PCR-BASED DETECTION AND PHYLOGENETIC CHARACTERIZATION OF CHICKEN INFECTIOUS ANEMIA VIRUS (CIAV) IN PHILIPPINE NATIVE CHICKENS (*Gallus gallus domesticus)* FROM SELECTED LIVE BIRD MARKETS IN BATANGAS, PHILIPPINES

Fehrgie Dion V. Aseoche[1] and Dennis V. Umali[1*]

ABSTRACT

Chicken infectious anemia virus (CIAV) is an economically important immunosuppressive pathogen of poultry. At present, very minimal information is available regarding CIAV in the Philippines. In this study, a total of forty-nine Philippine native chickens from selected live bird markets from the municipalities of Rosario, Padre Garcia, and Lemery in Batangas were randomly collected and analyzed for the presence of CIAVs. All birds showed varying signs of non-specific disease ranging from respiratory signs, traumatic wounds and integumentary disorders. Five out of ten pooled tissue samples (10.20-51.02%) from the native chickens were confirmed to be positive for CIAV using polymerase chain reaction. Nucleotide sequencing and phylogenetic analyses showed that the field CIAV strains were genetically diverse and belong to genotypes A2, D1, and D2. Comparison of the nucleotide sequences of the VP1 gene showed that the Philippine CIAV strains had sequence similarity of 98-99% to field strains from South Korea, Taiwan, and China. Data obtained in this study are useful references in the development of molecular diagnostic tools and vaccine design to prevent CIAV in Philippine native chickens.

Keywords: chicken infectious anemia, Batangas, live bird markets, nucleotide sequencing, Philippine native chickens, polymerase chain reaction

INTRODUCTION

Chicken infectious anemia (CIA) is an economically important immunosuppressive disease of chickens that may result to increase in mortality, vaccination failures, and production losses (Ganar *et al*., 2017). The disease was first reported in Japan in 1979 as a new disease of poultry caused by a novel virus called chicken infectious anemia virus (CIAV) (Imai *et al.,* 1990). Since its first discovery in Japan, the virus has been detected in most countries worldwide (Eltahir *et al.*, 2011). The virus causes an acute, immunosuppressive health condition with elevated mortality characterized by aplastic anemia, gangrenous dermatitis, subcutaneous petechial hemorrhages, retarded growth, abnormal feather development, weight loss, bone marrow aplasia, and generalized lymphoid atrophy (AboElkhair *et al*., 2014). Secondary viral, bacterial, or fungal infections are also frequently observed in affected chickens due to

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna 4031, Philippines (*email: dvumali@up.edu.ph).

the compromised immune system (Oluwayelu, 2008). Moreover, CIAV can be transmitted vertically, leading to congenital infections in progeny chicks (Yuan *et al.,* 2021). CIAV has high resistance to treatment with common disinfectants, which limits the effects of routine cleaning and disinfection practices in poultry facilities (Rosenberger and Cloud, 1998).

The terminology for the etiologic agent of CIAV has changed through the years. It was originally referred to as chicken anemia agent (CAA) but was later renamed to chicken anemia virus (CAV) after its biochemical and virologic characterization (Noteborn *et al.*, 1992). However, because the name of the disease was termed chicken infectious anemia, the etiologic agent is presently referred to as chicken infectious anemia virus (CIAV). Moreover, although the chicken is considered as the only recognized natural species of CIAV (Pope, 1991), recent studies have detected CIAV genomic fragments in human feces (Zhang *et al., 2012),* the feces of stray mice and dogs (Li *et al.,* 2017) and serum of dogs and cats suggesting possible cross-species transmission of the virus to mammals and its potential public health concern (Liu *et al.,* 2022a*).*

CIAV is a non-enveloped, icosahedral DNA virus from the family Circoviridae. It has a negative-sense, single-stranded circular genome consisting of 2.3 kb nucleotides, arranged in three partially overlapping open reading frames (Todd *et al.,* 1990). With the advances in molecular techniques and the addition of more nucleotide sequences in the GenBank, CIAV sequences worldwide have been classified into four different genotypes (A-D) with five subtypes (A1, A2, A3, D1, and D2). Despite the varying genotypes, all CIAV strains are classified into only one serotype (Koch *et al.,* 1995; Rosario *et al.* 2017).

Knowledge and understanding of the occurrence and distribution of CIAV in live bird markets in the Philippines, particularly in the province of Batangas, is of significance. With the recent outbreak of avian influenza particularly in Central Luzon, Batangas is currently the top poultry-producing province in the Philippines (PSA, 2023). Live bird markets are animal health concern because these areas are conducive environments for disease transmission. Moreover, multiple species and age of domestic birds such as chickens, turkeys, ducks, pigeons, and other waterfowl are normally marketed in close proximity to each other in live bird markets. As an immunosuppressive agent with potentially diverse direct and indirect adverse health consequences, CIAV can result in significant economic losses. In the Philippines, information on CIAV is limited, hence studies related to the field detection and genetic characterization of CIAV in live bird markets are essential.

MATERIALS AND METHODS

All procedures performed on domestic chickens were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños (UPLB) with IACUC Protocol Number 2018-0026.

Live Bird Markets

According to the operation size, three live bird markets from the municipalities of Rosario, Padre Garcia, and Lemery in Batangas were selected in this study. Pertinent information about their time of operation, the source and destination of the live birds, and the number of birds being supplied in the market were obtained from the Regional Animal Disease and Diagnostic Laboratory (RADDL) IV-A.

Philippine Native Chickens

A total of forty-nine (49) Philippine native chickens (Rosario = 15 birds; Padre Garcia = 14 birds; Lemery = 20 birds) were obtained randomly from the identified live bird markets. Samples were collected via convenience sampling method wherein the samples that were obtained depended on the availability and accessibility of study units at the time of sample collection. The age of interest is between five to eight months old, regardless of sex. All birds were purchased at a maximum of five birds per stall. Birds were transported in crates to the University of the Philippines Veterinary Teaching Hospital – Los Baños for physical examination, necropsy, and organ sample collection.

Sample Collection

All animals were weighed individually and were examined for any abnormalities. All chickens were euthanized humanely through cervical dislocation. During necropsy, approximately 5g of internal organs such as the spleen, kidney, cecal tonsils, bone marrow, trachea, lungs, and liver were collected from each bird. Collected samples were packed in individual plastic containers and stored at -20°C until analysis.

DNA Extraction

Approximately 1g of liver, spleen, kidney, cecal tonsils, and bone marrow from each bird were pooled and manually homogenized using sterile mortar and pestle. The homogenized tissue samples were mixed with a normal saline solution containing penicillin (10,000 units/ml) and streptomycin (10 mg/ml at a concentration) to form a 30% tissue homogenate. The samples were then centrifuged at 6,000 rpm for 10 min. An aliquot of 40-50ul of homogenized tissue samples from four to five chickens belonging to the same stall of the same live bird markets were pooled together. A total of 10 pools (Rosario = 3, Padre Garcia = 3, and Lemery = 4) were obtained from the sampled chickens. Viral DNA from homogenized tissue samples were extracted using a commercial kit (QIAamp® Viral DNA Mini Kit, Qiagen, West Sussex, UK). DNA extraction was performed according to the manufacturer›s recommendations.

Polymerase Chain Reaction (PCR)

DNA extracts were tested for CIAV using PCR as reported previously (Eltahir *et al.,* 2011). Amplification of the VP1 region of the CIAV strains was performed using the primer pairs VP1F: 5'-AGCCGACCCCGAACCGCAAGAA-3'and VP1R: 5'-TCAGGGCTGCGT CC CCAGTACA-3'. Amplification of 1390 bp of the VP1 region was carried out through initial denaturation of 94°C for 4 min, followed by 34 cycles of denaturation, annealing, and extension at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, respectively. The final extension was carried out at 72°C for 15 min. Electrophoresis using 1.2% agarose gel stained with Gel Red® (California, USA) was performed to visualize the PCR products.

Nucleotide Sequencing and Phylogenetic Analysis

Nucleotide sequencing and phylogenetic analyses were performed as reported previously (Eltahir *et al.,* 2011). In brief, positive PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR products were submitted to the Philippine Genome Center for Sanger sequencing. PCR products were sequenced from both directions. Sequence assembly was performed using CodonCode

Aligner® (version 11.0.1, CodonCode Corporation, MA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 11. Phylogenic analysis of nucleic acid and deduced amino acid sequences were performed using the neighbor-joining method with the Kimura 2-parameter model at 1000 bootstrap replicates.

RESULTS AND DISCUSSION

CIAV is a significant viral disease of poultry because of its potential to induce immunosuppression by targeting the T-cell progenitor of the thymus and the hemocytoblasts of the bone marrow of the infected host. Moreover, CIAV can be transmitted vertically by the transovarian route resulting in disease in the progeny chicks characterized by depression, wasting, anemia, and increased mortality due to secondary infections (Rosenberger and Cloud, 1998). The presence of CIAV can be confirmed by the detection of either CIAV antibodies or CIAV genome, or both, in breeder, broiler, or layer flocks (AboElkhair *et al.,* 2014).

Table 1. PCR detection and nucleotide sequencing of CIAV from live bird markets in Batangas, Philippines

Live bird market	Number of birds	Number of pools	PCR-positive pools	% Positivity		Genotype
				by pool	by bird	
Rosario	14	3	2	66.67	14.29-71.43	D1
Padre Garcia	15	3	0	0	0	
Lemery	20	4	3	75.00	15.00-75.00	A2, D1, D2
TOTAL	49	10	5	50.00	10.20-51.02	

In this study, genomic surveillance of CIAV from various live bird markets in Batangas using PCR and nucleotide sequencing was performed. All the 49 Philippine native chickens that were used in this study were around five to eight months of age according to the vendors. All of the birds were unvaccinated against CIAV. All birds showed varying signs of non-specific disease ranging from respiratory signs (20.41%), to integumentary disorders (6.12%); and traumatic wounds (6.12%) upon physical examination. Laboratory analyses (Table 1) showed that five out of ten pooled tissue samples tested positive for CIAV (50.00% positivity rate) in PCR; in which two were from Rosario (66.67% positivity rate) and three from Lemery (75.00% positivity rate). On a bird level, CIAV positivity rate ranged from 14.29 to 71.43% in Rosario and 15.00 to 75.00% in Lemery. The high positivity rate may indicate that there was an either ongoing active infection or a subclinical infection in the sampled native chickens. This is in congruence with the study made by Ducatez *et al.* (2008), wherein almost 80% of the samples collected from live bird markets in southeastern China were positive for CIAV using PCR. In other countries in Asia, the prevalence of CIAVs in chicken flocks is reported to range from 47% to 62% in Vietnam (Dao *et al.,*

2018; Van Dong *et al.,* 2019; Huynh *et al.,* 2020) and 40 to 70% in Thailand, and Malaysia (Hailemariam *et al.,* 2008; Chansiripornchai, 2016).

Genetic characterization of field CIAV strains was performed using nucleotide sequencing of the VP1 gene. VP1 is the main gene for phylogenetic studies because it is the major capsid protein of CIAV that is associated with virulence and antigenicity (Renshaw *et al.,* 1996). It also contains abundant neutralization antigenic epitope and it plays a key role in the growth and transmission of the virus. The VP1 protein also has the highest variability at the amino acid level, with a hypervariable region located at nt 139 to 151 (Liu *et al.* 2022b). Phylogenetic analysis using the VP1 gene showed that the field CIAV strains from native chickens in selected LBMs in Batangas, Philippines were from genotypes A and D. The Philippine CIAV strains were given the following identification codes: Philippine/Batangas/Lemery/KVI-CAVLT2/2018 (KVI-CAVLT2); Philippines/Batangas/Lemery/KVI-CAVLT3/2018 (KVI-CAVLT3); Philippine/Batangas/Lemery/KVI-CAVLT4/2018 (KVI-CAVLT4); Philippine/Batangas/Rosario/KVI-CAVRT1/2018 (KVI-CAVRT1); and Philippine/Batangas/Rosario/KVI-CAVRT2/2018 (KVI-CAVRT2). The field CIAV strains were assigned with accession numbers OR933736 to OR933740 in NCBI GenBank.

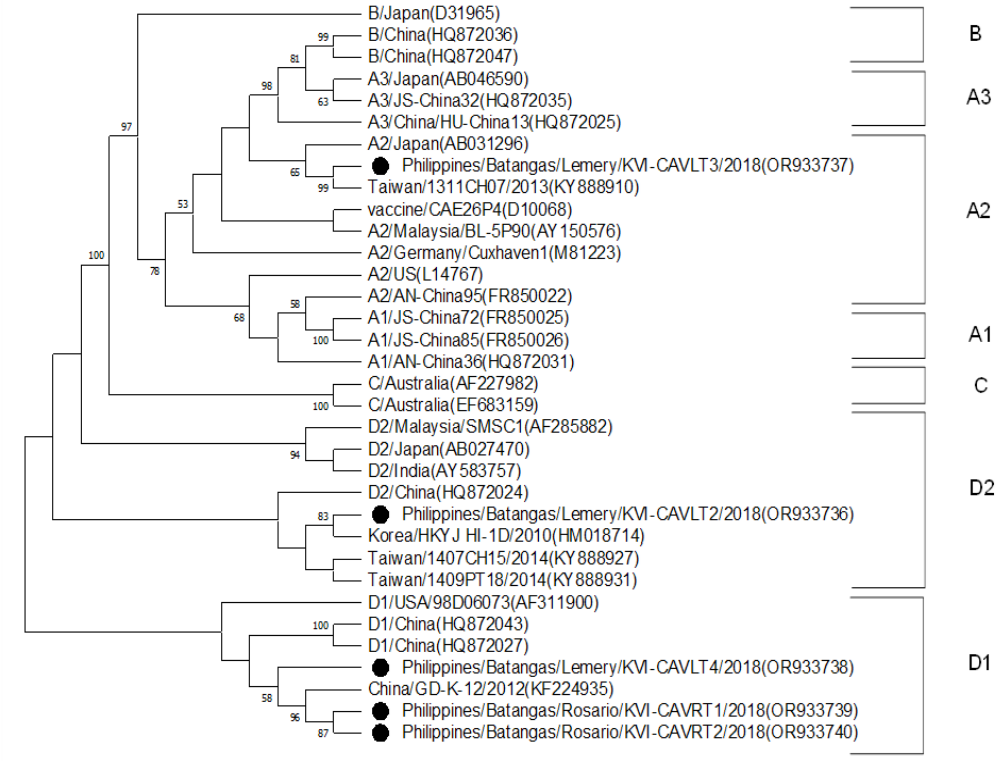

Figure 1. Sub-genotypic analysis of CIAV strains from native chickens in selected live bird market in Batangas, Philippines using the VP1 gene.

[Phylogenic analysis of nucleic acid and deduced amino acid sequences were performed using the neighbor-joining method with Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed There were a total of 1293 nucleotide positions in the final dataset.]

Sub-genotypic analyses of the field CIAV strains showed that KVI-CAVLT3 belongs to genotype A2 while strains KVI-CAVRT1, KVI-CAVRT2, and KVI-CAVLT4 were from genotype D1. Strain KVI-CAVLT2 was analyzed as a CIAV strain from genotype D2 (Figure 1). In China, the predominant CIAV strains were reported to belong to genotypes A1, A2, A3, D1, and D2 (Eltahir *et al.,* 2011), whereas CIAV sequences from Japan, Australia, Italy, Malaysia, Tunisia, and Argentina accounted for approximately 62.5% of the total number of CIAV sequences in Group D (Zhang *et al.,* 2022). CIAV isolates in Iran were reported to belong to D and A3 genotypes (Hosseini *et al.,* 2021) while CIAV strains in India were observed to belong to genotype A (Ganar *et al.,* 2017). Analysis of nucleotide sequences showed that the Philippine field strains had high homologies to strain HKYJ_HI-1D/2009 isolated from chickens in South Korea (99%) in 2009, to strain 1407CH15/2014 isolated from chickens in Taiwan in 2014 (99%) and to strain AH4/2005 isolated from chickens in China (98%).

During the conduct of the study, the selected live bird markets were observed to have various market practices that may play a crucial role in transmitting viral poultry pathogens. These practices include the intermingling of different species of poultry, insufficient cleaning and disinfection of cages wherein the birds are kept, and improper disposal of wastes. In contrast to other countries where operations of live bird markets are on a continuous basis, the live bird market in this study transiently operates according to a particular schedule normally early in the morning once or twice a week. However, due to the recent outbreak of avian influenza, all live bird markets in Luzon are temporarily prohibited from operating. In terms of biosecurity, the location of the live bird markets is on the side of the streets where high human and vehicle traffic occurs. The intermingling of birds such as game fowls, mallard ducks, muscovy ducks, native chickens, and pigeons was observed. Live bird markets are good avenues for poultry viruses to emerge and rapidly spread to different species of poultry and geographic areas resulting in outbreaks (Ducatez *et al.*, 2008).

Overall, the data generated from this study can help in tracking the risks and threats of CIAV in the Philippines. It can be concluded that the field CIAV strains obtained from the live bird market in Batangas were genetically diverse and belonged to different genotypes. Furthermore, it was observed that the field strains may have common origins with CIAVs circulating in Far East Asia. Vaccination against CIAV in Philippine native chicken breeders should be explored as part of the vaccination program for these birds. The strict application of biosecurity protocols and proper cleaning and disinfection of live bird markets in the Philippines is highly recommended. Disease surveillance in live bird markets is a great tool to monitor and prevent any potential poultry disease outbreaks in the future, especially of CIAV.

REFERENCES

AboElkhair M, Abd El-Razak AG and Metwally AEY. 2014. Molecular characterization of chicken anemia virus circulating in chicken flocks in Egypt. *Adv Virol* 797151: 1–6.

Chansiripornchai N. 2016. Field study of seroconversion of three different commercial vaccines of chicken infectious anemia virus in Thailand. *Thai J Vet Med* 46(4):699-704.

Dao D, Cao T, Vu T, Nguyen V and Huynh T. 2018. Prevalence of chicken infectious anemia virus (CIAV) circulating in Hanoi and surrounding provinces. *Vietnam J Agri Sci*

16:36–45.
Ducatez MF, Chen H, Guan Y and Muller CP. 2008. Molecular epidemiology of chicken anemia virus (CAV) in southeastern Chinese live birds markets. *Avian Dis* 52(1): 68-73.
Eltahir YM, Qian K, Jin W, Wang P and Qin A. 2011. Molecular epidemiology of chicken anemia virus in commercial farms in China. *Virol J* 8:145.
Ganar K, Shah M, Kamdi BP, Kurkure NV and Kumar S. 2017. Molecular characterization of chicken anemia virus outbreaks in Nagpur province, India from 2012 to 2015. *Microb Pathog* 102:113-119.
Hailemariam Z, Omar AR, Hair-Bejo M and Giap T.C. 2008. Detection and characterization of chicken anemia virus from commercial broiler breeder chickens. *Virol J* 5:128.
Hosseini H, Majidi S, Ziafati Kafi Z, Esmaeelzadeh Dizaji R and Ghalyanchilangeroudi A. 2021. Molecular characterization and the first full sequencing genome of chicken infectious anemia virus (CIAV) in Iran. *Iran J Vet Res* 22(4):331-336.
Huynh LTM, Nguyen GV, Do LD, Dao TD, Le TV, Vu NT and Cao PTB. 2020. Chicken infectious anaemia virus infections in chickens in northern Vietnam: epidemiological features and genetic characterization of the causative agent. *Avian Pathol* 49(1):5-14.
Imai K and Yuasa N. 1990. Development of a microtest method for serological and virological examinations of chicken anemia agent. *Nihon Juigaku Zasshi* 52(4): 873-875.
Koch G, van Roozelaar DJ, Verschueren CA, van der Eb AJ and Noteborn MH. 1995. Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus. *Vaccine* 13(8):763-770.
Li Y, Fang L, Cui S, Fu J, Li X, Zhang H, Cui Z, Chang S, Shi W and Zhao P. 2017. Genomic characterization of recent chicken anemia virus isolates in China. *Front Microbiol* 10:8:401.
Liu L, Li Y, Yin M, Zhao P, Guo L and Wang Y. 2022a. Genomic characterization of chicken anemia virus in broilers in Shandong province, China, 2020–2021. *Front Vet Sci* 9:816860.
Liu Y, Lv Q, Li Y, Yu Z, Huang H, Lan T, Wang W, Cao L, Shi Y, Sun W and Zheng M. 2022b. Cross-species transmission potential of chicken anemia virus and avian gyrovirus 2. *Infect Genet Evol* 99: 105249.
Noteborn MH, Verschueren CA, Van Roozelaar DJ, Veldkamp S, Van Der Eb AJ and de Boer GF. 1992. Detection of chicken anaemia virus by DNA hybridization and polymerase chain reaction. *Avian Pathol* 21(1):107-118.
Oluwayelu DO, Todd D and Olaleye OD. 2008. Sequence and phylogenetic analysis of chicken anaemia virus obtained from backyard and commercial chickens in Nigeria. *Onderstepoort J Vet Res* 75(4):353-357.
Philippine Statistics Authority [PSA]. 2023. *Chicken Situation Report (January – December 2022)*. Retrieved on 18 September 2023 https://psa.gov.ph/sites/default/files/2022_Chicken%20Annual%20Situation%0Report_v6_signed.pdf
Pope CR. 1991. Chicken anemia agent. *Vet Immunol Immunopathol* 30(1):51-65.
Renshaw RW, Soiné C, Weinkle T, O'Connell PH, Ohashi K, Watson S, Lucio B, Harrington S, and Schat KA. 1996. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *J Virol* 70(12):8872-8878.

Rosario K, Breitbart M, Harrach B, Segalés J, Delwart E, Biagini P and Varsani A. 2017. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. *Arch Virol* 162(5):1447-1463.

Rosenberger JK and Cloud SS. 1998. Chicken anemia virus. *Poult Sci* 77(8):1190-1192.

Todd D, Creelan JL, Mackie DP, Rixon F and McNulty MS. 1990. Purification and biochemical characterization of chicken anemia agent. *J Gen Virol* 71(4):819-823.

Van Dong H, Tran GTH, Van Nguyen G, Dao TD, Bui VN, Huynh L.T.M, Takeda Y, Ogawa H and Imai K. 2019. Chicken anemia virus in northern Vietnam: molecular characterization reveals multiple genotypes and evidence of recombination. *Virus Genes* 55(5):643–653.

Zhang M, Deng X, Xie Z, Zhang Y, Xie Z, Xie L, Luo S, Fan Q, Zeng T, Huang J and Wang S. 2022. Molecular characterization of chicken anemia virus in Guangxi Province, southern China, from 2018 to 2020. *J Vet Sci* 23(5): e63.

Zhang X, Xie Q, Ji J, Chang S, Liu J, Chen F, Ma J and Bee Y. 2012. Complete genome sequence analysis of a recent chicken anemia virus isolate and comparison with a chicken anemia virus isolate from human fecal samples in China. *J Virol* 86(19):10896-10897.