*Original Article*

# Development of Enzyme-Linked Immunosorbent Assay to Detect Antibodies against Chicken Infectious Anemia Virus

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***Abstract***

A standardized and robust indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against chicken infectious anemia virus (CIAV) was developed. Thai CIAV was isolated and used for the entirety of this study. For virus cultivation, a cell density of MDCC-MSB1 cells at 3 x $10^6$ cells/ml and a fetal bovine serum concentration at 10 percent were the most appropriate for Thai CIAV. Then, crude virus of CIAV was prepared using ultracentrifugation technique. To develop the ELISA test, optimal dilution of goat anti-chicken IgG was 1 : 200 and antigen concentration in the ELISA was 1.00 µg/ml, as determined by checkerboard titration. In the present study, a comparison was made between commercial and in-house ELISA for the detection of antibodies to CIAV. Relative sensitivity, specificity and accuracy of in-house ELISA were 93%, 78% and 86%, respectively. Agreement between commercial and in-house ELISA was substantial (Kappa value = 0.71). Consequently, the in-house ELISA was as good as the commercial ELISA in screening chicken serum samples for antibodies against CIAV.

**Keywords:** chicken infectious anemia virus, ELISA, MDCC-MSB1 cells, test kit

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## บทคัดย่อ

# การพัฒนา enzyme-linked immunosorbent assay ในการตรวจสอบแอนติบอดีต่อไวรัสเลือดจางติดต่อในไก่

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การศึกษานี้ได้พัฒนาวิธี indirect enzyme-linked immunosorbent (ELISA) ซึ่งเป็นวิธีมาตรฐานในการตรวจหาแอนติบอดีต่อไวรัสเลือดจางติดต่อในไก่ (CIAV) โดยใช้ CIAV ที่แยกได้จากประเทศไทย เพาะเลี้ยงไวรัสในเซลล์ MDCC-MSB1 cells ที่ความหนาแน่น 3 x $10^6$ เซลล์/มล. และ fetal bovine serum ที่ความเข้มข้นร้อยละ 10 ซึ่งเหมาะสมในการเลี้ยง CIAV และเตรียมไวรัสด้วยอัลตราเซนตริฟิว จากนั้นเตรียมการทดสอบอีไลซาด้วยการทดสอบ checkerboard titration ได้ความเข้มข้นที่เหมาะสมของ goat anti-chicken IgG ที่ 1 : 200 และแอนติเจนที่ 1.00 มค.ก./มล. เปรียบเทียบระหว่างชุดอีไลซาเชิงพาณิชย์และชุดอีไลซาที่เตรียมขึ้นเพื่อใช้ตรวจหาแอนติบอดีต่อ CIAV พบว่าค่าความไว ความจำเพาะ และความถูกต้องสัมพัทธ์ของชุดทดสอบอีไลซาที่เตรียมขึ้น คือ ร้อยละ 93 78 และ 86 ตามลำดับ และพบความสอดคล้องระหว่างชุดทดสอบเชิงพาณิชย์และชุดทดสอบที่เตรียมขึ้น ในระดับสำคัญ (Kappa value = 0.71) จากผลการทดลองสามารถสรุปได้ว่า ชุดทดสอบอีไลซาที่ผลิตขึ้นเพื่อใช้ตรวจคัดกรองซีรัมไก่ที่มีแอนติบอดีต่อ CIAV มีคุณภาพดีทัดเทียมกับชุดทดสอบเชิงพาณิชย์

**คำสำคัญ:** ไวรัสเลือดจางติดต่อในไก่อีไลซา เซลล์ MDCC-MSB1 ชุดทดสอบ

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## *Introduction*

Chicken infectious anemia virus (CIAV) is a causative agent of chicken infectious anemia (CIA) (Hailemariam et al., 2008). The CIAV is a ubiquitous and highly resistant chicken virus causing anemia and death in chicks less than 3 weeks old and immunosuppression in chickens older than 3 weeks old (Miller et al., 2003; Chansiripornchai et al., 2012). The disease is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression with secondary viral, bacterial or fungal infections (Schat and van Santen, 2008). Worldwide, there are approximately 50 billion chickens raised every year (Kaiser, 2010). The CIA causes serious economic loss to commercial broiler industry (Simeonov and Doumanova, 2005). Furthermore, the disease also affects producers of specific-pathogen-free (SPF) eggs. As a consequence of seroconversion, the flock is considered positive and the eggs are no longer SPF (Schat, 2008). Therefore, it is necessary to ensure that those SPF chicken flocks that supplying eggs for vaccine production are free of CIAV infection (Todd et al., 1990[b]). Vertical transmission of the virus plays an important role in infection among young chickens (Miller et al., 2003). Detection of seroconversion in breeder flocks should be conducted before egg production (Iwata et al., 1998). A vaccine-based strategy to control clinical and subclinical disease associated with CIAV infection is also necessary (Todd et al., 1995). Various serological assays have been developed including indirect immunofluorescence assay (IFA) (Simeonov and Doumanova, 2005), virus neutralization (VN) test, ELISA-based assays (Schatand van Santen, 2008) and indirect immunoperoxidase assay (IIP) (Lamichhane et al., 1992). The VN test is more sensitive than other assays (Otaki et al., 1991), but the procedure is time-consuming, expensive and laborious. The continuous passage of the virus makes the test too cumbersome for use in large serologic surveys (Lamichhane et al., 1992). The IFA requires expensive equipment and experienced personnel (Tod et al., 1990[b]). The ELISA is in close agreement with the IFA test for detecting CIAV antibodies (Goodwin et al., 1992). The ELISA is quick and simple to perform and is suitable for large-scale testing (Tod et al., 1990[b]). The advantage of the ELISA test may be the cost of antigen preparation (Lamichhane et al., 1990). Although various commercial ELISA kits are available, all of them have to be imported, making the cost too high for large scale screening of antibodies to CIAV in chickens. Therefore, the purpose of the present study was to prepare a reliable indirect ELISA for the detection of CIAV antibodies in monitoring infections or assessing vaccination in chickens.

## *Materials and Methods*

***Virus and appropriate viral cultivation:*** The CIAV used in this study was isolated from bone marrow of CIA-infected chickens in Thailand. A viral isolation was inoculated into MDCC-MSB1 cell line (CLS, Germany), which is a chicken-T cell line established from Marek's disease lymphoma, and incubated at $37^0$C for 3 days. Cytopathic effects typical of CIAV were observed with the use of an inverted microscope. Appropriate cell density and concentration of fetal bovine serum (FBS) (Gibco BRL, MD) were determined.

***Cell cultures:*** The CIAV field strain was cultivated for 5 passages in MDCC-MSB1 cells. The MDCC-MSB1 of $3x10^5$ or $3x10^6$ cells were cultured in RPMI 1640 medium containing 2 or 10% FBS in 5% $CO_2$ at $40^0$C to find the most suitable condition for the virus cultivation.

***ELISA antigens:*** The infected cultures were harvested and stored at $-70^0$C until use. Virus/cell lysate was frozen and thawed 3 times and left at $37^0$C for 3 days. Cellular debris was removed by refrigerated centrifuge (Centurion Scientific Ltd., UK) at 2,500 g for 30 min at $4^0$C. The supernatant was centrifuged at 80,000 g for 1.5 hour at $4^0$C in an ultracentrifuge (L-XP Series ultracentrifuge class: R (50.2 Ti), Beckman, USA). The crude virus pellets were thoroughly resuspended using phosphate buffered saline (PBS). Aliquots of the antigen were stored at $-70^0$C.

***Protein analysis:*** Protein concentration was determined by the method of Bradford using Quick start™ (Bio-Rad Lab, USA). Samples containing the CIAV virus were analyzed using SDS-PAGE by electrophoresis in gels containing 12.5% polyacrylamide as described by Laemmli (1970). Precision Plus Protein™ Standards (Bio-Rad Lab, USA) were included in the analysis. Gels were stained with Coomassie blue R for 1 hour and washed with destaining solution.

***Optimization of in-house ELISA test:*** An ELISA protocol was modified from Todd et al. (1990[b]). Briefly, CIAV-positive and negative reference sera (Biochek, Holland) were diluted at 1 : 1000 dilutions and then added to a microtiter plate coated with CIAV antigen at different concentration 0.125, 0.250, 0.500, 1.00, 2.00 and 4.000 mg/ml of crude virus, followed by overnight incubation at room temperature. The fluid was then poured from the antigen-coated plates and the plates were tapped dry. A blocking (300 µl/well) solution containing 1 % bovine serum albumin in PBS was added and incubated for 60 min. After 3 times of washing with 300 µl of washing buffer, the goat anti-chicken IgG (H+L) horseradish peroxidase (Synbiotics Corporation, USA) at different dilutions, 1 : 50, 1 : 100, 1 : 200, 1 : 400 and 1 : 800, was added and then incubated at room temperature for 30 min. Following the incubation period, the unreacted conjugate was removed by 3 washing times. ABTS-Hydrogen peroxidase (Synbiotics Corporation, USA) substrate was added into the wells followed by 15 min incubation at room temperature. The reaction was stopped by adding 100 µl of the stop solution and the absorbance value was measured at 405 nm using a 6-well ELISA reader (Biotek Instruments, USA). The commercial CIAV ELISA test kits were obtained from BioChek Poultry Immunoassay (Holland).

***Determination of positive/negative cut-off and tested serum samples:*** A mean of sample-to-positive (SP) ratio value plus 2 standard deviations (SD) of the control sera was calculated according to the formulation below as described by Crowther (2002).

Cut off =(X + 2SD (Negative)) – (X - 2SD (Positive))/2

Reference sera against infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), *Pasteurella multocida* (PM), *Mycoplasma synoviae* (MS) (Synbiotics Corporation, USA) were used for test kit analysis. CIAV antibody positive and negative sera were obtained from BioChek (Holland). The field serum samples of CIAV-infected flocks were collected from 180 commercial broiler chickens.

***Statistical analysis***: Cut-off value, relative sensitivity, specificity and accuracy of the in-house were calculated (Chansiripornchai, 2007). A Kappa test was also applied for agreement between in-house and commercial ELISA tests according to Goodwin et al. (1992). A Kappa value of greater than or equal to 0.40 represents good agreement.

## *Results*

***Cultivation of virus:*** An MDCC-MSB1 cells suspension containing 3.0 x $10^6$ cells/ml was infected with the Thai CIAV strain. The cells showed typical cytopathic effects (CPE) (Fig 1).

***Determination of appropriate viral cultivation:*** The present study indicated that a cell density of MDCC-MSB1 cells at 3 x $10^6$ cells/ml and FBS concentration at 10% were the most appropriate for Thai CIAV cultivation. These cell density and FBS concentration revealed the highest 50% tissue culture infectious dose (TCID50) (Table 1).

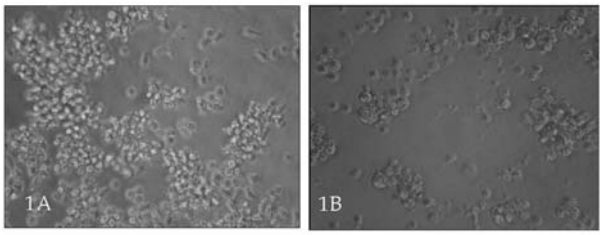


**Figure 1** Cytopathic effects in MDCC-MSB1 cells after 3-day incubation. A. Uninfected cells. B. MDCC-MSB1 cells infected with CIAV.

**Table 1** Determination of appropriate viral cultivation

| No. | Cell density (cells/ml) | FBS concentration | $TCID_{50}$/ml |
|---|---|---|---|
| 1 | 3 x $10^5$ | 2 % | 4 x $10^{2.8}$ |
| 2 | 3 x $10^5$ | 10 % | 4 x $10^{3.8}$ |
| 3 | 3 x $10^6$ | 2 % | 4 x $10^{3.2}$ |
| 4 | 3 x $10^6$ | 10 % | 4 x $10^{8.9}$ |

Note: FBS: fetal bovine serum; $TCID_{50}$ : 50% tissue culture infectious dose

***Protein analysis***: The protein concentration of crude CIAV was 2.382 mg/ml. The SDS-PAGE analysis revealed a single band of 57.3 kDa of CIAV (Fig 2).

***Optimization of ELISA test:*** Different concentrations of conjugate and antigen were titrated in combination using the CIAV-antibody positive and negative sera. The results were evaluated for the maximal positive-to-negative (S/N) ratio between positive and negative sera. One to two hundred of conjugate dilution and 1 µg/ml of antigen concentration were chosen throughout the study according to a subsequent dilution indicating a satisfactory difference between the references of positive and negative sera (Table 2).

***Determination of positive/negative cut-off:*** A cut-off value calculated by the mean of sample-to-positive (SP) ratio value and standard deviation (SD) of the control sera was set at 0.265. There was no cross reaction of the in-house ELISA test to various respiratory avian pathogens including IBV, ILTV, PM and MS (Table 3). The relative sensitivity, specificity, and accuracy of 180 CIAV collected serum samples were 93%, 78% and 86%, respectively. The positive and negative predictive values were 81% and 92%, respectively. Agreement between the commercial and in-house ELISA was highly significant (Kappa value = 0.71).

## *Discussion*

Since the development of various commercially available ELISA kits for the detection and measurement of antibodies to CIAV, improvements to this technique have been carried out. These ELISA test kits are widely used by poultry diagnostic laboratories. The CIAV-specific antibodies to natural infection can be detected by ELISA within 2 to 3 weeks of initial exposure (Michalski et al., 1996). The principal aim of the study was to determine the value of in-house ELISA test using Thai CIAV isolate in detecting CIAV infection in chickens in Thailand. In the present study, the Thai CIAV was isolated and used for the entirety of the study. Recently, MDCC-MSB1 cell cultures have been preferred for in vitro cultivation, although sublines of MDCC-MSB1 differ in their susceptibility to infection (Schat, 2008). For the Thai CIAV isolate cultivation, a cell density of MDCC-MSB1 cells at 3 x $10^6$ cells/ml and aFBS concentration at 10% gave the highest yield of virus. The crude virus was used to prepare the in-house ELISA test instead of purified preparations such as sucrose-density-gradient centrifugation because it would not be economically viable due to the losses incurred (Todd et al., 1990b).

**Table 2** Checkerboard titration of ELISA test. The S/N ratio is shown to determine the optimal conjugate dilution and antigen concentration.

| Conjugate dilution/ Antigen Concentration | 1 : 50 | 1:100 | 1:200 | 1:400 | 1:800 |
|---|---|---|---|---|---|
| 0.125 µg/ml | 3.92 | 4.96 | 5.08 | 4.40 | 3.59 |
| 0.250 µg/ml | 3.68 | 4.42 | 5.09 | 6.15 | 2.47 |
| 0.500 µg/ml | 3.73 | 4.95 | 4.57 | 5.87 | 1.94 |
| 1.000 µg/ml | 3.13 | 4.89 | 6.60* | 6.39 | 2.43 |
| 2.000 µg/ml | 1.29 | 4.30 | 5.89 | 5.37 | 2.23 |
| 4.000 µg/ml | 3.00 | 4.55 | 4.26 | 4.57 | 2.30 |

Remarks: *represents the highest S/N ratio from this study

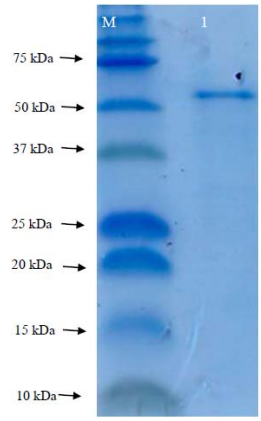


**Figure 2** SDS-PAGE of CIAV. Lane M is the marker (Precision Plus Protein™ Standard Kaleidoscope, Biorad, USA). Lane 1 is the CIAV crude virus.

In general, the antigens used for CIAV enzyme immunoassay were prepared from partially purified virus preparation grown in MDCC-MSB1 cells (Schat and van Santen, 2008). After ultracentrifugation, the crude CIAV was used for preparing a coating antigen for ELISA. The SDS-PAGE analysis revealed a single band of 57.3 kDa of CIAV. Previous reports had demonstrated that a 50 kDa viral protein (VP1) was the only protein detected in highly purified virus particles (Todd et al., 1990[a]). The molecular weights of VP1, VP2 or VP3 were 50, 30/27 and 16 kDa by SDS-PAGE, respectively (Iwata et al., 1998).

The ELISA using the crude lysate from Thai CIAV-infected cells as an antigen was examined. At present, the substantial agreement (Kappa value = 0.71) between the in-house ELISA and the commercial ELISA means that it is possible to use this ELISA to screen chicken serum samples for antibodies against CIAV. The in-house ELISA using Thai CIAV coating

**Table 3** Cross-reaction to other avian pathogens' antibodies

| Positive sera | Mean ± Standard deviation |
|---|---|
| IBV | 0.133 ± 0.013 |
| ILTV | 0.172 ± 0.014 |
| PM | 0.115 ± 0.002 |
| MS | 0.234 ± 0.014 |
| Reference negative sera | 0.145 ± 0.007 |

**Table 4** Contingency table for comparison of commercial and in-house ELISAs.

| In-house ELISA | Commercial ELISA | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 84 | 20 | 104 |
| Negative | 6 | 70 | 76 |
| Total | 90 | 90 | 180 |

virus provides a simple means of detecting anti-CIAV antibodies for monitoring CIAV infections or evaluating vaccination in breeder farms in Thailand.

This in-house ELISA technique has the advantage of the same basic principle being able to be used for detecting antibodies against other chicken pathogens using the same reagent except the coating antigen.

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