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Development of a blocking latex agglutination test for the detection of antibodies to chicken anemia virus

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A B S T R A C T

A blocking latex agglutination test (b-LAT) developed in this study was evaluated for the detection of antibodies against chicken anemia virus (CAV) in chickens. Polystyrene latex beads were coupled with a neutralizing monoclonal antibody (mAb) to CAV (mAb-beads). When mAb-beads were mixed with antigens prepared from the lysate of MDCC-MSB1 cells infected with CAV, agglutination occurred. A short pre-incubation of CAV antigens with CAV-specific antiserum inhibited the agglutination of mAb-beads. The test results were obtained within 5 min. The specificity of b-LAT was evaluated using sera from specific pathogen-free chickens and sera containing antibodies to avian influenza virus, Newcastle disease virus, infectious bursal disease virus, and Marek's disease virus; nonspecific agglutination and cross-reactivity with antibodies to unrelated viruses were not observed. The examination of 94 serum samples collected from commercial breeder chickens of various ages (17–63 weeks) revealed good agreement (93.6%, Kappa value = 0.82) between b-LAT and a virus neutralization test, known to be most sensitive and specific in the detection of antibodies to CAV. These results indicate that b-LAT, a simple and rapid test, is a useful and reliable tool in CAV serology.

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1. Introduction

Chicken anemia virus (CAV), first reported by Yuasa et al. (1979), belongs to the genus *Gyrovirus* of the family *Circoviridae*. The absence of antigenic differences among CAV strains of different origin suggests that these strains belong to a single serotype (McNulty et al., 1990; Yuasa and Imai, 1986).

Clinical disease is associated with increased mortality, severe anemia, growth retardation, intramuscular and subcutaneous hemorrhage, whitish-yellow or pink bone marrow, and severe thymic atrophy in young chicks (Taniguchi et al., 1983; Yuasa et al., 1979). In chickens of more than 2 weeks of age, however, CAV usually causes subclinical disease (Yuasa and Imai, 1986), which likely leads to immunosuppression resulting in enhanced susceptibility to other avian pathogens as well as reduction in response to vaccines (Adair, 2000).

CAV is known to be ubiquitous among chicken flocks throughout the world (Schat and van Santen, 2008). Usually field flocks become infected with CAV horizontally after diminishing maternal antibodies at about 3 weeks of age without showing any clinical signs. This is followed by seroconversion in most breeder chicken flocks from 8 to 12 weeks of age (McNulty et al., 1988). However, the exposure of antibody-negative breeder flocks to CAV during the laying period results in vertical transmission of the virus, and causes severe disease in the progeny (Chettle et al., 1989; Hoop, 1993; Yuasa et al., 1987). Therefore, vaccination of breeder flocks that fail to show seroconversion should be considered prior to the collection of eggs. Even specific pathogen-free (SPF) chicken flocks reared under very strict hygienic conditions have been reported to become infected with CAV (Cardona et al., 2000; McNulty et al., 1989; Yuasa et al., 1985). Therefore, ensuring that SPF chicken flocks that supply eggs for vaccine production are free of CAV infection is

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extremely important (Todd et al., 1990), in turn highlighting the importance of monitoring seroconversion in commercial and SPF breeder flocks.

Currently, three serological tests are routinely available for the detection of antibodies to CAV: an enzyme-linked immunosorbent assay (ELISA), an indirect fluorescent antibody test (IFAT), and a virus neutralization test (VNT) (Todd et al., 1990, 1999; von Bülow et al., 1985; Yuasa et al., 1983b, 1985). Of these, VNT has the highest sensitivity and specificity; however, the test is time-consuming and laborious. In contrast, IFAT is a relatively simple test to perform, but requires experienced personnel and frequently yields false-positive results, particularly when sera are tested at lower dilutions (Otaki et al., 1991). Both VNT and IFAT are unsuitable for testing a large number of samples. In contrast, the ELISA is well-suited for the routine screening of large numbers of samples (Lamichhane et al., 1992; Tannock et al., 2003; Todd et al., 1990, 1999), but requires approximately 2.5–5 h following the overnight incubation of antigens or a monoclonal antibody (mAb) for its completion. Moreover, the ELISA necessitates the purification or semi-purification of antigens from infected cells. Compared to the IFAT, higher rates of false-positive reactions were obtained with certain commercial ELISA kits (Michalski et al., 1996; Tannock et al., 2003). Therefore, the development of a reliable, simple, and rapid test for the detection of antibodies to CAV is of utmost importance.

In this study, the development of a highly sensitive and specific blocking latex agglutination test (b-LAT) for the detection of antibodies to CAV in chickens is reported. The test is based on the ability of CAV-specific antibodies present in the test sera to block the binding of CAV antigens. The CAV antigens were prepared from infected cell lysates and coupled with latex beads using mAb against CAV. The results were scored as antibody-positive (no agglutination of mAb-beads) or antibody-negative (agglutination observed).

2. Materials and methods

2.1. Cell culture and virus

MDCC-MSB1 (MSB1) cells were cultured in the growth medium (GM) RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS) and 10% Daigo's GF21 growth factor (Wako Junyaku, Osaka, Japan), as described previously (Imai et al., 1993).

The CAV strain A2/76 (Yuasa and Imai, 1986) was used in the current study. Viral titers were determined by a micro-test method, as described by Imai and Yuasa (1990).

2.2. Indirect fluorescent antibody test (IFAT)

For the detection of antibodies to CAV, IFAT was performed using CAV-infected MSB1 cells, as detailed in Yuasa et al. (1985). In brief, the infected cells were harvested at 36 h post-infection, and were smeared on to a microscope slide, dried, and fixed with cold acetone for 10 min. The slides were incubated with chicken serum and then with FITC-conjugated rabbit anti-chicken IgG (Rockland, Gilbertsville, PA) at 37 °C for 30 min each, followed by observation under a fluorescence microscope (Biorevo BZ-9000, Keyence, Japan) for the presence of fluorescent signals.

2.3. Preparation of CAV antigens

MSB1 cells infected with the CAV strain A2/76 were prepared as described previously (Yuasa et al., 1985), with slight modifications. In brief, 2 ml CAV suspension (titer of approximately 10^7 $TCID_{50}$/ml) was mixed with 10^7 MSB1 cells, incubated at 39.5 °C for 1 h, and suspended in GM at a concentration of 10^6 cells/ml.

Infected cells that showed a cytopathic effect (enlargement of the infected cells) were harvested at 72 h post-infection by centrifugation at 1500 × *g* for 5 min, and washed with phosphate buffered saline (PBS, pH 7.4). The supernatant was then removed, and the cells were resuspended in 1 ml of PBS, followed by three freeze-thaw cycles. The cell lysate was subjected to sonication, and then to centrifugation at 10,000 × *g* for 15 min. The supernatant containing the CAV antigens was collected, and the pellet of cellular debris was discarded. The titer of CAV in the antigen solution was approximately $10^{9.5}$ $TCID_{50}$/ml. The cell lysates from uninfected MSB-1 cells were prepared as negative antigens in the same manner. The infected and uninfected cell lysate antigens were stored at −30 °C until use.

2.4. Monoclonal antibody (mAb)

A neutralizing mAb (MoCAV/F11, IgG2b) generated by our research group (Trinh et al., 2015) was used in the current study. Ascites fluid containing MoCAV/F11 was obtained following the intraperitoneal injection of approximately 10^7 hybridoma cells into a BALB/c mouse that had been primed with incomplete Freund's adjuvant, as described previously (Harlow and Lane, 1988). The mAb was semi-purified using 50% saturated aluminum sulfate. Protein concentration of the semi-purified mAb IgG, which was determined by Lowry method (Lowry et al., 1951), was 6.9 mg/ml.

2.5. Sensitization of latex beads

Polystyrene latex beads (1.0 μm; Polysciences, Inc., Warrington, PA) were coupled with mAb in accordance with the manufacturer's instructions. In brief, 0.5 ml of a 2.6% (w/v) suspension of the beads was coupled with 400 μg of mAb IgG. The coupled beads (mAb-beads) were blocked with 1.0 ml of bovine serum albumin (BSA, 10 mg/ml) for 30 min at room temperature. Then the mAb-beads were suspended in 1 ml of storage buffer (1% BSA, 5% glycerol, and 0.1% sodium azide in PBS), and stored at 4 °C until use.

2.6. Blocking latex agglutination test (b-LAT)

First, 2-fold serial dilutions of CAV antigens in PBS were incubated with mAb-beads for determining the highest dilution that permitted complete agglutination (1 unit). In this study, 8 units contained in 5-μl volume (the viral titer: approximately $10^{6.6}$ $TCID_{50}$) were used in a single test of b-LAT.

The final b-LAT protocol was as follows: Initially, a mixture containing 5 μl of CAV antigens and an equal volume of chicken serum was incubated at room temperature for 15 min. Subsequently, 5 μl of mAb-beads were mixed on a plastic surface with an equal volume of the CAV antigens and chicken serum mixture. This was then spread out circular with a diameter of approximately 1 cm, followed by gentle agitation for 5 min. The results were scored as antibody-positive (no agglutination of mAb-beads) or antibody-negative (agglutination observed).

2.7. Serum samples

A total of 94 serum samples from chickens, collected from 4 different layer breeder flocks in different areas of Japan, were used for the comparative evaluation of b-LAT, VNT, and IFAT (Tables 1 and 3a). The characteristics of these flocks and the results of antibody detection in these serum samples using VNT and IFAT have been previously reported (Imai et al., 1993). In brief, sera were collected at a single time point from flocks 2, 3, and 4, and periodically collected from the same individual chickens of flock 1 at 19, 52, and 63 weeks of age.

Table 1
Field chicken serum samples used in this study.

Serum samples			Remarks
Source	Age at sampling	No. of samples	
Sera from 4 breeder farms without CAV problems in Japan			
Flock 1	19, 52, and 63 weeks[a]	10, 19, 19	No outbreak of CAV-induced disease in the progeny of breeders
Flock 2	25 weeks	18	
Flock 3	17 weeks	10	
Flock 4	48 weeks	18	
Sera from 2 breeder farms with CAV problems in Japan			
Farm 1			
Flock A	103 and 270 days	10 each	CAV outbreak in the progeny of breeders at 240 days of age
Flock B	116, 180, and 259 days	10 each	CAV outbreak in the progeny of breeders at 218 days of age
Farm 2			
Flock C[b]	240 and 481 days	10 each	Vaccination in breeders at 70 days of age. Sampling after CAV outbreak in the progeny[c]
Sera in Vietnam			
Chicken flock	4–6 weeks	74	Sampling from 16 broiler chicken flocks in 2 provinces (Hanoi and Hanam)
LBM[d]	>8 weeks	237	Sampling from 4 LBMs in Hanoi

[a] Sera were periodically collected from the same individual chickens of Flock 1.
Detailed information about the flocks was described previously (Imai et al., 1993).
[b] Flock C contained 3 groups of chickens with different ages, and sampling was conducted in 2 of them.
[c] It was not identified which age group was responsible for vertical transmission to the progeny.
[d] LBM: Live bird market.

CAV-induced disease was observed among the progeny of breeder chickens in a broiler breeder farm (designated as Farm 1) and a layer breeder farm (designated as Farm 2), which are located in different areas of Japan. Samples on Farm 1 were collected in 2008 and samples in Farm 2 were collected in 2013. CAV infection in the diseased chicks was confirmed by clinical signs and gross lesions, followed by viral isolation and gene detection using PCR from the livers of the diseased chicks, by methods described previously (Imai et al., 1998; Yuasa et al., 1983a).

CAV vaccination was not performed in Farm 1, and sera were collected from the breeder chickens of 2 flocks (flocks A, B) before and after the occurrence of CAV-induced disease. The CAV-induced disease was recorded among the progeny of these breeder chickens at the age of approximately 240 (flock A) and 218 (flock B) days. Sampling time and the number of serum samples collected are shown in Tables 1 and 4.

Flock C of Farm 2 included 3 groups of breeder chickens of different ages (196, 448, and 476 days) that had been vaccinated at the age of 70 days, despite which CAV-induced disease was recorded among their progeny. Sera from the breeder chickens were not collected before or during the occurrence of the disease; therefore, identification of the age group responsible for the vertical transmission of CAV is not possible. Sera were collected from the breeder chickens, with the exception of the oldest age group, after CAV-induced disease was no longer observed among the progeny. Sampling time and the number of serum samples collected are shown in Tables 1 and 4.

Sera from field chicken in Vietnam were kindly provided by the National Institute of Veterinary Research (NIVR) in Hanoi, Vietnam. Seventy four serum samples were collected in 2013 from 16 flocks with 4–6-week-old chickens in the Hanoi and Hanam Provinces. Sera were also collected in 2013 from 237 chickens >8 weeks old from 4 live-bird markets (LBM) in Hanoi (Tables 1 and 5).

Sera from SPF chickens were kindly provided by the National Institute of Animal Health (NIAH), Japan, and the Advanced Technology Development Center of Kyoritsu Seiyaku (Tsukuba, Japan). Antisera to CAV, avian influenza virus (AIV) subtype H9N2, Newcastle disease virus (NDV), and infectious bursal disease virus (IBDV) produced in SPF chickens were also provided by NIAH. As antisera to Marek's disease virus (MDV) were unavailable, sera collected from 10-week old breeder chickens inoculated with a MDV vaccine were used in this study. These sera were examined for the presence of antibodies to MDV using an IFAT with uninfected MSB1 cells, known to express MDV antigens (Schat and van Santen, 2008; Yuasa et al., 1985), and fixed with acetone as described above. The sera were positive for antibodies to MDV (data not shown). The number of sera used is given in Table 2.

2.8. Data analysis

Data were analyzed using the chi-square test. Kappa value was determined using Graphpad (http://graphpad.com/quickcalcs/kappa2/).

Table 2
Evaluation of the specificity of b-LAT.

Origin of serum	No. of serum samples	Non-specific agglutination[a]		No. of antibody-positive serum samples
		Dilution of serum		
		1:1	1:2[b]	
SPF chicken serum	107	6	0	0
Chicken anti serum to AIV	10	0	0	0
Chicken anti serum to NDV	15	0	0	0
Chicken anti serum to IBDV	5	0	0	0
Chicken anti serum to CAV	5	0	0	5
Positive chicken serum to MDV[c]	10	0	0	0

[a] PBS was used instead of CAV antigens.
[b] Dilution in PBS.
[c] The serum samples were collected from breeder chickens vaccinated with a MDV vaccine.

Table 3a
Comparison of the incidence of CAV antibody in sera from field chickens using VNT, IFAT, and b-LAT.

Chicken flocks (Age of chicken)	Incidence of CAV antibody		
	VNT (%)[a]	IFAT (%)[b]	b-LAT (%)
Flock 1			
(19 weeks old)	0/10[c] (0.0)	0/10 (0.0)	0/10 (0.0)
(52 weeks old)	19/19 (100.0)	19/19 (100.0)	19/19 (100.0)
(63 weeks old)	19/19 (100.0) a[d]	6/19 (31.5) b	17/19 (89.4) a
Flock 2 (25 weeks old)	18/18 (100.0)	18/18 (100.0)	18/18 (100.0)
Flock 3 (17 weeks old)	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)
Flock 4 (48 weeks old)	18/18 (100.0) a	9/18 (50.0) b	14/18 (77.7) a
Total	74/94 (78.7%) a[e]	52/94 (55.3%) b	68/94 (72.3%) a

[a] Data from the report previously described (Imai et al., 1993).
[b] Data from the report previously described (Imai et al., 1993).
[c] No. of positives/no. of sera examined.
[d] Data within flocks followed by a different alphabetical letter were significantly different ($P < 0.05$).
[e] Data of the total incidence of CAV antibody followed by a different superscript letter was significantly different ($P < 0.05$).

3. Results

3.1. Specificity of b-LAT

Prior to evaluating the specificity of b-LAT, the serum samples were examined for nonspecific agglutination of mAb-beads in the absence of CAV antigens. Of the 152 undiluted serum samples tested, 6 sera (3.9%) from SPF chickens showed nonspecific agglutination of mAb-beads (Table 2); however, nonspecific reactions were completely eliminated upon 2-fold dilutions of the serum samples in PBS. Therefore, 2-fold dilutions of the sera were employed in subsequent experiments.

All the serum samples from SPF chickens and the sera containing antibodies to AIV, NDV, IBDV, and MDV showed negative results in the b-LAT, while 5 chicken antisera to CAV showed positive results (Table 2). In addition, when the b-LAT was prepared using uninfected cells (not antigen), no reactions were observed when testing chicken serum positive for CAV antibodies (data not shown).

3.2. Comparison of b-LAT with VNT and IFAT

To evaluate the usefulness of the b-LAT in detecting antibodies to CAV, a comparison was made between b-LAT, VNT, and IFAT using sera from 94 layer breeder chickens (Table 3a).

The total incidence of antibody to CAV, as determined using the three tests, was found to be 78.7% (VNT), 72.3% (b-LAT), and 55.3% (IFAT). The incidence of antibody to CAV was not significantly different between b-LAT and VNT, but there was a significant difference between the b-LAT and the IFAT ($P < 0.05$).

Antibodies to CAV were detected in serum samples from flock 1 by the 3 tests in all the chickens at 52 weeks of age, while the incidence of CAV antibody using IFAT (31.5%) was significantly lower as compared to that using VNT (100%) and b-LAT (89.4%) in 63-week-old chickens ($P < 0.05$). Similarly, in 48-week-old chickens of flock 4, the incidence of antibody to CAV using IFAT (50%) was found to be significantly lower as compared to that using VNT (100%) and b-LAT (77.7%) tests ($P < 0.05$).

The results of VNT and b-LAT tests showed 93.6% agreement (Kappa value = 0.82; Table 3b). The sensitivity of b-LAT in comparison with VNT was 91.8% (95% confidence interval [CI]: 83.4%–96.2%). In contrast, the results of IFAT and b-LAT showed 78.7% agreement (Kappa value = 0.55; Table 3c). The sensitivity of IFAT in comparison with b-LAT was 76.4% (95% CI: 65.1%–84.9%). In contrast, the results of VNT and IFAT showed 76.5% agreement (Kappa value = 0.50). The sensitivity of IFAT in comparison with VNT was 70.2% (95% CI: 59.0%–79.4%; data not shown).

3.3. Use of b-LAT for the serological examination of breeder chicken flocks with CAV-induced disease among their progeny

As shown in Tables 1 and 4, in Farm 1 (flocks A and B), CAV vaccination was not performed, and the CAV-induced disease was identified in the progeny of these breeder chickens at the age of approximately 240 (flock A) and 218 (flock B) days. The serum samples collected from breeder flocks A and B prior to the incidence of CAV-induced disease among their progeny were all found to be negative for CAV antibodies using b-LAT (Table 4). However, seroconversion to CAV-positive was detected in these flocks when the chickens were examined at 270 days old (flock A) and 259 days old (flock B) after the vertical transmission of CAV to their progeny ceased. These results were also supported by IFAT analysis.

The serum samples of chickens inoculated with CAV vaccines at 70 days of age in flock C of Farm 2 were collected at 240 and 481 days of age after the vertical transmission of CAV to their progeny ceased (Tables 1 and 4). The results showed that the serum samples were positive for antibodies to CAV by both b-LAT and IFAT tests.

Table 3b
Agreement in antibody detection between b-LAT and VNT.

VNT (74/94)[a]	b-LAT (68/94)[b]	No. of serum samples	Agreement (%)	Kappa value
+[c]	+	68	93.6	0.82
–[d]	–	20		
+	–	6		
Total		94		

[a] No. of positives/no. of sera examined in the VNT (data from Table 3a).
[b] No. of positives/no. of sera examined in the b-LAT (data from Table 3a).
[c] (+) Positive result.
[d] (–) Negative result.

Table 3c
Agreement in antibody detection between b-LAT and IFAT.

IFAT (52/94)[a]	b-LAT (68/94)[b]	No. of serum samples	Agreement (%)	Kappa value
+[c]	+	50	78.7	0.55
–[d]	–	24		
+	–	2		
–	+	18		
Total		94		

[a] No. of positives/no. of sera examined in the IFAT (data from Table 3a).
[b] No. of positives/no. of sera examined in the b-LAT (data from Table 3a).
[c] (+) Positive result.
[d] (–) Negative result.

Table 4
Detection of CAV antibodies in breeder chicken flocks with the outbreak of CAV-induced disease.

Farm	Chicken flocks[a] (Age at serum collection)	Vaccination	Antibody detection		Serum collection time before or after the outbreak of CAV-induced disease
			b-LAT	IFAT	
1	Flock A (103 days)	No	0/10[b]	Nt[c]	Before
	(270 days)		10/10	7/10	After
	Flock B (116 days)		0/10	Nt	Before
	(180 days)		0/10	Nt	Before
	(259 days)		10/10	10/10	After
2	Flock C (240 and 481 days)	Yes (70 days old)	20/20	20/20	After

[a] CAV-induced disease was observed in the progeny of the breeder chickens at the age of approximately 240 (Flock A) and 218 (Flock B) days. In Flock C including 3 different age groups, it was unidentified which age group was responsible for CAV vertical transmission.
[b] No. of positives/no. of sera examined.
[c] Not tested.

3.4. *Use of b-LAT for the serological surveillance of CAV infection in chickens from Vietnam*

To examine the occurrence of CAV in Vietnam, 311 serum samples randomly collected from 4 to 6-week-old chickens from 16 flocks located in Hanoi and Hanam Provinces, and from chickens of more than 8 weeks of age from 4 LBMs in Hanoi, were analyzed by b-LAT (Table 5). Only 2.7% (2/74) of serum samples from 4 to 6-week-old chickens were positive for antibody to CAV, as opposed to 70.4% (167/237) of the samples from chickens of more than 8 weeks age.

4. Discussion

VNT is known to be the most specific, sensitive, and reliable serological test (Otaki et al., 1991; Yuasa et al., 1983b) for the detection of antibodies to CAV. However, the test is laborious and time-consuming, requiring as many as 7–9 passages of cells for completion (Schat and van Santen, 2008). In contrast, IFAT is not as sensitive as VNT for the detection of antibodies in older chickens (Imai et al., 1993) and shows nonspecific staining, particularly with the use of a lower dilution of the serum (Otaki et al., 1991). Therefore, well-trained observers are required for differentiating specific results from nonspecific ones. In addition, both VNT and IFAT are unsuitable for testing a large number of serum samples. ELISA has a distinct advantage in this aspect (Lamichhane et al., 1992; Tannock et al., 2003; Todd et al., 1990, 1999); however, ELISA is also laborious and time-consuming both for setting-up and for completion. Commercial ELISA kits are available for the detection of antibodies to CAV, albeit not in Japan; however, instances of false-positive or false-negative results have been reported (Michalski et al., 1996; Tannock et al., 2003). All these tests require specialized equipment or facilities.

In the present study, a b-LAT was developed for the detection of antibodies to CAV in order to overcome the drawbacks of the currently available serological tests. This test is based on the principle that serum (antibody) from CAV-infected chicken blocks the binding of CAV antigens to mAb-beads. The b-LAT test does not require specialized equipment, and appears to be advantageous in terms of simplicity and speed as compared to IFAT, VNT, or ELISA. The results of the b-LAT are obtained within minutes. Therefore, b-LAT is readily utilizable under field conditions.

Nonspecific reactions, often observed in serological tests performed for detecting antibodies in sera, are likely to lead to erroneous diagnoses. In this study, a very low incidence of nonspecific agglutination of mAb-beads in the absence of CAV antigens was observed with the use of undiluted sera from SPF chicken. However, nonspecific agglutination disappeared when 2-fold dilution of the chicken sera was used (Table 2). In addition, nonspecific reaction and cross-reactivity were not observed upon analysis of sera from SPF chicken and sera that contained antibodies to AIV, NDV, IBDV, or MDV using b-LAT, with positive results obtained only with antisera to CAV. These results indicate the high specificity of b-LAT for the detection of antibodies to CAV in chicken serum.

VNT and b-LAT showed a significantly higher sensitivity for the detection of antibodies to CAV as compared to that by IFAT, although this difference in sensitivity was observed only with older chickens (Table 3a). The results of b-LAT and VNT were in good agreement (93.6%) with a Kappa value of 0.82 (Table 3b), which could be weighted into the category of almost perfect agreement (Kappa = 0.81–0.99; Viera and Garrett, 2005). Because a neutralizing mAb was employed in b-LAT, the detected antibodies likely corresponded with neutralizing antibodies found in the sera of CAV-infected chickens. Moderate agreement was observed between the results of b-LAT and IFAT (Kappa value = 0.55, Table 3c).

The vertical transmission of CAV from breeder flocks to their progeny has been known to play a major role in CAV infections in young chicks. Antibody-negative breeders could be infected with CAV by horizontal transmission or the semen of infected cocks during the laying period (Chettle et al., 1989; Hoop, 1993; Yuasa et al., 1987). Vertical transmission of CAV was observed 7–14 days following the infection of hens under experimental conditions (Hoop, 1992; Yuasa and Yoshida, 1983). In the present study, the applicability of b-LAT in the diagnosis of field CAV cases was evaluated. CAV antibodies were not detectable in sera collected from breeder chicken of flocks A and B in Farm 1 prior to the occurrence of

Table 5
Use of b-LAT for the detection of CAV antibodies in sera collected from field chickens in Vietnam.

Location	Samples from	Age (weeks)	No. of samples	Antibody detection by b-LAT (%)		
				Positive	Negative	Suspected[a]
Hanoi, Hanam	Chicken flock	4–6	74	2 (2.7%)	72 (97.3%)	0
Hanoi	LBM[b]	>8	237	167 (70.4%)	56 (23.6%)	14 (5.9%)

[a] Suspected: Samples showed unclear agglutination.
[b] LBM: Live bird market.

CAV-induced disease (Table 4); this observation also indicates good health management programs in the farm. After the occurrence of CAV-induced disease, b-LAT clearly showed the seroconversion of tested breeder chickens to CAV-positive, which was also supported by IFAT results. This finding demonstrates the suitability of b-LAT for serological diagnosis in the field.

CAV vaccination of breeder flocks has been successfully employed for the prevention of vertical transmission of the virus to progeny chicks (Schat and van Santen, 2008). Although breeder chickens of Farm 2 were vaccinated at the age of 70 days, severe CAV-induced disease in their progeny resulted from the vertical transmission of the virus from these breeders. This observation raises the question of why progeny chicks from the vaccinated breeders remained susceptible to CAV infection. Two different scenarios, such as antigenic mismatching of the vaccine strain to CAV isolate or failure of vaccination procedure, could explain this situation. First, the antigenicity of the CAV strain that infected the breeder chickens could have been different from that of the vaccine strain; however, the reactivity of the CAV strain (HK1/13) isolated from the diseased chicks was not different from the polyclonal antibody raised against the A2/76 strain (data not shown), and amino acid properties of the strain (accession no. KJ126838) were comparable with those of the other reported strains (Trinh et al., 2015). It has been reported that antigenic differences were not observed among CAV isolates using chicken polyclonal antibodies (McNulty et al., 1990; Yuasa and Imai, 1986). Second, the vaccine was not adequately inoculated using the route that the vaccine company recommends (personal communication). Therefore, the incidence of CAV-associated disease among the progeny of the vaccinated breeders was most likely due to the failure of vaccination procedures.

In the field where CAV is prevalent, horizontal transmission of CAV to chicks could occur as early as 8–9 weeks of age when diminished levels of maternal antibodies are found (McNulty et al., 1988). In the present study, only a small number of serum samples (2 of 74) collected from 4–6-week-old field chickens in Vietnam yielded positive result in b-LAT (Table 5). In contrast, most chickens of >8 weeks old demonstrated the presence of antibodies, probably as a result of horizontal transmission of CAV; this result also suggests that CAV is highly prevalent in Vietnam, and that CAV transmission among field chickens appears to be similar to that in other countries. To the best of our knowledge, this is the first study to present evidence of CAV infection in Vietnam.

5. Conclusions

It needs to be emphasized that serological monitoring of breeder flocks for CAV infection is important prior to the laying period in order to protect chicks from vertical transmission of CAV and for ensuring the CAV-free status of SPF chicken flocks. The results of b-LAT developed in the present study were in almost complete agreement (93.6%, Kappa value = 0.82) with those of VNT, known to be the most specific and sensitive test for the detection of antibodies to CAV, and moreover, could be obtained within 5 min. Thus, the simple, rapid, highly specific, and sensitive b-LAT technique is expected to have a potentially high application in CAV serology.

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References

Adair, B.M., 2000. Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24, 247–255.
Cardona, C.J., Oswald, W.B., Schat, K.A., 2000. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. J. Gen. Virol. 81, 2067–2075.
Chettle, N.J., Eddy, R.K., Wyeth, P.J., Lister, S.A., 1989. An outbreak of disease due to chicken anaemia agent in broiler chickens in England. Vet. Rec. 124, 211–215.
Harlow, E., Lane, D., 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, pp. 274–275.
Hoop, R.K., 1992. Persistence and vertical transmission of chicken anaemia agent in experimentally infected laying hens. Avian Pathol. 21, 493–501.
Hoop, R.K., 1993. Transmission of chicken anaemia virus with semen. Vet. Rec. 133, 551–552.
Imai, K., Yuasa, N., 1990. Development of a microtest method for serological and virological examinations of chicken anemia agent. Jpn. J. Vet. Sci. 52, 873–875.
Imai, K., Mase, S., Tsukamoto, K., Hihara, H., Matsumura, T., Yuasa, N., 1993. A long term observation of antibody status to chicken anaemia virus in individual chickens of breeder flocks. Res. Vet. Sci. 54, 392–396.
Imai, K., Mase, M., Yamaguchi, S., Yuasa, N., Nakamura, K., 1998. Detection of chicken anaemia virus DNA from formalin-fixed tissues by polymerase chain reaction. Res. Vet. Sci. 64, 205–208.
Lamichhane, C.M., Snyder, D.B., Girschick, T., Goodwin, M.A., Miller, S.L., 1992. Development and comparison of serologic methods for diagnosing chicken anemia virus infection. Avian Dis. 36, 725–729.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
McNulty, M.S., Connor, T.J., McNeilly, F., Kirkpatrick, K.S., McFerran, J.B., 1988. A serological survey of domestic poultry in the United Kingdom for antibody to chicken anaemia agent. Avian Pathol. 17, 315–324.
McNulty, M.S., Connor, T.J., McNeilly, F., 1989. A survey of specific pathogen-free chicken flocks for antibodies to chicken anaemia agent, avian nephritis virus and group a rotavirus. Avian Pathol. 18, 215–220.
McNulty, M.S., Connor, T.J., McNeilly, F., McLoughlin, M.F., Kirkpatrick, K.S., 1990. Preliminary characterisation of isolates of chicken anaemia agent from the United Kingdom. Avian Pathol. 19, 67–73.
Michalski, W.P., O'Rourke, D., Bagust, T.J., 1996. Chicken anaemia virus antibody ELISA: problems with non-specific reactions. Avian Pathol. 25, 245–254.
Otaki, Y., Saito, K., Tajima, M., Nomura, Y., 1991. Detection of antibody to chicken anaemia agent: a comparison of three serological tests. Avian Pathol. 20, 315–324.
Schat, K.A., van Santen, V.L. 2008. Chicken Infectious Anemia. In: Saif, Y.M., A.M.F., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne D.E. (Eds.), Diseases of Poultry. Blackwell Publishing, Iowa, USA, pp. 211–235.
Taniguchi, T., Yuasa, N., Maeda, M., Horiuchi, T., 1983. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. Natl. Inst. Anim. Health Q. (Tokyo) 23, 1–12.
Tannock, G.A., Tan, J., Mawhinney, K.A., Todd, D., O'Rourke, D., Bagust, T.J., 2003. A modified blocking ELISA for the detection of antibody to chicken anaemia virus using an Australian strain. Aust. Vet. J. 81, 428–430.
Todd, D., Mackie, D.P., Mawhinney, K.A., Connor, T.J., McNeilly, F., McNulty, M.S., 1990. Development of an enzyme-linked immunosorbent assay to detect serum antibody to chicken anemia agent. Avian Dis. 34, 359–363.
Todd, D., Mawhinney, K.A., Graham, D.A., Scott, A.N., 1999. Development of a blocking enzyme-linked immunosorbent assay for the serological diagnosis of chicken anaemia virus. J. Virol. Methods 82, 177–184.
Trinh, D.Q., Ogawa, H., Bui, V.N., Baatartsogt, T., Kizito, M.K., Yamaguchi, S., Imai, K., 2015. Characterization of monoclonal antibodies to chicken anemia virus and epitope mapping on its viral protein, VP1. J. Gen. Virol., http://dx.doi.org/10.1099/vir.0.000042
Viera, A.J., Garrett, J.M., 2005. Understanding interobserver agreement: the kappa statistic. Fam. Med. 37, 360–363.
von Bülow, V., Fuchs, B., Bertram, M., 1985. [In vitro studies of the causative agent of infectious anemia (CAA) in chickens: multiplication, titration, serum neutralization test and the indirect immunofluorescence test]. Zentralbl Vet. B 32, 679–693.
Yuasa, N., Yoshida, I., 1983. Experimental egg transmission of chicken anemia agent. Natl Inst. Anim. Health Q. (Tokyo) 23, 99–100.
Yuasa, N., Imai, K., 1986. Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). Avian Pathol. 15, 639–645.
Yuasa, N., Taniguchi, T., Yoshida, I., 1979. Isolation and some characteristics of an agent inducing anemia in chicks. Avian Dis. 23, 366–385.

Yuasa, N., Taniguchi, T., Goda, M., Shibatani, M., Imada, T., Hihara, H., 1983a. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. Natl. Inst. Anim. Health Q. (Tokyo) 23, 75–77.

Yuasa, N., Taniguchi, T., Imada, T., Hihara, H., 1983b. Distribution of chicken anemia agent (CAA) and detection of neutralizing antibody in chicks experimentally inoculated with CAA. Natl. Inst. Anim. Health Q. (Tokyo) 23, 78–81.

Yuasa, N., Imai, K., Tezuka, H., 1985. Survey of antibody against chicken anaemia agent (CAA) by an indirect immunofluorescent antibody technique in breeder flocks in Japan. Avian Pathol. 14, 521–530.

Yuasa, N., Imai, K., Watanabe, K., Saito, F., Abe, M., Komi, K., 1987. Aetiological examination of an outbreak of haemorrhagic syndrome in a broiler flock in Japan. Avian Pathol. 16, 521–526.