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# Application of Real-time Polymerase Chain Reaction for Quantitative Detection of Chicken Infectious Anemia Virus

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## *Abstract*

The present study aimed to develop a quantitative method for the detection of chicken infectious anemia virus (CIAV). A real time polymerase chain reaction (real-time PCR) was developed to determine CIAV. The developed real-time PCR was a rapid and reliable quantitative technique for CIAV DNA compared with the conventional PCR. The appropriate cut-off was at the 40<sup>th</sup> cycle. Results indicated that the C<sub>T</sub> value of 39.62 was shown at the 10<sup>-9</sup> dilution or 0.454 fg/μl or 3.86 × 10<sup>3</sup> DNA copies. This data suggests that the limit of detection for real-time PCR is 0.454fg/μl. The quantitative real-time PCR will be useful for studying CIAV pathogenesis and determining the viral yield.

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**Keywords:** chicken infectious anemia virus, minimal detection limit, quantitative method, real-time PCR

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## บทคัดย่อ

### การประยุกต์ real-time polymerase chain reaction สำหรับการตรวจหาเชิงปริมาณของไวรัสเลือดจางติดต่อนไก

นิวัตร จันทร์ศิริพรชัย วิษณุ วรรณแสง นันทวัน วงศ์ชิตวรรณ สุภาวดี ชัยโชติ จิโรจ ศศิปรีย์จันทร์

การศึกษามีวัตถุประสงค์ในการพัฒนาวิธีเชิงปริมาณในการตรวจพบไวรัสเลือดจางติดต่อนไก วิธี real-time polymerase chain reaction (real-time PCR) ซึ่งถูกพัฒนาเพื่อการตรวจไวรัสเลือดจางติดต่อนไก วิธี real-time PCR ที่พัฒนาขึ้นเป็นเทคนิคเชิงปริมาณที่มีความรวดเร็วและเชื่อถือได้ในการตรวจหาดีเอ็นเอของไวรัสเปรียบเทียบกับวิธี PCR พื้นฐาน ค่า cut-off ที่เหมาะสม คือ รอบที่ 40 ผลการทดสอบแสดงให้เห็นว่า ค่า  $C_T$  ที่ 39.62 พบการเจือจางที่  $10^{-9}$  หรือ 0.454 fg/ $\mu$ l หรือ  $3.86 \times 10^3$  DNA copies จากข้อมูลนี้แสดงว่าข้อจำกัดของการตรวจหาไวรัสในระดับต่ำสุด คือ 0.454 fg/ $\mu$ l วิธี real-time PCR เป็นวิธีที่มีประโยชน์ในการศึกษาพยาธิกำเนิดและการตรวจหาไวรัสเลือดจางติดต่อนไก

**คำสำคัญ:** ไวรัสเลือดจางติดต่อนไก ปริมาณการตรวจพบน้อยที่สุด วิธีเชิงปริมาณ real-time PCR

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### Introduction

A prevalence of chicken infectious anemia virus (CIAV) has been found in most countries that have an intensive poultry industry (McNulty et al., 1991). The first observation of CIAV infection was revealed in Japan (Yuasa et al. 1979). The virus causes immunosuppressive disease characterized by severe anemia and hemorrhages, leading to mortality, increased susceptibility to secondary infections and a decreased responsiveness to vaccines (van Santen et al., 2001). This virus is ubiquitous and is highly resistant to various kinds of disinfectant. The CIAV causes anemia and death in chickens of less than 3 weeks old and immunosuppression in chickens of more than 3 weeks old (Miller et al., 2003). The cause of immunosuppression comes from a depletion of the cortical thymocytes resulting in enhanced concurrent infections and vaccination failure (Noteborn and Koch, 1995).

Current flock testing for CIAV infection or vaccination is usually based on the presence of antibodies detected by enzyme-linked immunosorbent assay (ELISA) test. There are a lot of commercial ELISA kits for CIAV such as the Flocksreen Chicken Infectious Anaemia Antibody ELISA kit (Guidhay Ltd. Guildford, Surrey, UK), the Chicken Anemia Virus ELISA test kit (IDEXX Laboratories, Inc., Westbrook, Maine) and the BioChek Poultry Immunoassays: Chicken Anaemia Disease Antibody Test Kit. However, the presence of antibodies can no longer be considered to be an exact indication of the presence or absence of CIAV (Cardona et al., 2000). Sometimes DNA testing is

necessary to assess the CIAV status of birds (Miller et al., 2003; Hailemariam et al., 2008). Several molecular techniques have been developed for the identification of CIAV such as dot blot hybridization (Todd et al., 1992), conventional polymerase chain reaction (PCR) (Todd et al., 1992; Oluwayelu and Todd, 2008) and nested PCR (Miller et al., 2003), but they may be inappropriate to quantify the viral load for pathogenesis studies and evaluate the viral yield, particularly for specific purposes such as the preparation of antigen for vaccine production or antibody test kit preparation.

Quantification of CIAV has been performed by infectivity titration with MDCC-MSB1 cell line (Goryo et al., 1987; Todd et al., 1990). However, this method is laborious and time consuming because the results will come out at least 3 weeks after seven to nine subcultures (Yamaguchi et al., 2000). To quantify the DNA of CIAV by real-time PCR may assist to determine the clinico-pathological significance of CIAV in clinical problem (McNulty and Todd, 2008). It is possible to quantify the CIAV DNA copies by adding a known quantity of DNA template and determining the amount of PCR products in the sample tube using a standard curve. So far, real-time PCR can be considered as the most sensitive and rapid method of detection and also quantification. Herein, we describe the development and application of the quantitative real-time PCR of CIAV. Additionally, the minimal and maximal detection limits are determined.

### Materials and Methods

**Virus strains:** The virus from Circomune® W (Biomune) vaccine has been analyzed.

**DNA extraction:** The CIAV DNA was extracted using DNA Trap II (DNA Technology Laboratory, Kasetsart University, Thailand) according to the manufacturer's instruction.

**Design of primers:** The primers were designed by Beacon Designer™ (Version 7, Premier Biosoft International). The primer sequences are listed in Table 1.

**DNA amplification:** Amplification reactions (25 µl) contained 2 µl DNA template, iQ™ SYBR® Green Supermix (Biorad Laboratories, CA), and 30 nM of each CIAV specific primer. Before amplification, the PCR mixture was heated to 95°C for 2 min. The amplification profile was as follows: 40 cycles of 95°C for 15 sec and 57°C for 15 sec.

**Determination of DNA copy number:** The DNA copy numbers were determined using a NanoDrop2000/200c spectrophotometer (Thermo Scientific, USA) and calculated with the following formulation. Number of DNA copies = (amount \* 6.022 x 10<sup>23</sup>) / (length \* 1x10<sup>9</sup> \* 650). Then, the DNA template was performed with a serial 10-fold dilution from 10<sup>-1</sup> to 10<sup>-9</sup>.

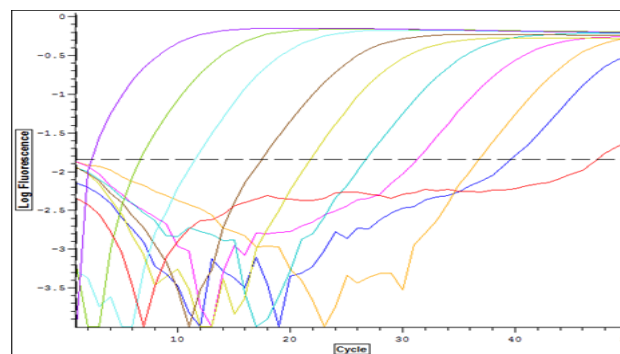
**Quantitative PCR assay:** Each dilution of DNA template was amplified according to what had previously been described in DNA amplification. Then, reactions and data analysis were carried out using the Chromo4™ System (Biorad Laboratories, CA). The results of the reactions were analyzed for standard graphs using Opticon Monitor™ version 3.1 (Bio-Rad Laboratories, CA).

**Table 1** Sequence of CIAV primers

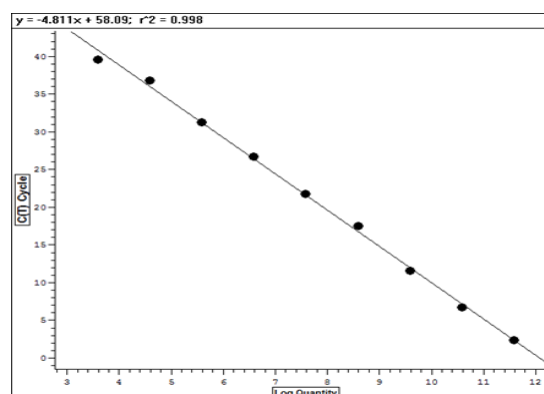
Primers	Nucleotide sequences	Product size (bp)
CAVq-1	5' GAG GAG ACA GCG GTA TCG 3'	109
CAVq-2	5' GCG GAT AGT CAT AGT AGA TTG G 3'	

**Table 2** DNA concentration of CIAV after PCR amplification, threshold cycle C<sub>T</sub> value and melting temperature

Dilution of DNA(log10)	DNA concentration (ng/µl)	DNA copies	C <sub>T</sub> value	Melting Temperature (°C)
-1	45.4	3.86 x10 <sup>11</sup>	2.34	82
-2	4.54	3.86 x10 <sup>10</sup>	6.72	82
-3	0.454	3.86 x10 <sup>9</sup>	11.54	82
-4	0.0454	3.86 x10 <sup>8</sup>	17.51	82
-5	0.00454	3.86 x10 <sup>7</sup>	21.78	82
-6	0.000454	3.86 x10 <sup>6</sup>	26.74	82
-7	0.0000454	3.86 x10 <sup>5</sup>	31.26	82
-8	0.00000454	3.86 x10 <sup>4</sup>	36.80	82
-9	0.000000454	3.86 x10 <sup>3</sup>	39.62	82



**Figure 1** Amplification curve for serial 10-fold dilutions of CIAV DNA in Chromo4™ System. Dilution of CIAV DNA in -1 log<sub>10</sub> (---); -2 log<sub>10</sub> (---); -3 log<sub>10</sub> (---); -4 log<sub>10</sub> (---); -5 log<sub>10</sub> (---); -6 log<sub>10</sub> (---); -7 log<sub>10</sub> (---); -8 log<sub>10</sub> (---); -9 log<sub>10</sub> (---); -10 log<sub>10</sub> (---).



**Figure 2** Standard curve of CIAV DNA. The linear equation was  $y = -4.811x + 58.09$ ,  $r^2 = 0.998$ . X: DNA copies of CIAV Fragment A recombinant plasmid. Y: CT Cycle of RT-PCR reaction.  $r^2$ : the coefficient of determination.

### Results and Discussion

DNA extraction from CIAV fragment was serial 10-fold diluted in nuclease-free water and subjected to PCR. There were 10<sup>-1</sup> to 10<sup>-9</sup> as shown in Fig 1 and Table 2. The results showed that C<sub>T</sub> value were 2.34, 6.72, 11.54, 17.51, 21.78, 26.74, 31.26, 36.80 and 39.62, respectively. The C<sub>T</sub> value gradually increased by decreasing the dilution of DNA. Based on the standard curve of CIAV DNA, it was possible to estimate the number of molecules of the CIAV in a specimen using the equation  $y = -4.811x + 58.09$  (Fig 2).

In this experiment, a rapid and reliable quantitative method for CIAV by real-time PCR was developed. This technique took 3 hours instead of more than 3 weeks by the conventional infectivity titration method. Additionally, no expensive

equipment, particularly CO<sub>2</sub> incubators and culture medium containing fetal bovine serum, were required (Yamaguchi et al., 2000). The primers were designed by Beacon Designer™ (Version 7, Premier Biosoft International) and were expected to be used for most of the CIAV isolates from the nucleotide sequence CIAV in the GenBank database. The appropriate cut-off was at the 40<sup>th</sup> cycle. Results indicated that the CT value of 39.62 was shown at the 10<sup>-9</sup> dilution or 0.454 fg/μl or 3.86 × 10<sup>3</sup> DNA copies. This data suggests that the minimal detection limit of real-time PCR was 0.454 fg/μl. McNulty and Todd (2008) earlier described that the sensitivity limit of PCR was around 10<sup>1</sup>-10<sup>3</sup> genome copies 0.1-10 fg. On the basis of the results obtained in this study, it can be concluded that the quantitative real-time PCR is a rapid and reliable quantitative technique for CIAV DNA molecules and can be useful for studying CIAV pathogenesis and determining the viral yield for viral preparation for specific purpose such as vaccine preparation and various diagnostic kits. Additionally, some real-time PCR formats can potentially be applied to genetically characterize the CIAV using high resonance melting curve analysis, which might give an insight into the understanding of the molecular epidemiology of disease.

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