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Original Research

Molecular Detection and Sequence Analysis of Chicken Infectious Anemia Virus from Commercial Chicken Flocks in Select Regions of the Philippines

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Abstract

Background: Chicken infectious anemia virus (CAV) is a ubiquitous pathogen that usually affects young chickens. Clinical infection leads to anemia, lethargy, immunosuppression, and increased mortality. **Methods**: In this study, 12 Philippine provinces from 5 regions were selected for sampling and testing against CAV using PCR assay. Oropharyngeal-cloacal swabs (OPCs), combined OPCs with tissues, and tissue samples were taken from cooperating breeder, broiler breeder, and layer farms. **Results**: The overall CAV positivity rate of the collected samples from commercial chicken flocks is 7.26% (34/468). Based on the flock type, a positivity rate of 29.79% (14/47), 11.19% (15/134), and 1.77% (5/282) were detected in layer, breeder, and broiler flocks, respectively. Based on the region, a positivity rate of 1.31% (4/305) and 21.74% (30/138) were detected from Regions III and IVA, respectively. Nucleic acid sequencing and phylogenetic analysis of selected samples showed clustering under genotype D1. **Conclusions**: The results describe the current CAV situation in commercial chicken flocks from different provinces and their relationship with other strains. Apparently, CAV detection was moderately associated with flock type and region; and weakly associated with flock age and sample type. The local CAV strains from this study were closely related (98.26-99.62%) to Chinese and Taiwanese strains.

Keywords
chicken anemia virus; nucleotide sequencing; poultry; polymerase chain reaction; swabs

1. Introduction

The Philippine chicken population is currently estimated to be at 200 million heads. Comprising this 180-billion-peso industry are around 69 million broilers (34.5%), 22 million layers (22.3%), and 86 million native or improved birds (43.4%) [1]. Chicken meat is the second most consumed animal protein source next to pork [2]. In fact, production has doubled in the past decades [3]. Table eggs are also a staple food in the Filipino household. It is not surprising that poultry husbandry is a promising venture. Along with the benefits, there are also hindrances that local producers face. These include climate change, waste management issues, high input costs, inefficient production, and animal diseases [2,3,4]. Among these threats to production, perhaps disease challenge is the most damaging. The recent Avian influenza outbreaks just shows how vulnerable the local flocks are [5]. In order to keep the local flocks safe from other disease epidemics, stake holders and the government

remain alert against notifiable poultry diseases [6]. However, vigilance must not be limited to the usual pathogens. Subclinical infections that lead to an immunocompromised flock, can also bring about significant losses. Chicken infectious anemia virus (CAV) is one example of an immunosuppressive agent that affect a flock [7,8].

The pathogen, CAV, belongs to the genus *Gyrovirus* under the family *Anneloviridae* [9]. It was first described and isolated in Japan by Yuasa and coworkers back in 1979 [10]. The virion is 20-25 nm in diameter, non-enveloped, spherical in shape, and with icosahedral symmetry. It has 60 capsid subunits that contain the single stranded DNA [11]. The viral genome is 2.3 kb in length with three partly overlapping open reading frames (ORFs) for VP1, the major structural protein; VP2, a scaffolding protein; and VP3, a non-structural protein called apoptin. Lymphocytic depletion in the thymic cortex, which leads to immunosuppression, is said to be mediated by apoptin [12]. The VP1, VP2, and VP3 genes have been used to detect and molecularly characterize CAV strains [13, 14].

This virus is ubiquitous and present in flocks worldwide. It is the causative agent of chicken infectious anemia disease (CIA) in young birds [10]. Clinically infected chicks suffer from anemia, leucopenia, thrombopenia, and immunosuppression. This leads to depression, wasting, increased mortality due to secondary infections, and reduced vaccination response [12, 15, 16]. In vivo, the virus targets the T-cell progenitors in the thymus and the hemocytoblasts in the bone marrow. Destruction of these cells, through apoptosis, leads to the disruption of hemopoiesis and lymphopoiesis [12]. Chicks that are three weeks old and younger are most susceptible, while older birds can remain subclinical infected [7, 11]. At present, the occurrence of clinical cases has been reduced due to the practice of vaccinating breeders [17].

There are numerous case reports and epidemiological studies regarding CAV infections from different Asian countries. Apparently, it is also an important concern in their poultry operations [13, 14, 18, 19, 20, 25]. In 2020, Dong and coworkers [21] described the first genetic analysis of CAV from Philippine chicken flocks. The strain, named L1531/17 (MT079855), was from a layer farm in Nueva Ecija province within the Central Luzon region. Phylogenetic analysis of the VP1 gene showed that the local CAV strain belonged to Genotype III [21] along with Chinese and Taiwanese strains. Perhaps there is a need to also check the other regions for CAV infection in order to better understand its local distribution and epidemiology.

In the present study, 12 provinces from five regions were selected for CAV detection. Oropharyngeal-cloacal swabs (OPCs), OPCs with tissues, and tissues samples were taken from participating farms for testing against CAV using PCR assay.

2. Materials and Methods

2.1 Collection of Samples

Prior to sample collection, certificate of approval (reference No. UPLB-2022-007) was received from the UP-Los Baños Institutional Animal Care and Use Committee. Samples were received from participating farms from the provinces of Batangas, Bulacan, Cebu, Davao, Iloilo, Isabela, Laguna, Nueva Ecija, Pampanga, Pangasinan, Quezon, and Rizal. Organ tissue samples from morbid birds and mortalities included parenchymatous organs namely the: spleen, liver, kidney, thymus, and bone marrow. Oropharyngeal-cloacal swabs (OPCs) were taken from morbid and healthy birds and immersed in transport media. A total of 468 tissue and OPC samples were collected from different broiler breeder, breeder, and layer flocks during the period of 2020 to 2023.

2.2 Sample Preparation

Each sample type (parenchymatous organs, OPCs, and combined OPCs-tissue samples) was pooled accordingly and pre-processed separately before DNA extraction. Briefly, parenchymatous organs were mixed with NSS containing Penicillin-streptomycin combination to create a 30% tissue solution and macerated until homogenous using a sterile mortar and pestle. All homogenized samples or swab solutions were then centrifuged at 6000 rpm for 5 minutes, and the supernatant was collected for viral DNA extraction.

2.3 Viral DNA Extraction

Viral DNA was extracted in all samples using QIAamp Blood and Tissue DNA DNeasy Kit (Qiagen, Germany) following the manufacturer's protocol with modification. Firstly, 25ul of the supernatant was mixed with 20 uL Proteinase K and 200 uL buffer and vortexed for 10 sec followed by incubation at 56° for 10 min. The manufacturer's protocol was followed until after the AW2 centrifugation step. The spin column was placed into a new tube and centrifuged again at 14,000 rpm (20,000 x g) for 1 min. Finally, the spin column was placed into a new 1.5 mL microcentrifuge tube. 200 uL of AE buffer was added followed by incubation at room temperature for 1 min, followed by centrifugation at 8000 RPM (6000 x g) for 1 min.

2.4 CAV-PCR Assay

Endpoint PCR assay was performed using Sapphire Amp® (Takara Bio, Japan) following manufacturer's instruction and as previously described methods [22]. The CAV specific primers Mk 10: 5'-GACTGTAAGATGGCAAGACGAGCTC-3' and Mk 11: 5'-GGCTGAAGG ATCCCTCATTC-3' were used for screening. These were expected to produce a 625 bp PCR product. A 20 µL reaction mixture was prepared comprised of 10 µL Sapphire Amp®, 8.6 µL ultrapure distilled water, 0.6 µL sample DNA, 0.4 µL forward primer, and 0.4 µL reverse primer. Conventional PCR assay was performed using the following conditions: initial denaturation at 94˚C for 30 sec, followed by 30 cycles of {denaturation at 94 ˚C for 30 sec, 60˚C for 30 sec, 72 ˚C for 30 sec} and final extension at 72˚C for 30 sec. The target amplicon was checked via gel electrophoresis using 1% agarose in 1X TAE buffer with 1% Gel red as nucleic acid stain. A 5 µL aliquot of the PCR products was loaded into each well followed by gel electrophoresis at 100V for 30-35 min. The gel was viewed under UV illumination using UVP High Performance UV transilluminator.

2.5 Sequence Analyses of VP1, VP2, and VP3 Genes

For nucleic acid sequencing, one DNA sample from four selected provinces with a CAV-positive result at screening were selected (Table 7). The VP1 and VP2 genes were amplified using previously described methods [14]. Primers VP1-F: 5'-AGCCGSCCCCGAACCGCAAGAA-3' and VP1-R: TCAGGGCTGCGTC CCCCAGTACA-3' were used to amplify the VP-1 region. These were expected to produce a 1390 bp PCR product. Primers VP2-F: 5'-GCGCACATACCGGTCGGCAGT-3' and VP2-R: 5'-GGGGTTCGGCAGCCTCACACTAT-3' were used to amplify the VP-2 region. These were expected to produce a 731 bp PCR product. For the amplification of VP1 and VP genes, 20 µL reaction mixtures were prepared separately comprising 10 µL Sapphire Amp®, 8.6 µL ultrapure distilled water, 0.6 µL sample DNA, 0.4 µL forward primer, and 0.4 µL reverse primer. Conventional PCR assay was performed using the following conditions for the VP1 gene: initial denaturation at 94˚C for 4 min, followed by 34 cycles of {denaturation at 94 ˚C for 1 min, 60˚C for 1 min, 72 ˚C for 1 min} and final extension at 72˚C for 15 min. Conventional PCR assay was performed using the following conditions for the VP2 gene: initial denaturation at 94˚C for 4 min, followed by 34 cycles of denaturation at 94 ˚C for 1 min, 63˚C for 1 min, 72 ˚C for 1 min} and final extension at 72˚C for 5 min.

Selected amplified products for VP1 and VP2 genes were submitted to KinoVet (Korea) for DNA sequencing. Generated sequences were quality checked and assembled using Codon Code Aligner ver. 7.1.2 to generate contiguous sequences. The DNA sequences of the isolates were BLAST searched in NCBI and aligned with other CAV strains available in GenBank. Multiple sequence alignment of nucleotide and amino acid sequences was done using MUSCLE v3.7 and neighbor joining phylogenetic tree was constructed using Jukes-Cantor model with 1000 bootstrapping value using MEGA 11. To compare the homology of the sequences, a percent identify matrix was created using Clustal Omega (EMBL-EBI) Multiple Sequence Alignment tool.

2.6 Statistical Analyses

CAV-positive samples were recorded and the CAV-positivity rates were computed according to: province, sample type, flock type, and age of flock. Cramer's V, a Chi-square-based measure of association between two categorical variables, was used to analyze the data. All tests were done at 5% level of significance.

3. Results

Out of a total of the 468 samples collected, 7.26% (34/468) were found to be CAV-positive. Among the different flock types, a positivity rate of 29.79% (14/47), 11.19% (15/134), and 1.77% (5/282) were detected in layer, breeder, and broiler flocks, respectively (Table 1). Across the different ages, a positivity rate of 0.72% (1/139) and 10.03% (33/329) were detected from flocks <3 weeks old and flocks >3 weeks old, respectively. Among the different sample types, a positivity rate of 7.07% (7/99), 9.38% (21/224), and 4.14% (6/145) were detected from tissue samples, OPCs, and mixed tissue and OPCs, respectively.

Table 1. CAV Positivity Rates per Flock Type

Per Flock Type				
Flock Type	Positive	Negative	Total	%
Broiler Breeder	5	282	287	1.74
Breeder	15	119	134	11.19
Layer	14	33	47	29.79
Total	34	434	468	7.26

Of the five regions sampled (II, III, IVA, VII, and XI), a positivity rate of 1.31% (4/305) and 21.74% (30/138) were detected from Regions III and IVA, respectively (Table 2).

Table 2. CAV Positivity Rates Per Region

Per Region				
Region	Positive	Negative	Total	%
2	0	2	2	0.00
3	4	301	305	1.31
4	30	108	138	21.74
7	0	4	4	0.00
11	0	19	19	0.00
Total	34	434	468	7.26

For Region III, a positivity rate of 1.2% (3/250) and 8.33% (1/12) were detected from broiler and layer flock samples, respectively. For region IXA, a positivity rate of 11.11% (2/18), 16.67% (15/90), and 39.39% (13/33) were detected from broiler, breeder, and layer flock samples, respectively (Table 3).

Table 3. CAV Positivity Rates Per Flock Type Per Region

Per Flock Type Per Region					
Region		Positive	Negative	Total	%
2	Broiler Breeder	0	0	0	0
	Breeder	0	0	0	0
	Layer	0	2	2	0
3	Broiler Breeder	3	247	250	1.20
	Breeder	0	40	40	0.00
	Layer	1	11	12	8.33
4	Broiler Breeder	2	16	18	11.11
	Breeder	15	75	90	16.67
	Layer	13	20	33	39.39
6	Broiler Breeder	0	2	2	0
	Breeder	0	0	0	0
	Layer	0	0	0	0
7	Broiler Breeder	0	2	2	0
	Breeder	0	0	0	0
	Layer	0	0	0	0
11	Broiler Breeder	0	15	15	0
	Breeder	0	4	4	0
	Layer	0	0	0	0
Total		34	434	468	7.26

Among the 12 provinces sampled, a positivity rate of 17.95% (7/39), 2.61% (3/115), 13.51% (5/37), 7.69% (1/13), 23.81% (10/42), and 40% (8/40) were detected from Batangas, Bulacan, Laguna, Nueva Ecija, Quezon, and Rizal, respectively (Table 4).

Table 4. CAV Positivity Rates Per Province

Per Province						
Region	Province	Positive	Negative	Total Per Province		Total Per Region
2	Isabela	0	2	2	0.00	2
3	Bulacan	3	112	115	2.61	305
	Pampanga	0	176	176	0.00	
	Pangasinan	0	1	1	0.00	
	Nueva Ecija	1	12	13	7.69	
4	Batangas	7	32	39	17.95	138
	Quezon	10	32	42	23.81	
	Rizal	8	12	20	40.00	
	Laguna	5	32	37	13.51	
6	Iloilo	0	2	2	0.00	2
7	Cebu	0	2	2	0.00	2
11	Davao	0	19	19	0.00	19
TOTAL						468

The association between CAV detection to flock type, age, sample type, and region was estimated using Cramer's V test. CAV vs flock type, garnered a value of 0.2245: moderate association; CAV vs age, a value of 0.1272: weak association; CAV vs sample type, a value of 0.0624: weak association; and CAV vs region, a value of 0.2199: moderate association. The Cramer's V coefficients indicated that CAV detection was moderately associated with flock type and region, and weakly associated with flock age and sample type (Table 5).

Table 5. Association of chicken anemia virus detection (CAV) to farm type, age, sample type, and region using Cramer's V test (0.00-0.10: Negligible; 0.10-0.20: Weak; 0.20-0.40: Moderate; 0.40-0.60: Relatively Strong; 0.60-0.80: Strong; 080-1.00: Very Strong).

Per Province						
Region	Province	Positive	Negative	Total Per Province		Total Per Region
2	Isabela	0	2	2	0.00	2
3	Bulacan	3	112	115	2.61	305
	Pampanga	0	176	176	0.00	
	Pangasinan	0	1	1	0.00	
	Nueva Ecija	1	12	13	7.69	
4	Batangas	7	32	39	17.95	138
	Quezon	10	32	42	23.81	
	Rizal	8	12	20	40.00	
	Laguna	5	32	37	13.51	
6	Iloilo	0	2	2	0.00	2
7	Cebu	0	2	2	0.00	2
11	Davao	0	19	19	0.00	19
TOTAL						468

Of the 34 positive samples, four were selected for nucleic acid sequencing and phylogenetic analysis; these were samples from Batangas, Laguna, Nueva Ecija, and Quezon provinces. These positive samples were prioritized because of the clinical observations in the sampled flocks (Table 6). Sequencing results of the VP1, VP2, and VP3 genes confirmed that these were in fact CAVs. The strain from Batangas was named: Philippines/Region4/ PHR4BLGS1/2020; the strain from Laguna: Philippines/Region4/PHR4LBBQSP5/2021; the strain from Nueva Ecija: Philippines/Region3/ PHR3NBRZAM/2021; and the strain from Quezon province: Philippines/ Region4/PHR4 QBBQL4/2021. All of the four strains clustered together under genotype D1 (Fig 1.) of which the majority included Chinese CAV strains.

Table 6. Clinical Profiles of field CAVs from select regions in the Philippines

	Field CAV Strain			
	PHR4BLGS1	PHR4QBBQL4	PHR4LBBQSP5	PHR3NEBRZAM
Origin	Region 4	Region 4	Region 4	Region 3
Year	2020	2021	2021	2021
Flock Type	Layer	Breeder	Breeder	Broiler Breeder
Farm Size	120,000	12,000	14,000	80,000
Housing System	Conventional	Conventional	Conventional	Tunnel Ventilated
Age	5 weeks old	6 weeks old	13 weeks old	6 weeks old
Clinical Profile	conjunctivitis, mild facial swelling, pale birds, tracheal rales	lethargy, tracheal rales, gasping, conjunctivitis	conjunctivitis, facial swelling, nasal discharge, snicking	runting-stunting, nasal discharge, poor uniformity, low ADG, increased FCR, elevated mortality
Vaccination History	No Vaccination	No Vaccination	Live FP+CAV (week 8)	No vaccination

The BLAST search used to determine the homology of the selected CAV-positive samples with published strains gave the following results: Philippines/Region4/PHR4BLGS1/2020 was found to be 99.23% similar with isolate GX1904A (MN103402.1.) which was from a chicken in Guangxi, China. Philippines/Region4/PHR4LBBQSP5/2021 was found to be 99.23% similar with isolate 1621TW (MT799750.1) which was from a chicken in Taiwan. Philippines/Region3/PHR3NBRZAM/2021 was found to be 99.62% similar with isolate 1860TW (MT799769.1) which was also from a chicken in Taiwan. Philippines/Region4/PHR4QBBQL4/2021 was found to be 99.04% similar with isolate GX1904A (MN103402.1) which was also from a chicken in Guangxi, China. The nucleic acid sequences of Philippines/Region4/PHR4BLGS1/2020, Philippines/Region4/PHR4LBBQSP5/2021, Philippines/Region3/PHR 3NBRZAM/2021, and Philippines/Region4/PHR4QBBQL4/2021, upon submission to Genbank, were assigned the accession numbers OR933741 to OR933744.

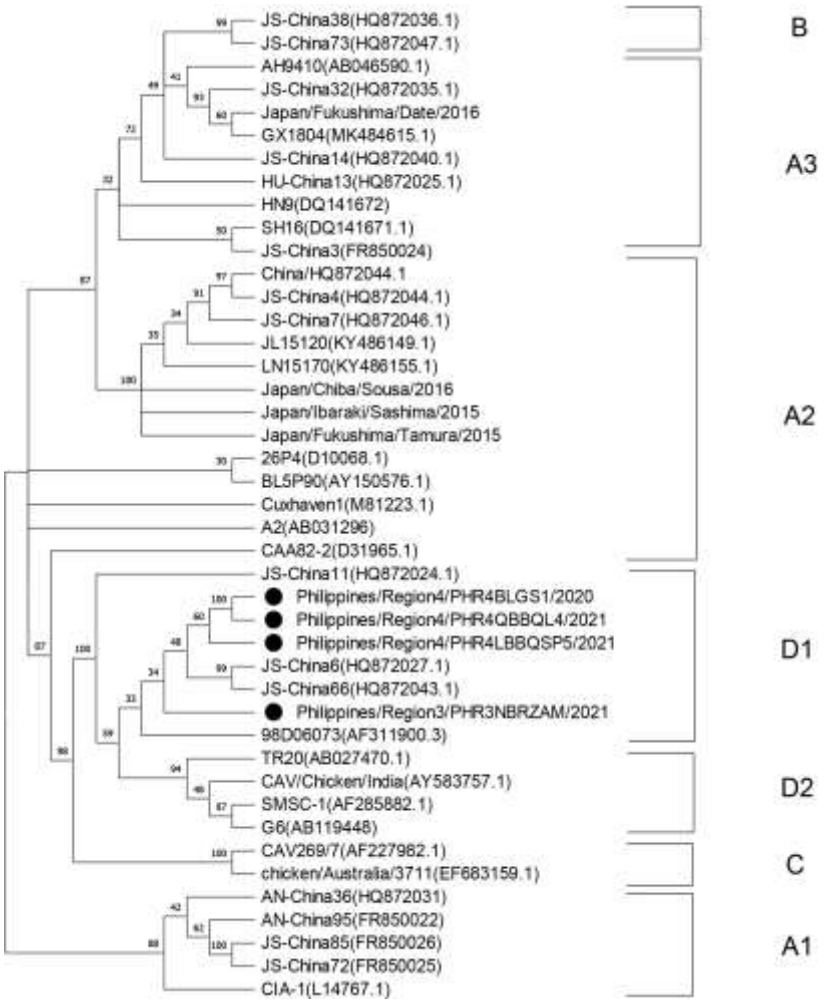

Figure 1. Figure 1. Phylogenetic analysis of field CAV strains from selected areas in the Philippines using the VP1, VP2, and VP3 genes. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA11.

The results of multiple sequence alignment, using Clustal Omega software, showed that the Philippine strains were indeed closely related with Chinese and Taiwanese strains. The homology between these strains were from 98.26% to 99.62% (Table 7).

Strain	Nucleotide sequence Identity (100%)						
	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)
Philippines/Region3/PHR3NBRZAM/2021 (i)	100.00	99.62	98.65	98.59	98.52	99.04	98.72
MT799769.1 (ii)	99.62	100.00	98.40	98.33	98.26	98.96	98.83
Philippines/Region4/PHR4BLGS1/2020 (iii)	98.65	98.40	100.00	99.55	98.58	99.23	98.59
Philippines/Region4/PHR4QBBQL4/2021 (iv)	98.59	98.33	99.55	100.00	98.39	99.04	98.52
Philippines/Region4/PHR4LBBQSP5/2021 (v)	98.52	98.26	98.58	98.39	100.00	99.10	99.23
MN103402.1 (vi)	99.04	98.96	99.23	99.04	99.10	100.00	99.30
MT799750.1 (vii)	98.72	98.83	98.59	98.52	99.23	99.30	100.00

Table 7. Nucleotide sequence identity of the strains in this study based on the VP1, VP2, and VP3 sequences using Clustal Omega multiple sequence alignment tool.

4. Discussion

Clinical CIA is generally a disease affecting chicks that are three weeks old or younger [7, 10, 29]. Morbid birds are lethargic, depressed, and have extensive cutaneous hemorrhages. There is prolonged recumbency leading to abrasions and bruises in the limbs. The necrotic dermatitis and bruises in the wings is where the term "Blue wing disease" originated from [10]. Infected flocks show poor growth, poor conversion, and increased mortality [7, 25]. The causative agent, CAV, targets the hemocytoblasts in the bone marrow, the destruction of which brings about anemia. One of the characteristic lesions at necropsy includes pale or yellowish bone marrow. The metabolic disruption brought about by anemia leads to the poor performance of infected birds [12,24]. CAV also targets the cells of primary lymphoid organs; the T-cell progenitor cells in the thymic cortex. Apoptin, which is coded by the VP3 gene, mediates the destruction of infected cells. This would account for the thymus and splenic atrophy observed upon necropsy of morbid birds. However, B-cells, stromal cells, and non-lymphoid leukocytes are not susceptible to infection [12]. The virus is also synergistic with other pathogens. This leads to the manifestation of mixed clinical signs. In many cases, clinical signs are overshadowed by concurrent contagions, thus, CIA infection can be overlooked [12, 15]. Concurrent infections become complicated because of the combined effects of anemia, immunosuppression, and the pathogens involved.

In the present study, out of 468 samples, a 7.26% positivity rate was garnered. Per flock type, broiler breeder flock samples were 1.77% positive; breeder flock samples were 11.19% positive; and layer flock samples were 29.79% positive. The results support the previous findings of Dong and coworkers [21]. Indeed, CAV infection is present in the Philippines; particularly Region III (Central Luzon region). Similar studies in other Asian countries have been published. Eltahir and co-workers [14] reported 10.72% positivity rate in samples collected from three Chinese provinces; Zhang and co-workers [26] reported 17.10% positivity rate in samples collected from one Chinese province; Ou and co-workers [27] reported 52.55% positivity rate in samples from various regions of Taiwan; Kye and co-workers [18] reported 9.09% positivity rate in samples from six Cambodian provinces; and Hailemarian and co-workers [20] reported 9.09% positivity rate in samples from six Malaysian provinces.

The results show that CAV infection in Philippine flocks is also present in Region IVA; namely the provinces of Batangas, Laguna, Quezon, and Rizal. It seems that the virus is more widespread than previously known.

Although convenience sampling was used in this study, some inferences can still be made. Based on the results of the Cramer's V test, it was shown that CAV detection is moderately associated with flock type and region. This implies that the likelihood of detecting CAV from a sample can be influenced by the flock type or the region where the samples originate. Breeder flocks are routinely given CAV vaccine. This practice, in fact, has helped reduce clinical disease occurrence. Maternal antibodies are passed on to the offspring which protects them during the vulnerable period [7, 17]. However, the animal can be at risk of getting infected once the protective antibody levels waned. Perhaps this occurs in flocks that suffer from clinical disease or come down with subclinical infection [8]. Regions III and IVA are neighbors that share boundaries. Human, animal, and fomite movement may contribute to the spread of CAV between these provinces. The virus is known to be hardy and ubiquitous in nature. It is said to be difficult to destroy and can persist in the environment for a long period of time [7, 11].

On the other hand, there is weak association between detection vs age, as well as detection vs sample type. This implies that the age at sampling or type of sample, whether organ or OPCs, may not influence the likelihood of detection. Sub clinically infected birds may shed the virus [7, 8, 11], thus, it can still be detected through sensitive assays (e.g., diagnostic molecular methods). Also, there may not be a need for conducting a necropsy for the purpose of collecting organs. It seems that OPCs, as samples, are enough to enable detection of CAV from infected birds.

Four CAV-positive samples were selected for nucleic sequencing because of the clinical observations in the source flocks. The birds exhibited respiratory signs, poor growth, and lethargy (Table 6). Although these clinical signs are not pathognomonic for CIA, the virus is known to amplify infections [8, 11], lead to poor performance, [10] and has been reported in flocks beyond 3 weeks of age [25, 28]. Sequencing results of these samples showed that the strains were all under genotype D1. These are closely related with each other, and to Chinese and Taiwanese strains as well. The strains JS-China11 (HQ872024.1), JSChina6 (HQ872027.1), JSChina66 (HQ872043.1), and 98D06073 (AF311900.3) are also found under this clade. On the other hand, the Philippines strains are distantly related to the vaccine strains 26P4 and Cuxhaven-1; which are both under genotype A2. This implies that the strains detected and identified are unlikely to be of vaccine origin. Philippines/Region4/ PHR4BLGS1/2020 was found to have a close homology with an isolate from a chicken in Guangxi, China; and so was Philippines/Region4/ PHR4QBBQL4 /2021. The same can be said of Philippines/Region4/PHR4LBBQSP5/2021 and Philippines/Region3/PHR3NBRZAM/ 2021; which were found to have a close homology with isolates from Taiwan.

Phylogenetic analysis indicates that the Philippine strains may have a shared common ancestry with Chinese and Taiwanese isolates. Perhaps geography could play a role in this genetic relatedness. Taiwan is just north of the Philippine islands, and China is just northwest of Taiwan. The L1531/17 strain from Nueva Ecija, as reported by Dong and co-workers [21], was also closely related to Chinese and Taiwanese strains. The fact that the Philippines also imports chicken from other countries, such as China, may also be contributory to CAVs' local epidemiology [21]. An often-unnoticed potential source of infection would be the use of contaminated vaccines in flocks. There are studies in China wherein CAV was detected in Fowl pox vaccines [30] and this has been implicated in the diseases' spread. This is probably another factor to the local spread that needs to be looked into. Future studies may give more valuable results if CAV is isolated. Perhaps infection trials may allow better characterization of the virulence and pathogenicity of local Philippine strains.

5. Conclusions

In the present study, CAV was detected in OPCs, combined OPCs with tissues, and tissue samples from different Philippine provinces. The results substantiate the findings of previous workers who reported CAV infection in a Philippine layer flock. Apparently, the infection is more widespread than previously known. Positive samples were not confined to Region III alone, samples from Region IVA provinces also showed positive results. Detection of CAV was found to be moderately associated with flock type and region; and less associated with the age of the sampled birds and the sample type collected. Nucleic acid sequencing and phylogenetic analysis revealed that the

Philippine strains are under the Genotype D1. These strains were found to be closely related to Chinese and Taiwanese isolates. It appears that the local strains are distantly related to vaccine strains.

Availability of Data and Materials

The data collected during the course of this research are already included as part of the submitted manuscript.

Author Contributions

Conceptualization, Methodology - D.V.U.; Investigation – D.V.U. and F.P.D.V.; Writing - Original Draft - D.V.U. and F.P.D.V.; Writing - Review & Editing - D.V.U. and F.P.D.V.; Resources - D.V.U.

Ethics Approval and Consent to Participate

All of the pertinent international, national, and/or institutional guidelines for the care and use of animals were adhered to during the conduct of this study. Certificate of approval, with reference No. UPLB-2022-007, was given by the UP-Los Baños Institutional Animal Care and Use Committee. -Los Baños Institutional Animal Care and Use Committee.

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Conflict of Interest

The authors declare no conflict of interest.

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