatovirus* of the family *Picornaviridae* (Knowles et al., 2012). It is an OIE notifiable disease infecting almost all ducklings less than 4 weeks of age, with a mortality rate as high as 50–95% (Woolcock and Tsai, 2013). Thus, the timely and accurate detection of infection is vital for disease control. The neutralization test is the most classical method among the currently approved methods (Hwang, 1969; Kim et al., 2006), but it is laborious and skill dependent. Other assays, such

as immunofluorescence technology and the immune colloidal gold technique, have much higher sensitivity and specificity but are costly. With increasing knowledge of molecular biology and following publication of the whole DHAV genome (Tseng et al., 2007), techniques based on PCR, such as RT-PCR and real-time TaqMan RT-PCR, have become available (Cheng et al., 2009; Yang et al., 2008). Although they are efficient, accurate and rapid, they rely on equipment that is not available in all settings, and they are far less convenient than detecting a virus-specific antibody in the serum (Liu et al., 2010). The indirect enzyme-linked immunosorbent assay (I-ELISA) is a good alternative to these techniques, and utilizing recombinant protein produced by molecular biological assays to establish an ELISA method is becoming a new trend for the efficient diagnosis and epidemiological surveillance of DHAV.

DHAV has three genotypes: DHAV-1, DHAV-2 and DHAV-3. Only DHAV-1 and DHAV-3 are prevalent in mainland China, and DHAV-1 is the most prevalent genotype worldwide (Soliman et al., 2015; Erfan et al., 2015). The DHAV genome consists of only one single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs), and it is translated into three mature structural proteins,

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VP0, VP3 and VP1 (Kim et al., 2006, 2007; Tseng et al., 2007). Among them, VP1 is the most external and important immunogenic protein and is thus the most widely studied. However, VP3 is also a main capsid protein with numerous antigenic sites that can potentially trigger host immune responses. Moreover, VP3 has a more conserved genome sequence than VP1 (Kim et al., 2006; Tseng et al., 2007; Tseng and Tsai, 2007), which may enable the development of novel diagnostic agents.

In this study, an I-ELISA method based on the recombinant VP3 protein of DHAV-1 (VP3-DHAV I-ELISA) was established. The parameters were optimized, and the sensitivity, specificity and repeatability were evaluated. Furthermore, I-ELISA based on the whole DHAV-1 particle (DHAV I-ELISA) was compared with the novel VP3-DHAV I-ELISA method. The newly established method may be a supplement to or even a substitution for the DHAV I-ELISA method for DHAV antibody detection and the monitoring of DHAV prevalence.

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 ducks that were artificially infected with DHAV-1, and the samples were stored at −80 °C until use. Negative serum samples were collected from DHAV-free healthy ducks, and their negative statuses were confirmed by RT-PCR. Confirmed antisera against several common duck-sensitive antigens were stored in our lab. Serum samples were obtained from naïve, DHAV-1-vaccinated and DHAV-1-infected ducks from different farms in Sichuan province, China, and they were used to compare VP3-DHAV I-ELISA with DHAV I-ELISA.

2.2. Antigen preparation

Recombinant VP3 protein of DHAV-1 was expressed using a pGEX-4T-1 vector in a prokaryotic expression host, BL21 (DE3), and purified by SDS-PAGE gel extraction by the author (results to be published). DHAV-1 particles were propagated in 11-day-old duck embryos (Liu et al., 2010) and purified by approaches described in a previous paper published by our lab (Jiang et al., 1989). The concentrations of both antigens were analyzed with a spectrophotometer (SmartSpec^{TM} 3000, Bio-Rad).

2.3. Establishment and optimization of VP3-DHAV I-ELISA

High-titer serum samples from artificially infected ducks and confirmed serum samples from DHAV-free healthy ducks were used as positive and negative working standards, respectively. The optimum dilutions of coating antigen and serum were determined by checkerboard titration in a 96-well microplate, according to an essentially classical indirect ELISA protocol (Crowther, 2009). The purified VP3 protein was serially diluted by two-fold (from 1:20 to 1:2560). The positive and negative serum samples were diluted from 1:40 to 1:1280 in the same manner in a separate plate. An HRP-labeled rabbit anti-duck IgG conjugate (1:5000 to 1:10,000 recommended, Beijing Kangbiquan BioScience) was optimized by preparing two-fold serial dilutions (from 1:500 to 1:4000). The optimal blocking buffer (dissolved in PBS containing 0.05% Tween-20) was chosen from 1% BSA, 5% BSA, 1% gelatin, 5% gelatin and 5% skim milk. All of the samples were tested in triplicate and measured with a microplate spectrophotometer (Model680, Bio-Rad) at two wavelengths (OD$_{450}$ and OD$_{630}$). A reaction with a corresponding positive value (P) of approximately 1.0, a negative value (N) of below 0.4, and the highest P/N value that was no less than 2.1, was considered optimal.

2.4. Validation of the method parameters

The cutoff value for the VP3-DHAV I-ELISA was determined using a panel of 40 negative serum samples under optimal conditions. The result was described as the mean of the total negative serum OD values plus three standard deviations (SDs) (Jia et al., 2009; Upadhyay et al., 2009). An OD value of above the cutoff value was considered positive for the serum sample.

A positive serum sample was used to evaluate the lower detection limit by performing end-point titration from 1:80 to 1:10,240 (Mahajan et al., 2015). Each dilution was tested three times. Positive and negative serum samples for DHAV-1 served as controls. The maximum dilution with the mean OD value that was above the cutoff value was considered to indicate the analytical sensitivity of the method. The analytical sensitivity of DHAV I-ELISA (mentioned below in Section 2.5) was evaluated in the same manner.

The specificity of VP3-DHAV I-ELISA was evaluated by an antigenic cross-reactivity test (Liu et al., 2014) and blocking test. Confirmed antisera to DHAV-1, DHAV-3, duck plague virus (DPV), avian influenza virus (AIV), duck swollen head septicemia virus (DSHSV), *Riemerella anatipestifer* (*R.A*), *Salmonella enterica* (*S.E*) and *Escherichia coli* (*E. coli*) were used to evaluate antigenic cross-reactivity. Each antiserum was tested three times, and DHAV-1-negative serum served as a control. A blocking test was conducted to further evaluate the specificity. Briefly, antiserum to DHAV-1 was mixed with DPV and DHAV-1 antigen separately at 10:1 (V/V), neutralized at 37 °C for 1 h, and then diluted to the optimal concentration and incubated as a primary antibody. Each serum sample was tested three times, and DHAV-1-positive serum that was not mixed with antigen served as a control. DHAV-3 antiserum was mixed with DHAV-1 antigen and neutralized in the same manner. The blocking rate was calculated as the evaluation index.

The repeatability evaluation was conducted as follows. Antigens from the same and different batches were used to test the same panel of positive ($n = 3$) and negative ($n = 3$) serum samples for DHAV-1. The intra-assay and inter-assay coefficients of variation (CVs) were calculated. Each serum sample was tested four times.

2.5. Comparison of VP3-DHAV I-ELISA and DHAV I-ELISA for antibody detection of DHAV-1 in clinical specimens

DHAV I-ELISA was conducted as previously described (Zhao et al., 1991) with some modifications. First, we prepared antigens as described in Section 2.2. Second, we blocked an ELISA plate with 5% skim milk (dissolved in PBS containing 0.05% Tween-20) for 1.5 h at 37 °C before adding diluted serum. Next, we adjusted the incubation time of the serum with an HRP-labeled rabbit anti-duck IgG conjugate to 45 min at 37 °C. Finally, we used a commercial TMB substrate and performed measurements at two wavelengths (OD$_{450}$ and OD$_{630}$).

Serum samples from different sources were tested by VP3-DHAV I-ELISA and DHAV I-ELISA separately to evaluate the similarities between the two assays to gain insights into the clinical applicability of the established VP3-DHAV I-ELISA. The sensitivity and specificity of the new method were determined. The similarity rate was calculated as follows: the sum of the true positive and true negative serum samples divided by the total number of samples.

3. Results

3.1. Standardization of the VP3-DHAV I-ELISA procedure

Purified recombinant VP3 protein was prepared and identified to satisfy the experimental requirements (data not shown). The

Table 1
P/N values corresponding to antigen and serum dilutions.

Dilutions of positive and negative sera	Antigen dilutions							
	1:20 (7.50 μg)	1:40 (3.70 μg)	1:80 (1.88 μg)	1:160 (0.94 μg)	1:320 (0.47 μg)	1:640 (0.24 μg)	1:1280 (0.12 μg)	1:2560 (0.06 μg)
1:40	2.56	3.1	2.99	2.54	2.71	2.79	2.14	1.43
1:80	3.31	3.46	3.42	2.03	3.17	3.21	2.55	1.50
1:160	3.17	3.08	3.47	**3.61**	2.97	3.57	2.55	1.30
1:320	3.25	3.42	3.17	3.01	2.99	3.10	1.88	1.23
1:640	2.47	2.82	2.76	2.84	2.73	2.81	1.60	1.10
1:1280	2.17	2.23	2.28	1.95	1.66	2.15	1.58	1.21

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

Table 2
Optimization of rabbit anti-duck IgG conjugate: average OD values at different dilutions of rabbit anti-duck IgG conjugate.

Dilutions	1:500			1:1000			1:2000			1:4000		
	P	N	P/N	P	N	P/N	P	N	P/N	P	N	P/N
OD values	1.17	0.52	2.26	0.87	0.37	2.35	0.56	0.21	**2.68**	0.36	0.12	2.64

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.

optimal concentrations of VP3 protein, serum and rabbit anti-duck IgG conjugate were 1:160 (0.94 μg), 1:160 (Table 1) and 1:2000 (Table 2), respectively. The optimal blocking buffer was 1% gelatin (data not shown). Low background, as well as significant differences, were found to exist between the positive and negative serum samples.

3.2. Validation of the method parameters

A panel of DHAV-1 antibody-negative serum samples was tested under the determined optimal conditions. The OD values ranged from 0.035 to 0.267, with a mean OD value of 0.122 and an SD of 0.070. The cutoff value was calculated using the following formula: $0.122 + 3 \times 0.07 = 0.332$.

The lower detection limit, also called the analytical sensitivity of the method, was evaluated by end-point titration and was determined to be 1:1280 ($OD_{450-630} = 0.37$) (Fig. 1). The analytical sensitivity was 1:2560 ($OD_{450-630} = 0.42$) for the DHAV I-ELISA (data not shown).

The results for the specificity evaluation showed that all of the serum samples, except for the DHAV-1- and DHAV-3-positive samples, were below the cutoff value (Fig. 2). The OD value of the DHAV-1-negative samples was 0.258 ± 0.010. The DPV-positive serum samples had the highest OD value (0.246 ± 0.023) among the tested samples, except for the DHAV-1- and DHAV-3-positive serum samples. A significant difference in OD values was detected between the seronegative and seropositive samples. For the blocking
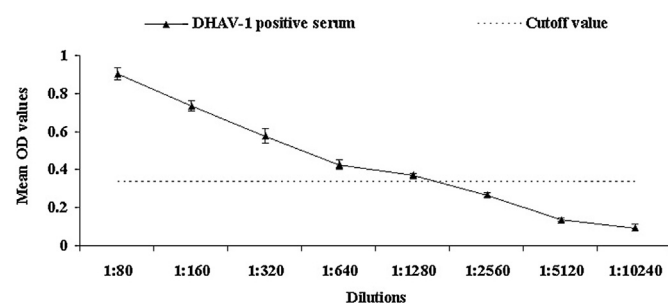
tests (data not shown), the OD values of the sera mixed with DHAV-1 and DPV particles were 0.230 ± 0.003 and 1.210 ± 0.003, respectively, while that of the control was 1.246 ± 0.038. The calculated blocking rate for DHAV-1 antiserum was 82%, indicating that the DHAV-1-positive serum reacted specifically with DHAV-1 rather than with DPV. The blocking rate for DHAV-3 antiserum was 56%, indicating that it cross-reacted with DHAV-1.

The repeatability of the assay was evaluated by determining the average intra-assay and inter-assay CVs. Serum samples with different OD values were tested (Fig. 3). The intra-assay CV ranged from 0.73% to 8.64%, with a mean of 5.36%, while the inter-assay CV ranged from 3.35% to 7.68%, with a mean of 5.53%. None of the tested serum samples had a CV of over 10%, indicating the stability and repeatability of the method.

3.3. Comparison of efficacies of VP3-DHAV I-ELISA and DHAV I-ELISA for detecting DHAV-1 antibody

Serum samples of different origins were tested simultaneously using the VP3-DHAV and DHAV-based I-ELISA for comparison. The cutoff value of the DHAV I-ELISA determined in this study was 0.313 (data not shown). The results (data not shown) showed that the positive rates for the VP3-DHAV and DHAV-based I-ELISAs were 50% (50/100) and 52% (52/100), respectively. However, one sample that tested negative using DHAV I-ELISA was found to be positive by VP3-DHAV I-ELISA. In contrast, 3 samples that tested negative by VP3-DHAV I-ELISA were found to be positive by DHAV I-ELISA. The diagnostic sensitivity and specificity of the new method were 94.2% (49/52) and 97.9% (47/48), respectively. The concordance between the two methods was 96% [(49 + 47)/100], indicating that the efficacy of VP3-DHAV I-ELISA for antibody detection was almost equal to that of DHAV I-ELISA.

4. Discussion

The pathogenic characteristics of DVH make it an intractable disease that must be identified and prevented during the early stages. I-ELISA can be performed to test sera from duck flocks with suspected DHAV infections to efficiently monitor the antibody levels in ducks, and it can provide a guide for preventive measures for timely control of the disease. ELISA is commonly used for the detection of numerous picornaviruses (Liu et al., 2010; Ma et al., 2008; Mahajan et al., 2013, 2015; Mohapatra et al., 2014; Yu et al., 2012) and not merely for identification of DHAV.
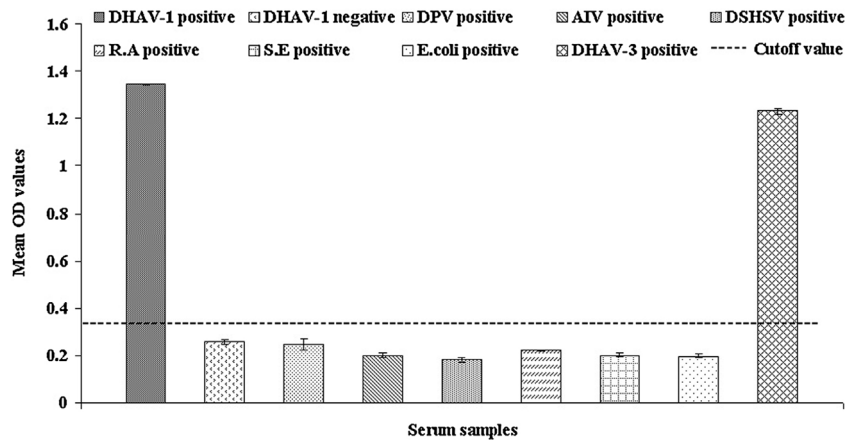

Fig. 1. The analytical sensitivity of the VP3-DHAV I-ELISA method. DHAV-1 antiserum was serially diluted by two-fold (from 1:80 to 1:10,240) and was detected using the VP3-DHAV I-ELISA method. The detected maximum dilution was 1:1280 according to the cutoff value of 0.332.

Fig. 2. Antigenic cross-reactivity analysis of the VP3-DHAV I-ELISA method. DHAV-1-negative serum and antisera to DHAV-1, DHAV-3 and other common duck pathogens were tested using the new method. Antisera to all of the duck antigens had OD values of below the cutoff value (0.332), except for the DHAV-1 and DHAV-3 antisera.

In theory, using a whole virus as a coating antigen in indirect ELISA is more advantageous than using its subunits because whole viruses have more comprehensive antigenicity. This advantage may account for the higher detection limit (1:2560) of DHAV I-ELISA than that of VP3-DHAV I-ELISA (1:1280). Nevertheless, purification of DHAV particles is costly and difficult (Liu et al., 2010); thus, it is urgent to identify a substitution for this antigen. Recombinant protein is a reasonable choice because it is readily available, easy to purify to any required concentration, and capable of immunoreactivity. Moreover, ELISAs based on recombinant proteins have been widely used (Ma et al., 2008; Zhang et al., 2015). To our knowledge, however, no VP3-DHAV I-ELISA method for DHAV detection has been reported.

In this study, an indirect ELISA method based on the recombinant VP3 protein was developed. A panel of DHAV-negative serum samples collected from healthy ducklings was confirmed by RT-PCR (Cheng et al., 2009). Although the samples had been filtered, the OD values varied greatly among them, possibly due to hemolysis or complex components present in the serum. The analytical sensitivity of the method was 1:1280 ($OD_{450-630} = 0.37$). The results of antigenic cross-reactivity testing indicated that the VP3-DHAV I-ELISA method failed to detect antisera to other duck-sensitive antigens but was capable of detecting antisera to both DHAV-1 and DHAV-3. This finding may be used to aid in the monitoring

of DHAV antibodies, but it fails to differentiate whether DHAV-1 or DHAV-3 is the pathogen, and it also indicates the existence of cross-reactivity between the two genotypes. VP3 is relatively conserved, and sequence BLAST analysis showed that the VP3 proteins of DHAV-1 and DHAV-3 share 79–80% identity, which is higher than that of the VP1 proteins (70.51–72.81%). However, a published VP1-based I-ELISA method has been demonstrated to be able to detect antisera to DHAV-1 and DHAV-3 simultaneously (Yang et al., 2014). The repeatability of the method was proven to be good because both the intra-assay and inter-assay CVs were lower than 10%. Taken together, these results indicate that the VP3-DHAV I-ELISA method is sensitive, specific and stable and that it can be used as a common approach to detect DHAV-1 and DHAV-3 antibodies.

To evaluate the efficacy of the novel I-ELISA method, it's critical and necessary to compare it with the standard method (Liu et al., 2014; Yang et al., 2014; Zhang et al., 2015). The neutralization test is not suitable for massive serological detection of the virus strain used in this study, which has not adapted to cell culture to date; moreover, no approved commercial ELISA kits for DHAV are available for clinical use. Even so, whole DHAV particle-based I-ELISA has been demonstrated to have good consistency according to the neutralization test (Zhao et al., 1991; Sun et al., 1997); therefore, a published DHAV I-ELISA method was modified and improved for use as a standard to evaluate the newly established VP3-DHAV
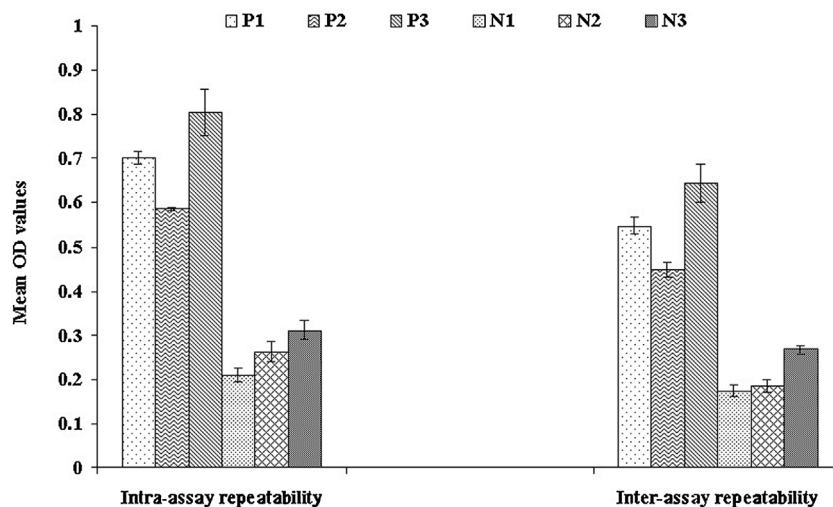

Fig. 3. Repeatability evaluation of the VP3-DHAV I-ELISA method. P1-P3, positive serum samples 1–3; N1–N3, negative serum samples 1–3. Antigens from the same and different batches were used to evaluate intra-assay and inter-assay repeatability, and the CVs of all samples were below 10%.

I-ELISA method. To imitate field conditions, serum samples were collected from ducks naturally infected with DHAV, and they were also obtained from vaccinated and naïve ducks were at the clinic. The results revealed strong similarities between the efficacies of the two assays (96%), suggesting that the immunoreactivity of the VP3 subunit is comparable to that of the whole DHAV-1 particle. It demonstrates that the VP3 subunit, in addition to VP1 (Oberste et al., 1999; Zhang et al., 2014), can also act as an indicator of DHAV-1 infection. Compared with the published VP1-based ELISA method developed by Liu et al. (2010), this new assay has lower sensitivity but higher specificity. This difference may be related to the increased sequence conservation of VP3 compared with VP1. It is difficult to accurately compare our results with those of Liu et al. because their diagnostic specificity and sensitivity were based on the neutralization test, while ours were based on the whole DHAV particle I-ELISA. Taken together, these results demonstrate that the VP3-DHAV I-ELISA is capable of serological detection.

This study is far from perfect, and we still need to evaluate a larger number of samples and to quantify antibodies. Even so, this method shows potential for the serological detection of DHAV antibodies.

In conclusion, we have developed a novel indirect ELISA method for detecting DHAV-1 and DHAV-3 antibodies cheaply, rapidly and accurately. The VP3-DHAV I-ELISA method has been demonstrated to be highly sensitive, specific, and equally capable of sero-surveillance compared with the DHAV I-ELISA method. It is a useful supplement for the monitoring of both vaccinated and non-vaccinated infected flocks.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.08.016.

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