Original Article

Molecular Characterization of Chicken Infectious Anemia Virus Outbreaks during 2008-2011 in Thailand

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Abstract

The present study aimed to characterize chicken infectious anemia virus isolated from various outbreaks in Thailand during 2008-2011, and compare them with other strains from the GenBank database using molecular and bioinformatic techniques. For this purpose, thirteen samples were collected from eight commercial farms affected by chicken infectious anemia outbreak. All were positive by PCR and grouped in 2 clusters with phylogenetic analysis based on nucleotide sequencing, consistent with amino acid sequence analysis of the VP1 gene in the hypervariable region. All of the thirteen strains were closely related to Chinese and Japanese strains with a high percentage of sequence similarity and was distantly related to vaccine strains used in Thailand.

Keywords: chicken infectious anemia virus, molecular characterization, outbreaks

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บทคัดย่อ

ลักษณะทางโมเลกุลของไวรัสเลือดจางติดต่อในไก่ที่ระบาดในประเทศไทยช่วง พ.ศ. 2551-2554

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาลักษณะของไวรัสเลือดจางติดต่อในไก่ที่แยกได้จากการระบาดของโรคในประเทศไทย ระหว่าง พ.ศ. 2551-2554 และเปรียบเทียบกับไวรัสไวรัสเลือดจางติดต่อในไก่จากประเทศไทยกับประเทศอื่นๆ ที่รายงานใน GenBank ด้วยเทคนิคทางโมเลกุลและข้อมูลทางชีวภาพ โดยทำการศึกษาไวรัสจำนวน 13 ตัวอย่าง ซึ่งรวบรวมได้จากฟาร์ม 8 แห่ง ที่พบการระบาดของโรคเลือดจางติดต่อในไก่ ไวรัส ทุกตัวอย่างให้ผลบวกต่อ PCR และสามารถจำแนกออกเป็น 2 คลัสเตอร์ ด้วยการวิเคราะห์สายสัมพันธ์เชิงพันธุกรรมโดยอาศัยลำดับเบสและการวิเคราะห์กรดอะมิโนของยีน VP1 ในบริเวณแปรปรวนไวรัสทั้ง 13 สเตรน มีความสัมพันธ์ใกล้ชิดกับไวรัสสเตรนจากจีนและญี่ปุ่น โดยมีความคล้ายของลำดับเบสในเปอร์เซ็นต์สูง และพบว่าไวรัสเหล่านี้มีความแตกต่างจากสเตรนของวัคซีนที่ใช้ในประเทศไทย

คำสำคัญ: ไวรัสเลือดจางติดต่อในไก่ ลักษณะทางโมเลกุล การระบาดของโรค

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Introduction

Chicken infectious anemia (CIA) causes economic loss throughout the poultry industry worldwide (Lee et al., 2009). The disease is characterized by aplastic anemia, hemorrhages in the muscles and subcutaneous tissue, thymus atrophy and immunosuppression (Chansiripornchai et al., 2012). CIA infection is identified based on pathognomonic signs and lesions exhibited by affected chickens. Chicken infectious anemia virus (CIAV) is a ubiquitous and highly resistant virus among chickens causing anemia and death in chickens less than 3 weeks old and immunosuppression in chickens more than 3 weeks old (Miller et al., 2003). Extensive lesions are found in the thymus and bone marrow between 10 and 17 days post infection, in younger chickens (Kuscu and Gurel, 2008). CIAV is a small circular DNA virus and the only member of the genus Gyrovirus in the family Circoviridae (Kim et al., 2010). The genome of CIAV is composed of three regions: viral protein1 (VP1), VP2 and VP3 (van Santen et al., 2001). CIAV has been detected and characterized using various techniques. Molecular techniques are highly specific and rapid tools for the initial detection of CIAV infected chickens, particularly polymerase chain reaction (PCR), which is a rapid and sensitive technique for the direct detection of CIAV DNA from various samples (Yamaguchi et al., 2001; Ducatez et al., 2006; Eltahir et al., 2011[a]). Moreover, it has been used for CIAV diagnosis and identification, even though it is no longer present in the field specimens, genetic differences can be detected and studied by sequence analysis of the amplicons (van Santen et al., 2007). In the past, CIAV was believed to have no significant antigenic or pathogenic difference among isolates. Until now, it has been well recognized that the virus has a single serotype with several genetic groups (Hailemariam et al., 2008). Molecular epidemiology of Chinese isolates of CIAV has revealed 4 distant sequence groups (Eltahir et al., 2011[b]). In Thailand, CIAV was first reported in 1996 (Tantaswasdi et al., 1996) and since then many outbreaks have been noticed. However, there has been no study conducted in the genetic characterization of Thai isolates based on sequence and phylogenetic analysis. The aim of the present study was to characterize CIAV isolated from various outbreaks in Thailand during 2008-2011 and compare them with other strains from GenBank database using molecular and bioinformatic techniques.

Materials and Methods

Samples and DNA extraction: Tissue samples were obtained from broiler and breeder chickens that had revealed symptoms of CIAV infection between the ages of 11-98 days. The samples were collected during the outbreaks of 2008 to 2011 (Table 1). DNA was extracted by viral RNA/DNA mini kit (Invitrogen, USA) from homogenized liver, thymus, spleen and bone marrow of chickens that showed clinical signs of CIA. The DNA was kept at -20^{0}C until use.

Virus detection by PCR: The extracted DNA was amplified using the primers CAV1 and CAV2 for PCR product of 724 bp (Todd et al., 1992), CAV-VP3F and CAV4 for PCR product of 633 bp (van Santen et al., 2001) of complete VP3 and partial V2 and VP1 genes (Table 2). The PCR amplification was performed in a 20 µl volume containing PCR buffer 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1µM of each primer and 2.0 Unit Taq DNA polymerase (Fermentas, USA). The amplification was performed with PTC-200 DNA Engine® Thermal Cycle (Bio-Rad, USA). The PCR

profile of CAV1 and CAV2 primers was pre-denaturated at 94^{0}C for 5 min, followed by 20 cycles of denaturation, annealing and extension at 94^{0}C for 30 sec, 60^{0}C for 30 sec and 72^{0}C for 30 sec for the first cycle. The next annealing step was decreased by 0.5^{0}C in each cycle until it reached 50^{0}C. The reaction was followed by 30 cycles of 94^{0}C for 30 sec, 50^{0}C for 30 sec, 72^{0}C for 30 sec, and a final extension of 72^{0}C for 7 min. The reaction of CAV-VP3F and CAV4 primers was performed by denaturation at 94^{0}C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 94^{0}C for 30 sec, 60^{0}C for 30 sec, 72^{0}C for 30 sec and a final extension 72^{0}C for 7 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized on Gel Documentation (UVItec, USA).

Sequencing and Phylogenetic analysis: The PCR products were purified with a PCR purification kit (Fermentus, USA) and direct sequence with a terminator cycle sequencing ready reaction kit using a genetic analyzer (Applied Biosystems, USA). Sequences of 4 primers were combined and analyzed using the Bioedit program (Hall, 2007). DNA Sequences of the 13 isolates in this study were submitted to GenBank with accession numbers: KC762955 [TH-PS1-08]; KC762956 [TH-PS2-08]; KC762957 [TH-PS3-08]; KC762958 [TH-PS4-09]; KC762959 [TH-PS5-10]; KC762960 [TH-PS6-10]; KC762961 [TH-PS7-10]; KC762962 [TH-BR1-11]; KC762963 [TH-BR2-11]; KC762964 [TH-BR3-11]; KC762965 [TH-BR4-11]; KC762966 [TH-BR5-11]; KC762967 [TH-BR6-11]. The sequence was compared with the GenBank database using the BLASTN program. The sequence datasets were aligned with clustalW (Thomson et al., 1994). Phylogenetic analysis was performed using Mega 4.0 (Tamura et al., 2007). Neighbor-joining (NJ) was used for generating phylogenetic trees with the Kimura 2-parameter model (Kimura, 1980). Bootstrap values (1000 replication) of greater than or equal to 50 were indicated in the tree.

Amino acid prediction: Translations of the nucleotide sequences to amino acid were done by the Bioedit program. Amino acid sequences were compared with other CIAV isolates from different countries and vaccine strains including CUX-1 and Del-Ros. Investigation of the amino acid positions 22, 139-151, 394 of VP1 and position 67 of VP3 region was performed.

Results

Samples and CIAV detection: Tissue samples were collected from breeder and broiler chickens in eight commercial farms in Thailand between November 2008 and September 2011 (Table 1). All diseased chickens showed subcutaneous hemorrhage, thymus atrophy and spleen, liver and anemic bone marrow. Thirteen samples gave positive results for PCR amplification using primers CAV1, CAV2 and CAV-VP3F, CAV4. CIAV was detected by PCR in the organs of infected breeders between the age of 11 to 98 days and broilers between 13 to 17 days.

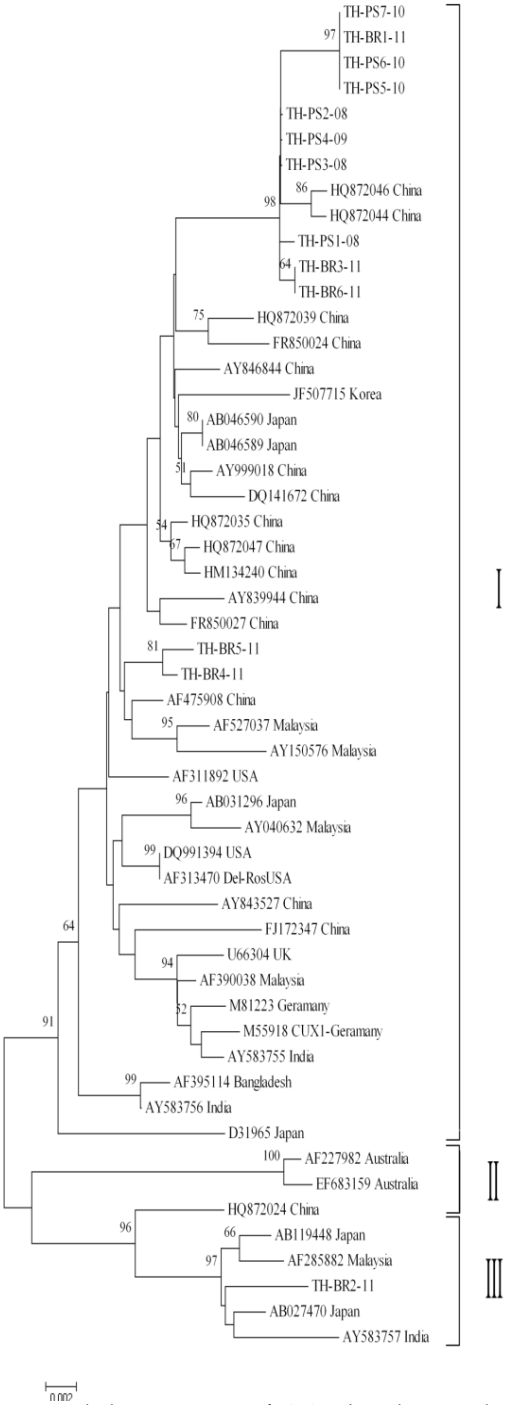

Figure 1. Phylogenetic tree of CIAV based on nucleotide sequence of partial VP1, VP2 and complete sequence of the VP3 genes from thirteen strains and 40 other CIAV strains from the GenBank database.

Nucleotide and Phylogenetic analysis: The nucleotide sequences of each isolate from two pairs of primers were combined and BLASTN searched in the GenBank database. The phylogenetic analysis of 13

CIAV nucleotide sequences and CIAV strains from other countries were placed in 3 major clusters (I, II and III) with high bootstrap values of 91, 100 and 96, respectively (Fig 1). The nucleotide of 12 strains placed in cluster I had a high sequence similarity (98.1%-100%). Strains TH-PS1-08, TH-PS2-08, TH-PS3-08, TH-PS4-09, TH-PS5-10, TH-PS6-10, TH-PS7-10, TH-BR1-11, TH-BR3-11 and TH-BR6-11 belonged to cluster I and were closely related to HQ872044 and HQ872046 from China with a bootstrap value of 98. Moreover, strains TH-BR4-11 and TH-BR5-11 were placed in cluster I with CIAV from China (AF475908) and Malaysia (AF527037, AY150576). TH-BR4-11 and TH-BR5-11 had high sequence similarity with AF475908 from China (99.4% and 99.3%, respectively). The strain TH-BR2-11 was placed in cluster III with Japan, Malaysia, India and China strains with 96% bootstrapping and showed 99.2% sequence similarity with the Japan strain (AB027470). The result revealed that TH-BR2-11 was closely related to CIAV from the Japan strain (AB027470). None of the 13 nucleotide sequences in this study was related to vaccine strains CUX1 (M55918, M81223) and Del-Ros (AF313470).

Table 1 CIAV strains isolated from different commercial farms in four provinces of Thailand.

Isolates*	Farm	Year of	Age	Tissue ¶
TH-PS1-08†	A	2008	15	B
TH-PS2-08†	A	2008	21	B
TH-PS3-08†	A	2008	17	B
TH-PS4-09†	B	2009	98	L, T, S
TH-PS5-10†	C	2010	-	B, T, S
TH-PS6-10†	C	2010	19	B, T, S
TH-PS7-10†	C	2010	11	B, T, S
TH-BR1-11‡	D	2010	17	B
TH-BR2-11‡	D	2011	15	B, T, S
TH-BR3-11§	E	2011	15	T
TH-BR4-11†	F	2011	13	B
TH-BR5-11†	F	2011	13	B
TH-BR6-11†	G	2011	14	B
BR7/11§	E	2011	16	B
BR8/11‡	D	2011	13	B
BR9/11ǀ	H	2011	15	B
BR10/11ǀ	H	2011	15	B
TH-BR7-11§	E	2011	16	B
TH-BR8-11‡	D	2011	13	B
TH-BR9-11ǀǀ	H	2011	15	B
TH-BR10-11ǀǀ	H	2011	15	B

* PS: Breeder, BR: Broiler, † : Lopburi province, ‡ : Petchaboon province, §: Chaiyapoom province,ǀǀ: Nakornsawan province, ¶ B: bone marrow, L: liver, S: spleen, T: thymus

Table 2 Primer pairs used for amplified CIAV.

Primers	Sequence	PCR products (bp)
CAV1	5´ GACTGTAAGATGGCAAGACGAGCTC-3´	724
CAV2	5´GGCTGAAGGATCCCTCATTC-3´	
CAV-VP3F	5´ TTAAGATGGACGCTCTCCAAGAAGATACTC -3´	633
CAV4	5´ CCTTGGAAGCGGATAGTCAT-3´	

Table 3 Polymorphisms in predicted CIAV amino acid sequences among Thai isolates.

Sequence	Amino acid position				
	VP1 22	VP1 75	VP1 97	HV region (VP1 139-151)	VP3 67
AF311887 (Albama, USA)	Q	I	L	QSQAAQNWPNCWL	S
AB119448 (Japan)	H	I	L	QSQAAQNWPNCWL	S
AF285882 (Malaysia)	H	I	L	QSQAAQNWPNCWL	S
AY583757 (India)	H	I	L	QSQAAQNWPNCWL	S
AB027470 (Japan)	H	I	L	QSQAAQNWPNCWL	N
AF395114 (Bangladesh)	H	I	L	QSQAAQNWPNCWL	N
AY846844 (China)	H	V	M	KSQAAENWPNCWL	N
AB046590 (Japan)	H	V	M	KAQAAENWPNCWL	S
AY583755 (India)	H	V	M	KSQAADNWPNCWL	S
JF507715 (South Korea)	H	V	M	KSEAAENWPNCWL	S
EF683159 (Australia)	H	V	M	KSQAAENWPNCWL	N
M55918 (Cux1 Germany)	H	V	M	KSQAADNWPNCWL	S
AF313470 (Del-Ros USA)	H	V	M	KSQAAENWPNCWL	S
TH-PS1-08	H	L	L	KSQAAENWPNCWL	S
TH-PS2-08	H	V	L	KSQAAENWPNCWL	S
TH-PS3-08	H	V	L	KSQAAENWPNCWL	S
TH-PS4-09	H	V	L	KSQAAENWPNCWL	S
TH-PS5-10	H	V	M	KSQAAENWPNCWL	S
TH-PS6-10	H	V	L	KSQAAENWPNCWL	S
TH-PS7-10	H	V	L	KSQAAENWPNCWL	S
TH-BR1-11	H	V	L	KSQAAENWPNCWL	S
TH-BR2-11	H	I	L	QSQAAQNWPNCWL	S
TH-BR3-11	H	V	L	KSQAAENWPNCWL	S
TH-BR4-11	H	V	L	KSQAAENWPNCWL	S
TH-BR5-11	H	V	L	KSQAAENWPNCWL	S
TH-BR6-11	H	V	L	KSQAAENWPNCWL	S

Amino acid prediction: Amino acid analysis of the partial VP1 and VP2 from 12 CIAV contained Lysine (K) and Glutamic acid (E) at positions 139 and 144 of VP1. Only TH-BR2-11 contained Glutamine (Q) at both positions (Table 3). The TH-BR2-11 strain showed amino acid sequence similarity at positions 139 and 144 of the VP1 region with the CIAV from Japan (AB027470, AB119448), Bangladesh (AF395114), the United States (AF311887), Malaysia (AF285882) and India (AY583757). The amino acid analysis at position 22 of the VP1 region of all 13 isolates was Histidine (H).

Discussion

In the present study, we detected CIAV in the bone marrow, thymus, spleen and liver from CIAV-infected chickens at various ages. The result of this study was similar to the Malaysian study suggesting that the spleen, thymus and bone marrow were excellent choices of organs for the diagnosis of CIAV infection (Eltahir et al., 2011[a]). Previous pathological studies of CIAV infection had revealed the pathological effect in different organs and ages of chickens; thymus (4-20 days), bone marrow (3-13 days) and spleen (4-20 days) (Smyth et al., 1993). Moreover, the clinical signs of CIAV were mainly found in young chickens of 2-4 weeks of age and the peak of mortality occurred at 17-21 days of age and decreased at the age of 23-26 days (Bülow et al., 1991).

Most of the CIAV infected chickens in this study were 11-21 days old (94.12%). Only the TH-PS4-09 was derived from CIAV infected breeders aged 98 days. The affected breeder and broiler chickens experiencing symptoms of CIAV infection were determined using PCR analysis. In this study, all samples revealed positive results for CIAV detection by PCR. Amino acid of CIAV from different countries was highly conserved and identified as only a single serotype (Renshaw et al., 1996). Generally, the amino acid at position 139 of the VP1 is lysine (K) and position 144 is glutamine (Q) (Hailemariam et al., 2008). The CIAV containing glutamine at positions 22, 139 and 144 of the VP1 is not replicated in all cell lines (Yamaguchi et al., 2001). Furthermore, CIAV containing Q-139 and/ or Q-141 has been reported to reduce the capacity of viral spreading (Renshaw et al., 1996).Therefore, the results suggest that TH-BR2-11 might not be replicated in cell lines or decrease the rate of spread.

The phylogenetic analysis of 13 nucleotide sequences could be separated into two groups based on the amino acid analysis of the VP1 genes in the hypervariable region (139-151) in this study. The nucleotide sequence analysis revealed that the 13 CIAV isolates of this study were closely related to the Chinese and Japanese strains (99.2-99.4% sequence similarity). We conclude that CIAV in the present study has a close relationship with Asian strains and is distantly related to vaccine strains (CUX1 and Del-Ros) used in Thailand. This means that the vaccine virus still conserves and does not cause clinical cases in field outbreaks and partial sequencing can help differentiate between field and vaccine strains of CIAV.

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