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Development of a DAS-ELISA for detection of H9N2 avian influenza virus

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ABSTRACT

H9N2 avian influenza virus is threatening animals and public health systems. Effective diagnosis is imperative to control the disease. Thus, we developed a panel of monoclonal antibodies (Mabs) against the H9N2 avian influenza virus (AIV) and implemented a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect the H9 viral antigen. Hybridomas 4D10 and 5G2 were screened to secrete immunoglobulin G (IgG) and IgA, respectively. Antibody 4D10 was used as the capture antibodies and HRP labeled 5G2 as the detector antibody. The specificity of the optimized DAS-ELISA was evaluated by using AIV subtypes H1, H3, H5, H9 and H10. Specimens containing AIV H9 subtype yielded a specific and strong signal above the background, whereas specimens containing all other subtypes yielded background signals. The detection limit of the DAS-ELISA is $10^{-2.3}$ $TCID_{50}$ (50% Tissue culture infective doses). Negative-positive threshold was 0.211 (OD630). In comparison with virus isolation the sensitivity and specificity of DAS-ELISA were found to be 98.9% and 98.1% respectively. Taken together, the newly developed Mab-based DAS-ELISA offers an attractive alternative to other diagnostic approaches for the specific detection of H9 subtype AIV.

1. Introduction

Avian Influenza (AI) H9N2 subtype was first reported to infect turkeys in the United States in 1966 (Homme and Easterday, 1970) and has widely circulated in Europe and Asia (Guo et al., 2000; Xu et al., 2012). H9N2 subtype viruses generally exist as low pathogenicity influenza viruses causing mild to moderate disease. However, they have been associated with severe morbidity and mortality in poultry as a result of co-infection with other pathogens (Brown et al., 2005; Nili and Asasi, 2002).

In 1994, H9N2 virus was isolated from diseased chickens in Guangdong province, China and later in domestic poultry in other provinces in China (Guo et al., 2000; Li et al., 2003; Liu et al., 2003; Xu et al., 2007).

The H9N2 viruses have also attracted great concern due to their possible avian-to-human transmission (Cong et al., 2007; Butt et al., 2005; Yu et al., 2011; Butt et al., 2010). Since the late 1990s, 12 cases of human H9N2 infection have been identified (Kimble et al., 2011). In addition, H9N2 viruses have been isolated sporadically but consistently from pigs in Hong Kong, China and South Korea (Maines et al., 2008; Peiris et al., 2001, 1999).

Given the great economic losses in poultry and the potential threat to human health, it is important for developing accurate and prompt diagnosis of H9N2 infection in birds. Currently, the “golden standard” for the diagnosis of H9N2 influenza is virus isolation (VI) in embryonating chicken eggs which is complicated and time-consuming. Another traditional method is the serological methods which includes Hemagglutination test (HA), Hemagglutination inhibition test (HI) and Immunity fluorescence (IF). In addition, molecular detection methods, such as RT-PCR, real-time PCR assays and a DNA microarray analysis for the detection of influenza virus have been developed (Boivin et al., 2001; Kaiser et al., 1999; Li et al., 2001). However, these methods are only suitable for laboratory diagnosis, but not practical in clinic practice, due to constraints such as accurate instruments, requirements for highly trained personnel, and time-intensive procedures. A rapid, simple and convenient method is a pressing need.

In this study, a DAS-ELISA which used a specific monoclonal antibody directed against the hemagglutinin of H9 subtype AIV, was developed and utilized to capture the specific antigens. The assay has undergone extensive field evaluation and has been found to be suitable for the detection of H9 subtypes influenza virus and the method showed a good specificity and sensitivity.

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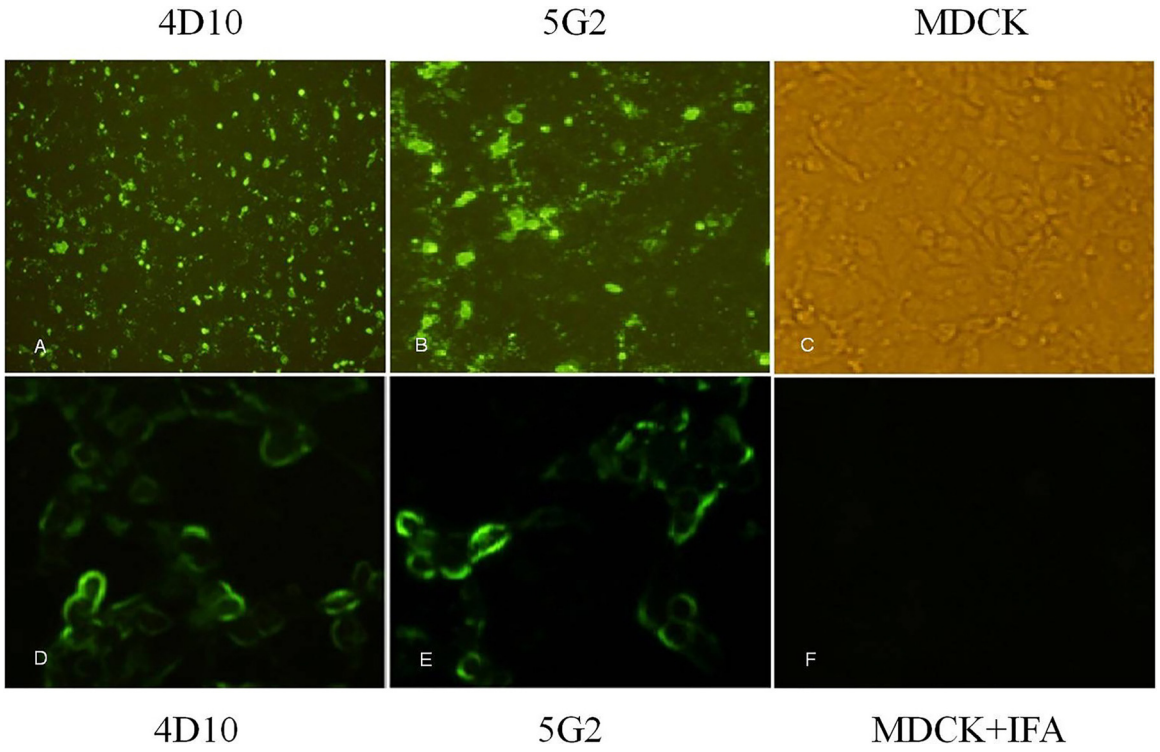

Fig. 1. Monoclonal antibodies raised against H9N2 virus detect H9N2 AIV by immunofluorescence. MDCK cells grown in 96-well plates to a confluence of 80% were infected with 100 HA units of H9N2 AIV. Antibodies 4D10, 5G2 and anti-H9N2 serum recognized the HA in H9N2 infected MDCK cells which showed the fluorescent signal. Control was PBS infected MDCK cells at 36 h post-infection, the cells were fixed and subjected to IFA.

Table 1
Coating antibody and HRP labelled antibody matching results.

		Virus antigen sample					
		Positive sample			Negative sample		
Coating antibody	4D10	2.351	2.323	2.672	0.132	0.094	0.083
		1.454	0.766	2.225	0.071	0.125	0.092
	5G2	2.641	1.806	2.445	0.106	0.124	0.126
		0.989	0.644	2.067	0.085	0.121	0.069

Twelve viral antigens were added (6 positive, 6 negative). The OD at 630 nm (OD630) was measured and recorded with an automated plate spectrophotometer. The optimal antibody pairing concentration was determined to be those when the greatest ratio of OD630 nm values between the positive and the negative antibody (P/N) were obtained.

2. Materials and methods

2.1. Preparation and purification of virus stock

All the influenza strains used in this study were : A/chicken/Tibet/S1/2009 (H9N2), A/chicken/Tibet/S2/2009 (H9N2), A/duck/Hubei/W1/2004 (H9N2), A/swine/Tianjin/3/04 (H1N1), A/swine/shanghai/6/03 (H3N2), A/chicken/Hubei/327/2004 (H5N1), A/swine/Hubei/10/2008 (H10N5).

Some other avian viruses were used in this study, and these pathogens were as follows: Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), infectious bursal disease virus (IBDV), egg drop syndrome-76 virus (EDSV).

Virus strain A/chicken/Tibet/S1/2009 (H9N2) were propagated in 9-day-old embryonated chicken eggs and the allantoic fluid was collected and purified by ultracentrifugation at 100,000 *g* for 2 h (Sala et al., 2003), and the concentrated virus pellet were resuspended in PBS and stored at −80 °C.

Table 2
Relative diagnostic sensitivity and specificity of DAS-ELISA and virus isolation.

		Virus isolation		
		Positive	Negative	Total
DAS-ELISA	Positive	435	4	439
	Negative	5	205	210
	Total	440	209	649

649 identical clinical samples were texted by DAS-ELISA and virus isolation parallelly. The inter-rater agreement was 98.4% (435 + 205 = 640,640/649 = 98.6%). Diagnostic sensitivity is 435/440 = 98.9%. Diagnostic specificity is 205/ 209 = 98.1%.

Table 3
Specificity test of the DAS-ELISA on different subtypes influenza virus and other pathogens.

HA subtypes of influenza A virus	H1	H3	H5	H10	H9(all clades)
OD value	0.059	0.045	0.048	0.058	1.223
Other pathogens	NDV	IBV	IBDV	ILTV	EDSV
OD value	0.142	0.143	0.122	0.136	0.145

The results showed that the DAS-ELISA had no cross reaction with other AIV subtypes or pathogeny.

MDCK cells were seeded in flat bottom 96-well plates with DMEM supplemented with 0.5% bovine serum albumin and 1ug/ml of trypsin for $TCID_{50}$ determinations. MDCK cells were incubated with A/chicken/Tibet/S1/2009 (H9N2) for 48 h and then the virus was collected from the cell-free supernatants.

2.2. Monoclonal antibody (Mab) against HA of H9N2 avian influenza virus

Four-week old BALB/c mice were immunized at 2-week intervals with purified inactive H9N2 avian influenza virus for three times. The

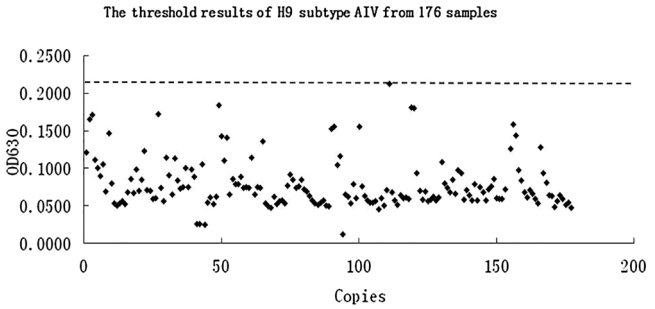

Fig. 2. According to the formula Critical value for $\overline{X}$ +3SD = 0.0815 + 3 × 0.043 = 0.21. The OD630 value ≤ 0.21was negative, OD630 value > 0.21 was positive.

amount of antigen is 90 μg for each time. Antigen with equal volume of Freund's complete adjuvant were injected for the first immunization. The mice were immunized with the same amount antigen with Freund's incomplete adjuvant 14 days after the first immunization. Antigen without adjuvant was injected intravenously in the third immunization. Mice were exsanguinated 5 days after the third inoculation and the serum were collected (mice anti-AIV H9 serum). The collected serum was purified and stored at −80 °C. Splenocytes were collected synchronously and fused to SP/2.0 myeloma cells. Hybridoma culture supernatants were screened by Hemagglutination inhibition (HI) assay and ELISA. Antigen used for HI assay was A/chicken/Tibet/S1/2009 virus (the HA titer of virus was 4 units), antigen used for ELISA assay was inactived A/chicken/Tibet/S1/2009 virus. The positive hybridoma cultures were cloned by two cycles of cloning in limiting dilution. Isotyping was performed by using a mouse Mab isotyping kit. We chose two monoclonal antibodies with high HI activity and specificity, namely 4D10 (IgG) and 5G2 (IgA), to develop the DAS-ELISA.

To produce large quantities of Mab, the hybrid cell line was grown as an ascitic tumor in Balb/c mice (Goding, 1980), and the mouse ascitic fluids was purified according to the method of McKinney and Parkinson (McKinney, 1987).

2.3. Hemagglutination inhibition test

The neutralization activity of antibodies was identified by hemagglutination inhibition assays (Wang et al., 2000; Webster et al., 1999). Antibodies were diluted serially in V-bottom 96-well plates. Inactived virus (the HA titer of virus was 4 units) was incubated with the antibodies for 30 min at room temperature, followed by the addition of 1% RBCs and incubation at room temperature for 40 min. The inhibition of hemagglutination at the highest antibody dilution was considered the HI titer of the antibody solution.

2.4. Immunofluorescence assay

MDCK cells were seeded in 96-well plates and incubated with H9N2 virus for 36 h. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature 36 h post-infection. All washes were performed with PBS, pH 7.4. Fixed cells were incubated with hybridoma culture fluid at 37 °C for 1 h, washed, and incubated with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig. Cells were washed and fluorescence visualized using on Olympus IX71 microscope with appropriate barrier and excitation filters.

Table 5
Comparison of the text results of DAS-ELISA and RT-PCR.

		DAS-ELISA		
		Positive	Negative	Total
RT-PCR	Positive	33	2	35
	Negative	0	25	25
	Total	33	27	60

60 samples of chickens previously by DAS-ELISA assay were tested by RT-PCR. In comparison with DAS-ELISA, RT-PCR had positive coincidence rate of 94.3% (33/35), negative coincidence rate of 100% (25/25).

2.5. Preparation of samples

As described previously (Zhang et al., 2006), all tissue samples were homogenized to give a 50% suspension (w/v) in sample buffer (PBS, pH7.2, containing 0.5% Triton X-100, penicillin G 5000 IU/mL and streptomycin 5 mg/mL). The suspensions were repeated thawing three times and centrifuged at 3000 *g* for 10 min, the supernatants were incubated for 15 min at 37 °C before application to DAS-ELISA. Tracheal and cloacal swabs of chickens were collected in 1 mL of sample buffer (PBS, pH7.2, containing 0.5% Triton X-100, penicillin G 5000 IU/mL and streptomycin 5 mg/mL) and incubated for 15 min at 37 °C before testing.

2.6. Procedures for DAS-ELISA

Microtiter plates were coated with 100 μL of capture antibody diluted in carbonate-bicarbonate coating buffer (pH9.6) for 1 h at 37 °C, then overnight at 4 °C. After incubation, plates were washed three times with washing buffer (0.01 M PBS containing 0.05% Tween-20, 200 μL/well), and then blocked with blocking buffer (5% nonfat dried milk +5%D-Trehalose, 200 μL/well) for 1 h at 37 °C. After washing, 100 μL of clinical samples prepared in PBS were added and incubated for 30 min at 37 °C. After incubation, the plates were washed with washing buffer, then the HRP labeled mouse secondary antibody (5G2) were added (100 μL/well) and the plates were incubated for 1 h at 37 °C. (The antibody was labeled with HRP through sodium periodate method.) After washing, substrate solution A (0.06%H_2O_2) and substrate solution B (1 mg/mL TMB) were added in each well (50 μL/well) and color reaction was developed for 10 min followed by stopping buffer with equal volume of 0.25% HF. The plates were read with the ELISA reader at 630 nm.

2.7. The determination of cut-off level

176 cloacal swabs were collected from chicken farms, and the samples were inoculated into chicken embryo for three passages, the result was negative, these samples were measured at 630 nm in the ELISA reader. The critical value was figured out by the formula of $\overline{X}$ + 3SD ("$\overline{X}$" represents the mean value of OD value of 176 samples, "3SD" represents three standard deviations).

Table 4
Sensitivity test of the DAS-ELISA on H9N2 virus detection.

HA titer	2^8	2^7	2^6	2^5	2^4	2^3	2^2	2^1	2^0
OD value	2.5010	2.3420	1.8510	1.3030	0.6820	0.4270	0.3020	0.2110	0.1350
$TCID_{50}$	$10^{-3.2}$						$10^{-2.3}$		

Through the sensitivity test, the results showed that the present method minimum detectable virus content was $10^{-2.3}$ $TCID_{50}$.

Table 6
The text results of DAS-ELISA on different tissues.

Tissue(chicken)	Heart	Liver	Spleen	Lung	Kidney	Brain	Muscle	Trachea	Cloaca
Total	50	50	50	50	50	50	50	50	50
Positive	35	43	46	50	48	10	30	25	8
Positive rate (%)	70	86	92	100	96	20	60	50	16

The positive rate of spleens, lungs and kidneys from H9N2-infected chickens was relatively high, and especially positive rate of the lungs was up to 100%. But the positive rate of such samples as brain and cloaca was very low (20%, 16%) by DAS-ELISA.

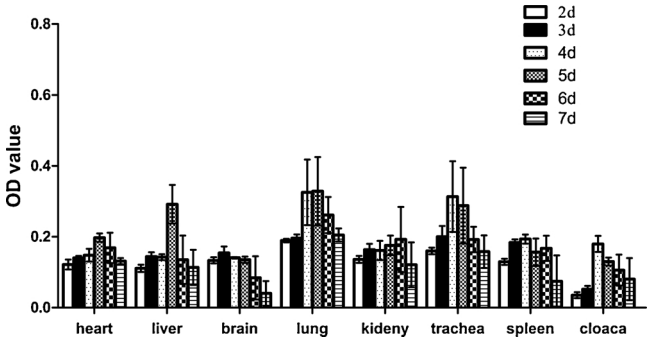

Fig. 3. The chickens were inoculated intramuscularly with H9N2 virus at dose of 0.5 mL $10^{-3.2}$ $TCID_{50}$. Tissue virus content of chicken infected by H9N2 AIV first high to low, rose significantly in 3–5 days, especially the virus of lungs and tracheal were relatively high in all detected tissues and organs, but the virus of brains was hardly detected.

2.8. Virus isolation (VI) in embryonated chicken eggs

Isolation of influenza virus was performed by inoculating the allantoic cavity of 9-day-old embryonated chicken eggs with 0.2 mL tissue suspensions or swab suspensions. Antibiotics were added to each sample to the following final concentrations: penicillin G, 5000 IU/mL and streptomycin, 5 mg/mL. The eggs were incubated for 4 days and candled daily for viability, embryos that died within 24 h of inoculation were discarded as nonspecific. Allantoic fluid from dead and surviving embryos was tested for the presence of HA activity. Samples that yielded no hemagglutination were passaged a second time (Allan and McNulty, 1985; Spackman et al., 2002).

2.9. Comparison with VI in embryonated chicken eggs

649 chicken samples were obtained from AIV-endemic areas during H9N2 avian influenza virus incident in Shandong province in 2012. The samples included heart, liver, spleen, lung, kideny, brain, tracheal and cloacal. All these samples were tested by VI and DAS-ELISA.

2.10. Comparison of DAS-ELISA and RT-PCR

60 cloacal swabs of chickens with unclear background from Hubei province of China were tested by DAS-ELISA and real time RT-PCR Comparison. The specific primers used in this study are P1: 5′-TCAAC AAACTCCACCGAAACTGT-3′, P2: 5′-TCCCGTAAGAACATGTCCATA CCA-3′. PCR was initiated by denaturation at 95 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 35 s and 72 °C for 2 min. The amplified products were further elongated at 72 °C for 10 min. PCR products were also identified by sequence.

2.11. Experimental infection of chickens

40 15-day SPF chickens were purchased from a commercial herd and the serum samples of chickens were collected for the HI assay, the results were negative. Four domestic chickens were randomly assigned as control group, the rest of the chickens as experimental group and were acclimated to isolaters biosafety level 3 housing for 1 week. Tracheal and cloacal swabs were collected from all chickens. The chickens of experimental group were then inoculated intramuscularly with influenza A/chicken/Tibet/S1/2009 (H9N2) virus at 0.5 mL $10^{-3.2}$ $TCID_{50}$ respectively. The uninfected controls chickens were mock inoculated with PBS. The tracheal and cloacal swabs from each live chicken were obtained from 1 to 7 days post inoculation. 4 samples of hearts, livers, spleens, lungs, kidneys, cloacas, tracheas, muscles and brains were collected from 2 to 7 days post inoculation, as well as the mock. All the experimental protocols were approved by the Laboratory Animal Monitoring Committee of Hubei province of China and performed accordingly.

2.12. Application of DAS-ELISA in clinical samples

The samples were collected from chickens suspected to be infected with AIV, these chickens were from Wuhan, Hebei and Fujian in 2012. Various viscera including hearts, livers, spleens, lungs and kidneys at a total of 258 were conducted to be tested by DAS-ELISA.

3. Results

3.1. Development of the antibodies

Immunofluorescence was used to screen the antibodies by infected MDCK cells with H9N2 virus. We screened two monoclonal antibodies, named 4D10 (IgG) and 5G2 (IgA), against H9N2 antigen (Fig. 1). The HI activity of 4D10 and 5G2 were 1:32 and 1:64 respectively.

3.2. Coating antibody and enzyme labelled antibody matching results

For select coating antibody and enzyme antibody in DAS-ELISA, the 4D10 and 5G2 were purified from mouse ascites were determined by antibody matching assay. Choosing 4D10 as coating antibody, the mean value of positive is 1.9652 (P1), the negative mean value is 0.0995 (N1), the value of P1/N1 is 19.7508, while chose 5G2 as coating antibody, the positive mean value is 1.7653 (P2) and the negative mean value is 0.1052 (N2), P2/N2 is 16.7804. We discovered that P1/N1 > P2/N2, so the 4D10 was selected as coating antibody (Table 1), and enzyme labelled antibody 5G2 was used as secondary antibody.

Checkerboard titration was used to determine the optimal concentration of coating antibody and the optimal dilution of HRP labelled secondary antibody. The results of tests showed that the optimal concentration of coating antibody was 2 μg/mL and the optimal dilution of HRP labelled secondary antibody was 1:400 (data not shown).

3.3. Analysis of sensitivity and specificity

To assess the diagnostic efficacy of the DAS-ELISA, 649 clinical samples including heart, liver, spleen, lung, kideny, brain, tracheal and cloacal were tested in parallel with the virus isolation. Among the 649 clinical samples, 200 samples were from AIV H9 suspected cases in field. Efficacy of the DAS-ELISA was compared with the virus isolation (VI) employing all the 649 clinical samples. Both the DAS-ELISA and VI identied 435 positive samples and 205 negative samples of the 649

clinical samples. The inter-rater agreement was 98.6% (435 + 205 = 640, 640/649 = 98.6%). The DAS-ELISA was found to be 98.1% (205/209 = 98.1%) specificity and 98.9% (435/440 = 98.9%) sensitivity as compared to virus isolation (Table 2), and it had no cross reaction with other AIV subtypes or pathogeny (Table 3).

Cut-off level (OD630 = 0.21) was calculated as three standard deviations above the mean optical density value (OD630 = 0.0815 + 0.129) obtained from 176 samples without antigen (Fig. 2). The samples were cloacal swabs collected from chicken farms, and they were detected to be negative of AIV antigen by VI assay.

The ability of the DAS-ELISA to detect a broad palette of AIV strains was tested on 5 AIV strains of different subtypes and some other pathogens (NDV, IBV, ILTV, IBDV, EDSV). Only all H9 subtype AIV strains were positive, whereas all other subtypes and other pathogens were tested negative (Table 3).

A/chicken/Tibet/S1/2009 viral antigen (the HA titer of the virus was 4 units) were diluted serially in ELISA Plate to evaluate the sensitivity of the developed DAS-ELAISA. The least detectable amount of viral proteins was approximately 1.6 ng/mL for influenza A/chicken/Tibet/S1/2009 (H9N2). The concentration of viral protein was measured by Bradford assay. In the parallel test, $TCID_{50}$ determination was performed with the viral antigens at the dilution of 2^8 HA titer and 2^2 HA titer (the least detectable amount) by using MDCK cell, the results were $10^{-3.2}$ and $10^{-2.3}$ respectively (Table 4).

3.4. Evaluation of the DAS-ELISA

3.4.1. Comparison of DAS-ELISA and RT-PCR

To compare DAS-ELISA with RT-PCR, 60 samples of chickens were tested by DAS-ELISA assay and RT-PCR simultaneously. In comparison with DAS-ELISA, RT-PCR had positive coincidence rate of 94.3% (33/35), negative coincidence rate of 100% (25/25) (Table 5).

3.4.2. Detection of experimentally infected chickens

In the artificial infection experiment, 40 15-day chickens inoculated with the amount of virus in 0.5 mL $10^{-3.2}$ $TCID_{50}$ by chest muscle injection. The chickens showed mild symptoms accompanied with clinical signs of influenza after 72 h and pathological lesions upon necropsy throughout the experiment. This DAS-ELISA was used to detect H9 subtype AIV in tissue samples of experimentally dead chickens and swabs samples. The positive rate of spleens, lungs, kidneys from H9N2-infected chickens was relatively high, and especially the positive rate of the lungs was up to 100%. But the positive rate of the samples as brain and cloaca was very low (20%, 16%) (Table 6). Some chickens infected with H9N2 AIV only showed trivial clinical signs and none of them died, but the DAS-ELISA was able to detect H9 virus from swabs of them from 1 to 3 days. The RT-PCR was unable to detect AIV H9 subtype virus of swabs from 3 to 7 days. Notably, the infected chickens shed more virus from 3 to 5 days post inoculation (Fig. 3).

3.4.3. Application of DAS-ELISA in clinical testing

In the clinical test, 258 chicken samples collected from various regions were tested by DAS-ELISA. The results showed that 3 samples were positive, and the positive rate was 1.16% (3/258).

4. Discussion

Avian influenza is considered a severe threat to human and animal health. AIV subtyping is based on antigenic variations in HA and NA proteins (Fouchier et al., 2005). Serological assays available for detection of AIV antigen are the hemagglutination assay and ELISA. The HI test, by using reference antisera, is a subtype specific test. But antibodies against the NA can interfere with the HI, leading to nonspecific inhibition and possible misidentification of an isolate (Shien et al., 2008). Due to their simplicity, high sensitivity, and economy, ELISA has been recognized as a suitable assay for diagnosis and seroepidemiological surveillance (Marché and Van Den Berg, 2010). The specificity and sensitivity of the assay for routine monitoring of AIV H9 antibodies were evaluated by HI and a commercial AIV ELISA kit.

In this test, H9N2 influenza viruses isolated from Tibet disease chickens were taken as antigen to immunized mice. Using serological methods, we got two monoclonal antibodies which were specific and high titer. Taken these antibodies as raw material, we got a DAS-ELISA method to test H9N2 subtypes of influenza virus, and it turned out to be highly sensitive, specific, convenient, rapid and cost effective.

This method was used to detect other types of flu viruses such as H1 subtypes, H3 subtypes, H5 subtypes and H10 subtypes of influenza virus. The results were negative. And we used the method to detect the main virus of livestock and poultry such as NDV, IBDV and EDSV, the results were negative. These results indicated that this method was specific.

The 4D10 monoclonal antibody is specific to H9 subtype virus and not cross-reactive to other subtypes of influenza. Though RT-PCR has been successfully applied to the detection of AIV in infected poultry previously (Chen et al., 2007; Dybkær et al., 2003), DAS-ELISA lends itself to incorporation into a convenient dipstick assay/lateral flow assay. In this work we have developed an DAS-ELISA that utilized H9N2-specific Mabs 4D10 and 5G2 of isotypes IgG and IgA, respectively. In the DAS-ELISA, the sensitivity of antibody 5G2 was below 4D10 (Table 1), while 4D10 showed HI activity. Control assays with non-H7 subtype avian influenzas revealed that our DAS-ELISA was specific for the H9 subtype and thus would be useful for diagnosis and potentially for incorporation into a dipstick assay kit for rapid and convenient H9N2 virus detection. Our DAS-ELISA can detect avain influenza virus in tracheal samples as early as 3 days post-infection until approximately a week post-infection. This was 2 days earlier than a previous study of H9N2 AIV that used a commercially available type A antigen-based AC-ELISA (Chen et al., 2007). This improved sensitivity may be the result of optimization for H9N2 detection, while the previous study utilized a broad-spectrum AC-ELISA. Antibody 4D10 was proved a little more sensitive than antibody 5G2 in the DAS-ELISA. Thus, antibody 4D10 can be developed to make a difference in H9N2 AIV surveillance.

Declaration of interest

The authors declared no conflict of interest.

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