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Development of a monoclonal antibody-based competitive enzyme-linked immunosorbent assay for detection of antibodies against duck adenovirus 3

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Abstract

Background Duck adenovirus 3 (DAdV-3), the emerging pathogen of a disease characterized by swelling and hemorrhage in liver and kidney, is widely distributed in China, resulting in serious economic losses to the duck farms. However, no vaccine is commercially available for the prevention of the disease. Thus, a precise and reliable diagnosis is of great value for the implementation of control measure to limit the spread of DAdV-3 infection. In this study, a competitive enzyme-linked immunosorbent assay (c-ELISA) was developed for detection of antibodies against DAdV-3.

Results In the c-ELISA, a monoclonal antibody (mAb) 3D9, which was specific to Fiber-2 protein of DAdV-3, was labeled with horseradish peroxidase (HRP) and then used as secondary antibody. The cut-off value of the c-ELISA was determined as 18% using 48 clinical negative duck sera. The preliminary concordance evaluation based on 24 duck sera revealed that the results of the c-ELISA were highly consistent with those of indirect immunofluorescence assay (IFA). The cross-reactivity assessment showed that the c-ELISA only reacted with the positive sera against DAdV-3, but not with positive sera against other duck-associated viruses tested. The coefficients of variation of intra-batch and inter-batch assays were all below 10%, suggesting a good repeatability of the c-ELISA. Moreover, 133 duck sera from animal experiments in our laboratory were detected by c-ELISA and indirect ELISA. The results showed that the coincidence rate between the two assays was 90.98% (121/133) and the Kappa value was 0.815, revealing almost perfect agreement of the two assays.

Conclusions In summary, the c-ELISA developed here was specific, repeatable and reliable. The minimal cross-reactivity and independence of species-specific enzyme-conjugated secondary antibody made the c-ELISA a promising tool for the clinical diagnosis, serological epidemiological investigation, and evaluation of vaccine immunogenicity to control the disease caused by the infection of DAdV-3.

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Keywords DAdV-3, c-ELISA, Detection of antibodies, Preliminary concordance evaluation, Cross-reactivity assessment, Diagnostics

Background

Duck adenovirus 3 (DAdV-3), the causative agent of a novel disease characterized by hemorrhage and swelling in liver and kidney, has caused substantial economic losses to the Muscovy duck farms in China [1]. DAdV-3, belonging to the genus *Aviadenovirus* within the family *Adenoviridae*, is a nonenveloped icosahedral virus with non-segmented and double-stranded genome, which is about 44 kb in length [2]. The genome encodes two distinct fiber proteins: a short fiber protein (Fiber-1) and a long fiber protein (Fiber-2) [3]. Those essential structural proteins, located on the surface of the virion, mediate viral attachment to host cell receptors and are closely associated with virulence [4]. Fiber-2 is considered a protective immunogen due to its capacity to induce significant neutralizing antibodies, providing effective protection against DAdV-3 infection [5, 6].

Since its outbreak of the disease in 2014, the extensive and comprehensive studies of DAdV-3 have been carried out to gain better insight into DAdV-3, such as the pathogen, pathogenicity, epidemiology, diagnostic method and subunit vaccine [1, 6–9]. Epidemiological investigation showed that DAdV-3 was prevalent in Guangdong, Guangxi, Fujian, Jiangxi, Zhejiang, Hubei, Anhui and Jiangsu provinces of China [9]. The morbidity of the disease was ranged from 40 to 55%, with a mortality rate of 35–43%, posing a great threat to the duck farms in China [10]. Recently, some novel DAdV-3 mutants were reported. The mutants had G to A transitions and resulted in a stop codon in ORF67, which exhibited increased virulence compared to the early strains [2, 11]. Therefore, there is an urgent need to develop diagnostic approaches to monitor the prevalence of DAdV-3. Effective diagnostic methods targeting the Fiber-2 protein include real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for viral detection and indirect enzyme-linked immunosorbent assay (ELISA) for detecting specific antibodies [7, 8]. The two methods have been reported as specific, sensitive and repeatable tools for the detection of DAdV-3, contributing to the control of the disease. Nevertheless, the qRT-PCR method, relying on the expensive instrument, is confined to laboratory detection. Indirect ELISA works based on the binding of the specific antibodies in serum sample to the coated protein. It requires a species-specific enzyme-conjugated secondary antibody and a highly purified coating protein. The bacterial compositions or the tags in the unpurified coating antigen may result in a high incidence of false-positive reactions [12]. By contrast, competitive ELISA (c-ELISA) is not limited by expensive instrument and species-specific secondary antibody, which reduces the cost. The practical advantages of c-ELISA are as same as those of indirect ELISA, and both methods are featured with simple operation process, clear result determination, low cost of production, and suitability for large-scale test. Moreover, c-ELISA was expected to exhibit high specificity due to the competition between specific antibodies and the monoclonal antibody for binding to the coated protein. Besides, c-ELISA exhibits good performance in the detection of numerous clinical sera at one time, contributing to its wide application in serological diagnosis. Up to now, some c-ELISA methods have been developed for the detection of antibodies against avian pathogens, such as avian influenza virus (AIV), Newcastle disease virus (NDV), goose astrovirus (GAstV), Tembusu virus (TMUV), avian hepatitis E virus (HEV), and avian reovirus (ARV) [13–19]. Those c-ELISA methods provided alternative tools for disease diagnosis and vaccine efficacy evaluation.

The aim of this study was to develop a mAb-based c-ELISA for the detection of antibodies against DAdV-3. This c-ELISA depends on the recombinant Fiber-2 protein expressed by prokaryotic expression system and the enzyme-conjugated mAb 3D9 specific to Fiber-2 protein of DAdV-3.

Methods

Cells, proteins, viruses and sera

Leghorn Male Hepatoma cell line (LMH) from the American strain collection center (ATCC) was cultured in DMEM/F12 (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Lonsera, Shanghai, China) at 37 °C with 5% CO_2. The Fiber-2 protein, expressed in a prokaryotic expression system and purified by Ni-NTA agarose as described previously, was stored in our laboratory and used for coating antigen [20]. The viruses DAdV-3, serotype 1 fowl adenovirus (FAdV-1), FAdV-2, FAdV-4, FAdV-5, FAdV-6, FAdV-7, FAdV-8a, FAdV-8b, FAdV-9, FAdV-10 and FAdV-11 were stored in our laboratory. The positive sera against DAdV-3, FAdV-4, NDV and H9N2 AIV were stored in our laboratory. The sera against duck hepatitis virus type 1 (DHV-1), DHV-3 and TMUV were provided by Prof. Qingtao Liu (Jiangsu Academy of Agricultural Science) kindly. The sera against duck reovirus (DRV) and novel duck parvovirus (NDPV) were offered friendly by Prof. Zongyan Chen (Shanghai Veterinary Research Institute). Positive sera against DAdV-3 were from Muscovy ducks infected with DAdV-3 (1×10^6 $TCID_{50}$ per duck) through intramuscular route and the

negative sera against DAdV-3 were from Muscovy ducks without infection. The Muscovy ducks were purchased from a commercial hatchery (Yangzhou Guangling Jiumuyuan Animal Husbandry Co., Ltd.) in Jiangsu province of China. All animal experiments were in accordance with the institutional animal care guidelines and the protocol (SYXY-32), which was approved by the Animal Care Committee of Yangzhou University. The sera used in the study were confirmed by indirect immunofluorescence assay (IFA) using LMH cells infected with DAdV-3. Briefly, the LMH cells infected with DAdV-3 was fixed with prechilled acetone and ethanol (3:2). Then the duck sera diluted with a ratio of 1:100 were served as primary antibodies and the FITC-conjugated goat anti-chicken IgG (Sigma-Aldrich, Missoula, MO, USA) was served as secondary antibody. Finally, the LMH cells were observed under a fluorescence microscope.

Indirect ELISA

The Fiber-2 protein was diluted to 0.5 μg/mL and coated into a 96-well ELISA plate (100 μL/well) at 4 ℃ overnight. The following morning, the coated 96-well ELISA plate was blocked with 5% skim milk for 2 h at 37 °C. After three washes with PBST, the tested sera were diluted with a ratio of 1:400 and added into the 96-well ELISA plate (100 μL/well) for 1 h. Then the HRP-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, Missoula, MO, USA), diluted with a ratio of 1:10000, was added into the 96-well ELISA plate (100 μL/well) for 1.5 h. After three washes with PBST, TMB single-component substrate solution (Solarbio, Beijing, China) was added to the 96-well ELISA plate and reacted at 37 °C for 15 min. The reaction was stopped with 2 M H_2SO_4 (50 μL/well) and the value of optical density at 450 nm (OD_{450nm}) was measured with an ELISA reader.

Generation of mAb 3D9

The mAb 3D9 specific to the Fiber-2 protein of DAdV-3 was produced as described previously [20]. Briefly, female BALB/c mice were immunized with purified Fiber-2 protein. After the immunization, the spleen cells and SP2/0 myeloma cells were fused. The positive hybridoma cells were screened and subcloned to generate the cell lines. The ascitic fluid was obtained through the intraperitoneal injection of monoclonal hybridoma cells and then purified using a protein-G-affinity column. The concentration of purified mAb 3D9 was measured using a One Drop™ OD-1000 + Spectrophotometer (WINS, Nanjing, China).

Indirect Immunofluorescence assay

The LMH cells, infected with DAdV-3 or other adenoviruses, were fixed with prechilled acetone and ethanol with a volume ratio of 3:2 for 5 min. Then the fixative solution was poured out and the fixed LMH cells were dried at room temperature. The LMH cells were incubated with mAb 3D9 for 45 min at 37 °C. After three washes with PBS, the LMH cells were incubated with FITC-labelled goat anti-mouse IgG (Sigma-Aldrich, Missoula, MO, USA) for 45 min at 37 °C. After another three washes with PBS, the LMH cells were observed with a fluorescence microscope.

Preparation of mAb 3D9 labeled with horseradish peroxidase

The mAb 3D9 labeled with horseradish peroxidase (3D9-HRP) was prepared by the Thermo Scientific™ EZ-Link™ Plus Activated Peroxidase Kit according to the manufacturer's instructions. In brief, 1 mg lyophilized EZ-Link Plus Activated Peroxidase, reconstituted with ultrapure water, was mixed with 1 mg mAb 3D9. In a fume hood, the reaction system was added with 10 μL Sodium Cyanoborohydride and then incubated for 1 h at room temperature. Then 20 μL Quenching Buffer was added and reacted for 15 min. Finally, the mAb 3D9-HRP was dialyzed and its concentration was measured using a spectrophotometer.

Establishment of c-ELISA using mAb 3D9-HRP

The concentration of the Fiber-2 protein and dilution of mAb 3D9-HRP were determined by a checkerboard titration as follows. The Fiber-2 protein, serving as coating antigen, was double-diluted and coated into a 96-well ELISA plate (100 μL/well) at 4 ℃ overnight. The concentration of Fiber-2 protein was ranged from 10 μg/mL to 0.156 μg/mL. The following morning, the coated 96-well ELISA plate was blocked with 5% skim milk for 2 h at 37 °C. After three washes with PBST, mAb 3D9-HRP was double-diluted and added into the 96-well ELISA plate (100 μL/well) for 1 h. The dilution of the secondary antibody was ranged from 1:2000 to 1:32000. After three washes with PBST, TMB single-component substrate solution was added to the 96-well ELISA plate and reacted at 37 °C for 10 min. The reaction was stopped with 2 M H_2SO_4 (50 μL/well) and the OD_{450nm} value was measured with an ELISA reader. The optimal concentration of Fiber-2 protein and dilution of mAb 3D9-HRP were selected when the OD_{450nm} value of the direct ELISA was approximately 1.0.

To determine the optimal dilution of the tested sera, three positive sera and three negative sera were used for the development of the c-ELISA. Three positive sera included two strong positive sera and one weak positive serum. The double-diluted sera, ranged from 1:2 to 1:128, were added to the coated and blocked 96-well ELISA plate (100 μL/well) for 1 h. After washes with PBST for three times, mAb 3D9-HRP was added into the 96-well ELISA plate (100 μL/well). The other steps were the same as described above. The optimal dilution of tested sera

was determined based on the percent inhibition (PI), which was calculated by the formula PI (%) = [(OD_{450nm} value of negative serum - OD_{450nm} value of tested serum) / OD_{450nm} value of negative serum] × 100%.

After the determination on the optimal dilution of tested sera, the other reaction conditions, including blocking buffer, reacting time of tested sera and reacting time of substrate, were respectively optimized based on the PI values of tested sera.

Determination of c-ELISA cut-off value

A total of 48 clinical negative duck sera were used in the c-ELISA to determinate the cut-off value. Meanwhile, three positive sera were served as positive control. The cut-off value of the developed c-ELISA was calculated with the 48 negative sera based on the mean PI value (X) plus threefold standard deviation (SD).

Preliminary concordance evaluation and cross-reactivity assessment

To further evaluate the preliminary concordance, a panel of 24 sequential sera from two infected Muscovy ducks and one noninfected Muscovy duck were tested by c-ELISA and IFA. The cross-reactivity of the c-ELISA was evaluated with positive sera against DAdV-3, FAdV-4, DHAV-1, DHAV-3, TMUV, DRV, NDPV, NDV and H9N2 AIV. The size of tested samples in the study is not large enough. The preliminary concordance evaluation and cross-reactivity assessment need further validation with more diverse and larger datasets in future studies.

Repeatability test

To assess the repeatability of the c-ELISA, three positive sera and three negative sera were applied to the repeatability test. The intra-batch assay based on three plates (coated at one point in time) was performed on different occasions and the inter-batch assay based on three plates (coated at different points in time) was performed on the same occasion. The coefficient of variation (CV) was calculated according to the PI values. Each serum had three experimental repeats.

Agreement of indirect ELISA and c-ELISA

The agreement between indirect ELISA and c-ELISA was achieved based on 133 sera from the Muscovy ducks infected with DAdV-3 and the noninfected Muscovy ducks. The coincidence rate (CR) was calculated using the formula CR (%) = [(number of total sera - number of inconsistent sera) / number of total sera] × 100%. Besides, the agreement of the two assays were estimated using the Kappa value, which was calculated using the software IBM SPSS Statistics 22. The Kappa value ≤ 0.00 was designated as poor agreement, 0.00 < Kappa value ≤ 0.20 slight agreement, 0.20 < Kappa value ≤ 0.40 fair agreement, 0.40 < Kappa value ≤ 0.60 moderate agreement, 0.60 < Kappa value ≤ 0.80 substantial agreement, and 0.80 < Kappa value < 1.00 almost perfect agreement [21].

Results

Generation of mAb 3D9 specific to the Fiber-2 protein of DAdV-3

After the immunization of Fiber-2 protein in mice, the spleen cells were fused with the SP2/0 cells. After screening and subcloning, the mAb 3D9 was successfully generated. As described in Fig. 1A, the mAb 3D9 only reacted with LMH cells infected with DAdV-3 but not with LMH cells infected with other adenoviruses. Above results reveals that the mAb 3D9 is generated.

Purification and HRP-labelling of mAb 3D9

After the generation of mAb 3D9, it was purified using a protein-G-affinity column and analyzed by SDS-PAGE. The concentration of the purified mAb 3D9 was 1.88 mg/mL and the purified mAb migrated at 50 and 25 kDa on SDS-PAGE without nonspecific bands (Fig. 1B), indicating that the mAb 3D9 was purified successfully. To develop a c-ELISA for detection of DAdV-3, the mAb 3D9 was labeled with HRP. The concentration of the mAb 3D9-HRP was measured as 0.59 mg/mL.

Establishment of c-ELISA based on mAb 3D9-HRP

To establish a c-ELISA, a checkerboard titration assay was performed to determine the concentration of coated Fiber-2 protein and the dilution of mAb 3D9-HRP. As shown in Table 1, the optimal concentration of Fiber-2 protein was 1.25 μg/mL, and the optimal dilution of mAb 3D9-HRP was 1:16000, indicating the high efficiency of mAb 3D9-HRP to bind to Fiber-2 protein. The following was the optimization of other reaction conditions. As described in Fig. 2, the optimal dilution of test sera was 1:4 considering the high PI value of the weak positive serum. The most suitable blocking buffer was 5% skim milk. The optimal serum and substrate reacting time was 60 min and 10 min, respectively. Above results indicate that the c-ELISA, after the optimization of the reaction conditions, shows good diagnostic performance for the detection of antibodies against DAdV-3.

Determination of cut-off value of the c-ELISA

A total of 48 clinical negative duck sera were assayed by the c-ELISA to determine the cut-off value and the PI values were shown in Table 2. The results indicated that the mean PI value was 3% and the SD was 5%. Therefore, the cut-off value for the developed c-ELISA was fixed on 18%. The tested serum with the PI value ≥ 18% was determined as positive, otherwise it was considered to be negative.

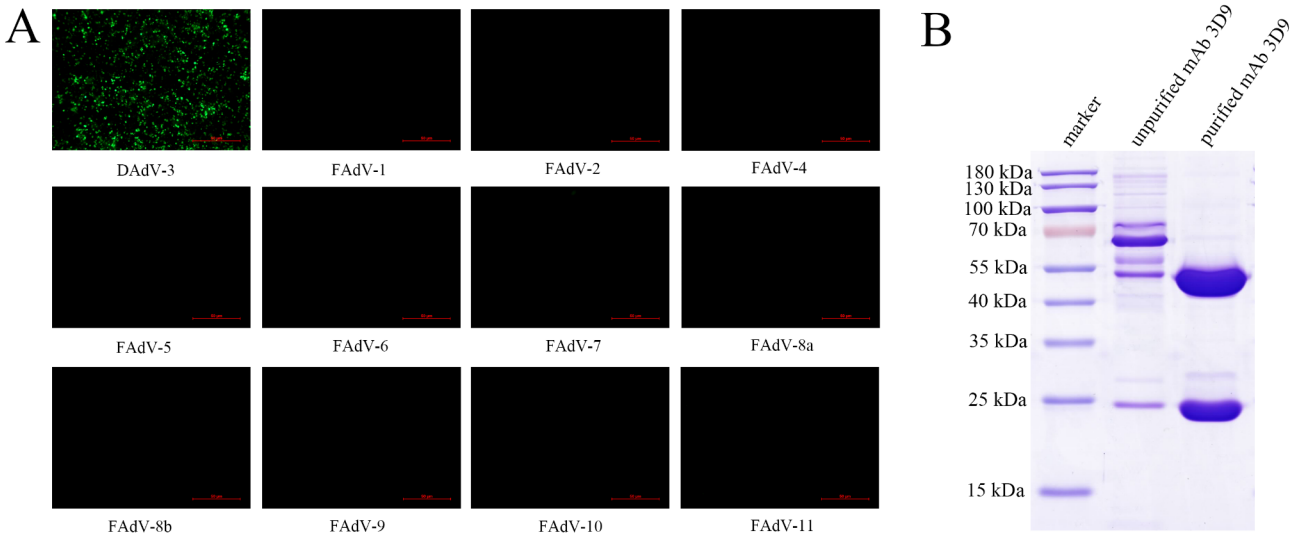

Fig. 1 Specificity and purification of mAb 3D9. (**A**) The specificity of mAb 3D9 was validated via IFA utilizing LMH cells infected with adenoviruses, showing its distinctive reactivity specifically to DAdV-3 and not to closely related viruses. (**B**) The purification of mAb 3D9 was confirmed by SDS-PAGE. The displayed gel was cropped from a full-length gel (Figure S1), which was included in the supplementary material file

Table 1 Determination on the concentration of Fiber-2 protein and Dilution of mAb 3D9-HRP by a checkerboard Titration

Different concentrations of Fiber-2 protein (μg/mL)	Different dilutions of mAb 3D9-HRP in medium			
	1:4000	1:8000	1:16000	1:32000
5	3.622	3.117	2.333	1.37
2.5	3.595	3.083	1.935	1.188
1.25	1.912	1.47	1.022[a]	0.536
0.625	0.936	0.793	0.53	0.27
0.313	0.477	0.43	0.21	0.169
0.156	0.153	0.143	0.093	0.083

[a] The optimal concentration of Fiber-2 protein and dilution of mAb 3D9-HRP were selected when the OD_{450nm} value of the direct ELISA was approximately 1.0

Preliminary concordance evaluation, cross-reactivity assessment and repeatability of the c-ELISA

The preliminary concordance of the c-ELISA and IFA was assessed with 24 duck sera. As described in Table 3, the detection result of c-ELISA was consistent with the result from IFA, which indicated that the c-ELISA could effectively recognize the positive sera against DAdV-3. To determine the discriminatory power of the c-ELISA for sera from ducks infected by DAdV-3 and closely related viruses, eight positive sera against FAdV-4, DHAV-1, DHAV-3, TMUV, DRV, NDPV, NDV and H9N2 AIV were tested. As shown in Fig. 3, the PI value of the DAdV-3 positive serum reached to 84.64%, nevertheless the PI values of nonspecific positive sera were ranged from 0 to 8.75% and less than the cut-off value. Above data demonstrate that the developed c-ELISA shows no cross-reactivity with other positive sera tested. To evaluate the repeatability of the c-ELISA, three positive sera and three negative sera were analyzed. The CVs of the PI values were measured to assess the repeatability of intra-batch and inter-batch assays. The results described in Table 4 showed that the CVs of intra-batch assay were ranged from 0 to 5.65%, and the CVs of inter-batch assay were within 6.57%, which were all below 10%, demonstrating an adequate repeatability of the c-ELISA. Above data suggest that the c-ELISA is an accurate and repeatable tool for the detection of antibodies against DAdV-3.

Comparison of the c-ELISA with indirect ELISA

To confirm the application of the c-ELISA in clinical detection, the developed c-ELISA was compared with the indirect ELISA based on 133 duck sera. The results displayed in Table 5 revealed that 121 sera (48 positive sera and 73 negative sera) were consistent between the two assays with the CR of 90.98% (121/133). In addition, the statistical analysis demonstrated that the Kappa value from the comparison of c-ELISA and indirect ELISA was determined as 0.815, indicating almost perfect agreement of the two assays. Above data indicate that the c-ELISA is a reliable tool for DAdV-3 detection and has the potential to apply to epidemiologic surveillance and evaluation of vaccine efficacy in the future.

Discussion

DAdV-3, a novel duck adenovirus, is an emerging infectious agent of a disease characterized by swelling and hemorrhagic liver and kidney in Muscovy ducks [1]. In recent years, the continual prevalence of DAdV-3 has become increasingly severe, constituting an enormous challenge to the Muscovy duck industry in China [2, 11]. Although an extensive effort has been made to understand DAdV-3, there is no effective commercial vaccine

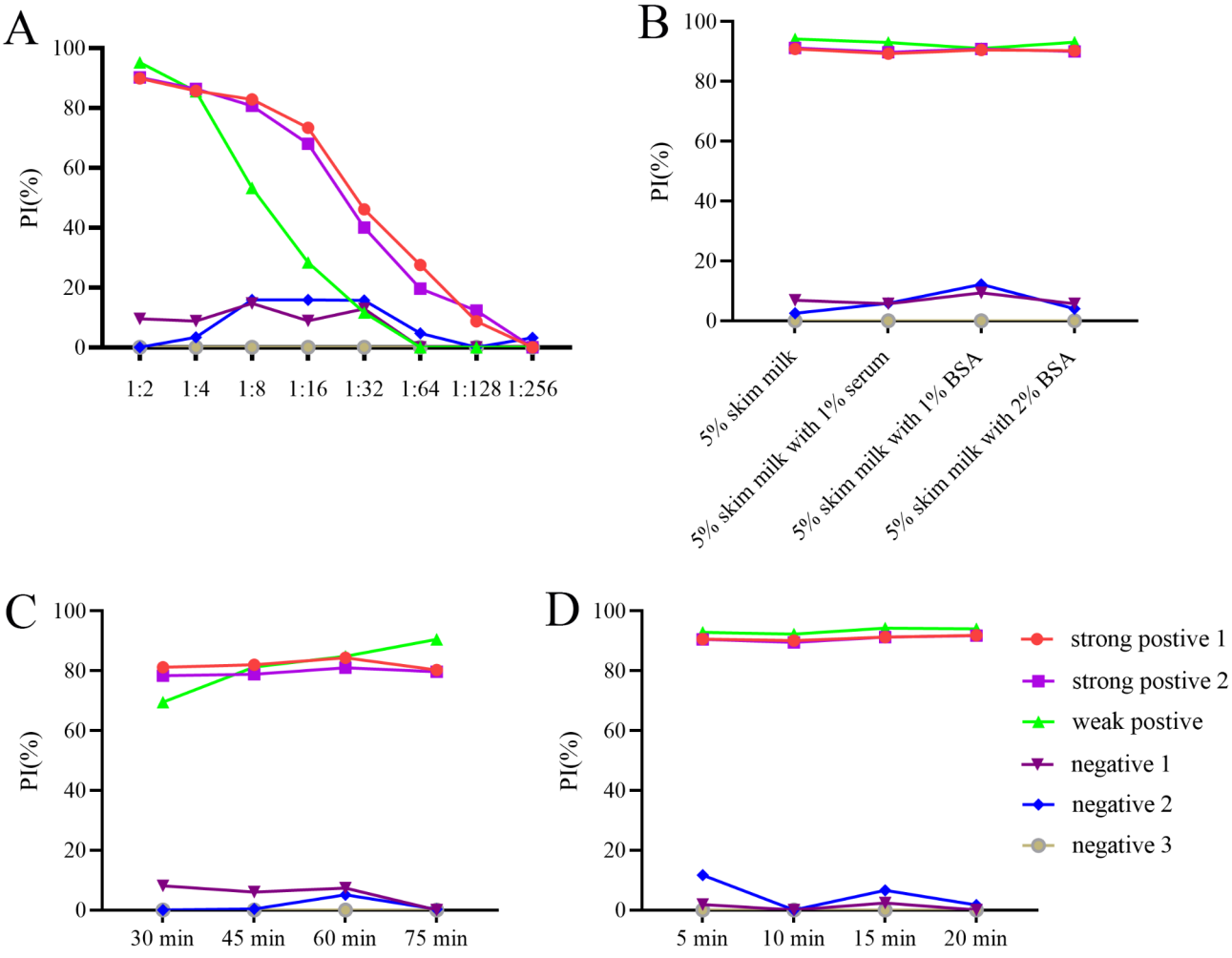

Fig. 2 Optimization of the c-ELISA. The dilution of tested duck sera (**A**), the blocking buffer (**B**), the reacting time of tested duck sera (**C**), and the reacting time of substrate (**D**) were respectively determined

Table 2 ELISA PI values from 48 negative Duck Sera for determining the cut-off value of the assay

PI values from 48 clinical negative duck sera							
6.8%	1.6%	0%	0%	0.2%	0.1%	6.8%	5.1%
0%	0%	7.3%	11.6%	0%	0%	1.6%	0%
0%	0%	0%	0.7%	2%	0%	6.6%	16.5%
0%	7.5%	5.1%	2%	3.4%	0%	0%	6.4%
0%	0%	4.1%	0%	0%	16.1%	0%	0%
0%	2.7%	17.5%	0%	0%	0%	7.1%	0.4%

available to prevent the disease. The effective control measurement primarily depends on the early accurate diagnosis. As the prerequisite for epidemiologic surveillance as well as evaluation of vaccine immunogenicity in the future, serological approach to detect specific antibodies against DAdV-3 is of instructive importance. Fiber-2 protein, a vital structural protein on the surface of the virion, is a good antigenic target for the development of detection method because of its high conservation and abundant expression during early stage of infection [2]. Indirect ELISA and c-ELISA are two main serological tests for the detection of antibody. The two ELISA methods have the same advantages, such as the capacity to detect numerous samples at one time and the rapid turnaround of testing results [15]. Indirect ELISA is more sensitive than c-ELISA. However, indirect ELISA has the potential to bring about false-positive results. The main benefit of c-ELISAs over indirect ELISAs is that c-ELISA has minimal cross-reactivity and is not limited by species-specific enzyme-conjugated secondary antibody. To date, an indirect ELISA targeting Fiber-2 protein has been developed [7]. However, a c-ELISA

Table 3 Concordance evaluation of the c-ELISA with IFA

wpi[a]	Sera of infected duck 1		Sera of infected duck 2		Sera of noninfected duck	
	IFA	c-ELISA	IFA	c-ELISA	IFA	c-ELISA
1	+[b]	+ (32.7%)	-[c]	– (0%)	-	– (0%)
2	+	+ (61.6%)	+	+ (78.7%)	-	– (0%)
3	+	+ (77.2%)	+	+ (79.5%)	-	– (0%)
4	+	+ (87.1%)	+	+ (88.1%)	-	– (4.4%)
5	+	+ (85.9%)	+	+ (89.1%)	-	– (9.1%)
6	+	+ (87.7%)	+	+ (91.8%)	-	– (4.1%)
7	+	+ (89.3%)	+	+ (89.9%)	-	– (0.3%)
8	+	+ (85.3%)	+	+ (93.1%)	-	– (0%)

[a] wpi means weeks post infection; [b] + means positive; [c]– means negative

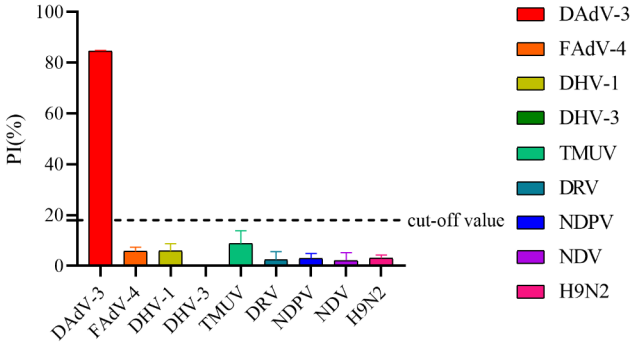

Fig. 3 Cross-reactivity assessment of the c-ELISA. The cross-reactivity of the c-ELISA was assessed with the positive sera against DAdV-3, FAdV-4, DHAV-1, DHAV-3, TMUV, DRV, NDPV, NDV and H9N2 AIV. Each serum had three experimental repeats

targeting Fiber-2 protein is not available currently. In this study, an efficient c-ELISA based on Fiber-2 protein and mAb 3D9-HRP was developed for detecting the antibodies against DAdV-3.

In this study, the recombinant Fiber-2 protein, used as the coating antigen, was expressed in a prokaryotic expression system and purified by Ni-NTA agarose as previously described [20]. The mAb 3D9, only reacting with DAdV-3 but not reacting with other adenoviruses, was labeled with HRP and then served as secondary antibody. To evaluate the efficiency of the mAb 3D9-HRP in ELISA, a checkerboard titration was carried out. The high OD_{450nm} values indicated that the mAb 3D9-HRP had high affinity with Fiber-2 protein. In our preliminary study, another mAb 6A3-HRP was also worked as the secondary antibody in the checkerboard titration. However, mAb 6A3-HRP was not as befitting as mAb 3D9-HRP, possibly owing to the difference of two epitopes recognized by the two mAbs. In this study, the tested sera were first added to the Fiber-2 coated plate, and then after incubation and washing, the mAb 3D9-HRP was added. In our previous study, we also tried the approach that the tested sera mixed with mAb 3D9-HRP were added to the coated plate simultaneously, which was known for the efficiency. Unfortunately, this efficient approach did not work well due to the reduced sensitivity.

In the development of the c-ELISA, the verification of negative and positive sera is extremely crucial. The three positive sera from Muscovy duck infected with DAdV-3 and the three negative sera from Muscovy duck without the infection of DAdV-3 were verified by IFA and indirect ELISA. Notably, a weak positive serum was determined here with lower OD_{450nm} in indirect ELISA and weaker fluorescence in IFA compared to the other two positive sera. After establishment and optimization, the c-ELISA could efficaciously distinguish the positive sera (two strong positive sera and one weak positive serum) from

Table 5 Agreement of the c-ELISA with indirect ELISA

Sera number	c-ELISA	Indirect ELISA		Agreement[a]	Kappa value
		Positive	Negative		
60	Positive	48 (A)	12 (B)	90.98%	0.815
73	Negative	0 (C)	73 (D)		

[a] Agreement = (A + D) / (A + B + C + D) × 100%

Table 4 Repeatability of the c-ELISA

Sera samples	Intra-batch			Inter-batch		
	X[a] (%)	SD[b] (%)	CV[c] (%)	X (%)	SD (%)	CV (%)
Strong positive 1	84.98	1.17	1.38	88.82	0.62	0.7
Strong positive 2	83.54	1.51	1.81	87.11	0.8	0.9
Weak positive	64.89	2.41	3.71	90.96	0.58	0.64
Negative 1	11.51	0.65	5.65	1.37	0.09	6.57
Negative 2	0	0	0	0	0	0
Negative 3	0	0	0	0	0	0

[a] X means mean PI value; [b] SD means standard deviation; [c] CV means coefficient of variation

the negative sera, indicating the potential of the c-ELISA to be a capable way for the detection of antibodies against DAdV-3.

Consequently, we assessed the analytical performance of the c-ELISA. The results of c-ELISA were highly accordant with those of IFA. Notably, the c-ELISA could efficiently detect the sera from the Muscovy ducks at the early stage of infection, contributing to the control of the disease. Moreover, the c-ELISA did not cross react with other positive sera tested in the study. The minimal cross-reactivity of the c-ELISA decreased the incidence of false-positive reactions. Repeatability was another important index for the reliability of the c-ELISA. In our study, the CVs of intra-batch and inter-batch assays were all below 10%. To further validate the reliability, a comparison between the c-ELISA and indirect ELISA was performed based on 133 duck sera. The CR of the two methods was 90.98% (121/133). Further statistical analysis revealed that the Kappa value was determined as 0.815, which indicated that the results of the two assays were highly consistent. The high CR and the almost perfect agreement between the two assays highlighted the accuracy and reliability of the c-ELISA, and the c-ELISA had the potential to be an alternative method for detecting the antibodies against DAdV-3. To date, some c-ELISA methods have been used for the serological detection of avian pathogens due to the minimal cross-reactivity and independence of species-specific secondary antibody [13–19]. They provided effective tools for the epidemiologic surveillance and vaccine evaluation, which contributes to the control of avian diseases.

In summary, a mAb-based c-ELISA was developed for the detection of antibodies against DAdV-3. The c-ELISA has no cross-reactivity with other positive sera tested, and the results of c-ELISA are highly concordant with those of IFA. The size of tested samples in the study is not large enough, and further validation with more diverse and larger datasets is needed in future studies. The c-ELISA is a promising serological diagnostic tool for large-scale surveillance of DAdV-3 in ducks and can be performed under the field diagnostic setting for the mass screening of serum samples. Moreover, if the Fiber-2 subunit vaccine or the inactivated vaccine for the prevention of DAdV-3 is available in the future, the c-ELISA will provide an effective tool for evaluating the level of antibody against Fiber-2 after vaccine immunization.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
JY and AQ designed the project. YL carried out the experiments. YL, YN, WZ, WW, SW, HS and ZW analyzed the data. YL, QX and TL drafted the manuscript. JY and AQ discussed and prepared the final report. All of the authors have read and approved the final manuscript.

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Data availability
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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