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The first genetic analysis of chicken anemia virus isolated in layer chicken flocks in the Philippines

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Abstract

Chicken anemia virus (CAV) exists worldwide; however, the infection of CAV in the Philippines has not been investigated. In this study, one out of three samples collected from diseased chickens farmed in Central Luzon, the Philippines, in 2017 was positive for the CAV genome based on real-time PCR testing. We conducted genetic characterization of the CAV strain. The phylogenetic analyses of the VP1 gene sequences and the protein-coding region of the CAV genome indicated that this CAV strain belongs to genotype III, which differs from vaccine strains 26P4, Cuxhaven 1, and Del-Ros that are already present in the country. The Filipino CAV strain is genetically related to the Polish, Chinese, and Taiwanese CAV strains.

Key Words: Chicken anemia virus, Genetic analysis, Philippines

CAV is a member of the genus *Gyrovirus* within the family *Anelloviridae* [20] and is a non-enveloped, circular, single-stranded DNA virus. Three overlapping genes, VP1, VP2, and VP3, encode the viral capsid and structural protein VP1 and two other non-structural proteins VP2 and VP3, respectively [15, 20]. As previously reported, the VP2 and VP3 genes are highly conserved among CAV strains. In contrast, the VP1 gene contains the hypervariable region at amino acid (aa) position 139–151. In addition, the VP1 protein is reported to contain eleven major variable substitutions [6, 19]. Therefore, the VP1 gene sequences were used to clarify the genetic characterization of CAV.

Based on phylogenetic analyses of the CAV full-length VP1 gene sequences, there are three genetically different genotypes I, II, and III [4, 11, 16]. Recent reports demonstrate that novel genotypes IV and V were also detected among CAV strains

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Table 1. Primers used in this study

Name	Nucleotide sequence (5'-3')	Position[a]
CAV-CQ1F	CAATCACTCTATCGCTGTGT	607–626
CAV-CQ1R	TTCGTCCATCTTGACTTTCT	46–65
CAV-CQ2F	GGCTACTATTCCATC(A/T)CCATTCT	13–34
CAV-CQ2R	GCTCGTCTTGCCATCTTACA	847–866

The primers were previously descried by Zhang and co-authors [27].
[a]The numbers correspond to positions within the Cux-1 strain genome (Accession No. M55918 [15])

[3, 17]. In addition, the recombination events were observed among CAV strains [3, 5, 17], which suggests that genetic evolution is ongoing among CAV strains. Therefore, it is important to assess the prevalence and genetic characterization of CAV strains in countries where few studies have been published.

CAV infection has been previously reported in several Southeast Asian countries [3, 7, 13] but not in the Philippines. Thus, there is little information on CAV infection in chickens in the Philippines on the viral genome sequences in GenBank. The aims of this study were to identify the CAV genome from samples of chickens farmed in the Philippines and genetically characterize the protein-coding region of the CAV genome.

This article does not contain any studies with human participants. The collection of chicken tissue samples was conducted by the Department of Agriculture-Regional Field Office III of the Philippines under institutional approval and with permission from the owners of the chickens.

In 2017, outbreaks of Newcastle disease were recorded in commercial layer chicken farms in Nueva Ecija Province in Central Luzon, the Philippines (Fig. 1). Chickens displaying nasal discharge, rales, ruffled feathers, pale comb, diarrhea, decreased egg production, and increased mortality were forwarded for sampling. In three distinct farms, three lung samples from 30-week-old chickens were collected. The samples were frozen and transferred to Obihiro University of Agriculture and Veterinary Medicine, Obihiro City, Japan. Then, the samples were homogenized in phosphate-buffered saline supplemented with kanamycin (1 mg/mL), gentamycin (100 µg/mL), and amphotericin B (10 µg/mL) as a 20% homogenate.

Viral DNA was extracted from the homogenized samples using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). An in-house real-time PCR for CAV VP1 gene detection was performed to detect the viral genome in the samples as previously described [3]. The CAV genome-positive samples were forwarded for nucleotide sequencing. The protein-coding region (1,823 bp) of the CAV genome was amplified by PCR using two pairs of primers, CAV-CQ1F/CAV-CQ1R and CAV-CQ2F/CAV-CQ2R, as previously reported [27] (Table 1). Separation of the amplified 1,778 and 831 bp PCR products was conducted on 1.5% agarose gels and followed by purification with a GeneClean® II Kit (MP Biomedicals, Santa Ana, CA). The BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and the Applied Biosystems 3500 Genetic Analyzer (Life Technologies) were used for the nucleotide sequencing.

Phylogenetic analyses were conducted for the full-length VP1 gene sequences and the protein-coding region of the CAV genome. The nucleotide and deduced aa sequences were aligned and analyzed by the ClustalW multiple alignment tool [24] in BioEdit v.7.2.5 [8]. The homology in the nucleotide and aa sequences of the CAV strains in this study was compared and examined by the BLAST program (https://blast.ncbi.nlm.nih.gov/) and GENETYX v.10 software (GENETYX

Corp., Tokyo, Japan). The phylogenetic tree at the nucleotide level was constructed based on a maximum likelihood method with a Hasegawa-Kishino-Yano model of nucleotide substitutions. A Jones-Taylor-Thornton model of amino acid substitutions was used to reconstruct the phylogenetic tree on aa sequences. The confidence values on phylogenetic trees were assessed by bootstrapping with 1,000 replications using MEGA 6 software [22]. The protein-coding region of the CAV genome obtained in this study was deposited to GenBank under accession number MT079855.

CAV isolation was performed in MDCC-MSB1 (MSB1) cells [1] as previously described [10] with a slight modification. Briefly, homogenized samples were inoculated into 2×10^5 MSB1 cells in 1 mL growth medium (GM) while PBS was inoculated in the control group. The cells were then passaged at three-day intervals through culturing of 200 µl of the cell suspension in a new well containing 1 mL GM. Then, the CAV-negative/positive wells were determined after ten passages. Cell death that resulted in the red color of the culture medium was regarded as CAV positive, whereas negative controls resulted in a yellow color and were considered CAV-negative [26]. The positive/negative results were confirmed by a conventional PCR method [9].

One of three samples obtained in this study was positive for the CAV genome based on real-time PCR (Ct value = 32.08) testing. The Filipino CAV strain obtained in this study was designated as Philippines/Nueva Ecija/L1531/17 and abbreviated as L1531/17. For the genetic characterization, the protein-coding region and full-length VP1 gene sequence of the L1531/17 strain were analyzed and compared with other CAV strains published in GenBank. The results indicate that there is no deletion and insertion in the protein-coding region of the L1531/17 strain. When comparing the full-length VP1 sequence of the L1531/17 strain with other CAV strains in GenBank, the nucleotide identity ranged from 93.87% (L1531/17 vs. Australia/S71488.1) to 99.33% (L1531/17 vs. Poland/KM458178.1/OPL3/13). At the aa level, the maximal identity of the predicted VP1 aa sequences was 99.55% (L1531/17 vs. Poland/KM458180.1/POD3/13, USA/L14767.1, and Egypt/MH001564.1/EG-26) while the minimum rate was 96.79% (L1531/17 vs. Malaysia/AF527037.1).



Fig. 1.
A map of the Philippines showing in what province the samples were collected in 2017. Nueva Ecija Province is located within the Central Luzon region in the Philippines.

Phylogenetic analyses of the full-length VP1 gene sequences (1,350 bp) (Fig. 2) and the protein-coding region of the CAV genome (Supplemental Fig. 1) indicated that the L1531/17 strain obtained in this study belongs to genotype III [3,17]. The L1531/17 strain was not clustered with vaccine strains USA/AF313470/Del-Ros, Netherlands/D10068.1/26P4, or Germany/M81223/Cuxhaven

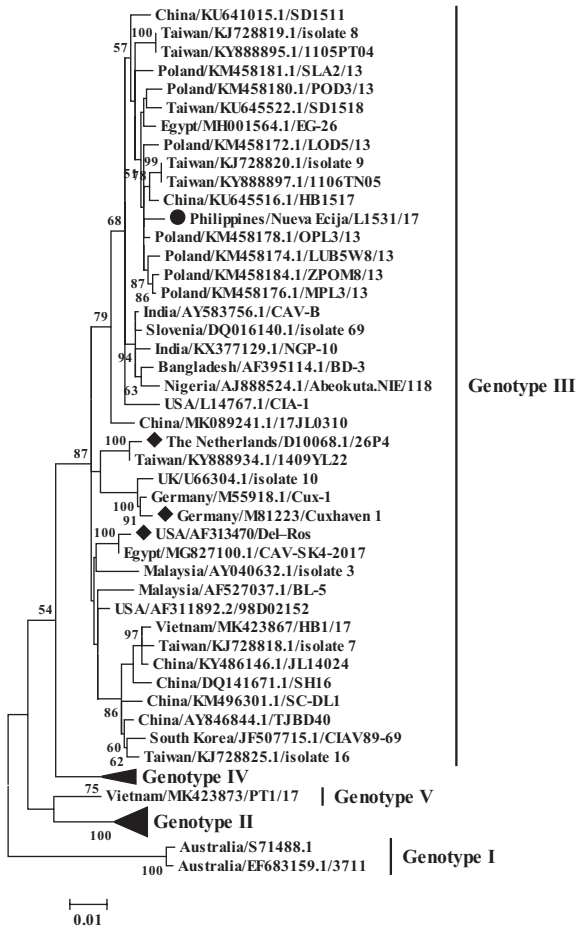


Fig. 2.
A phylogenetic tree of the full-length VP1 gene sequences (1,350 bp) of the Filipino CAV L1531/17 strain compared with those available in GenBank. The GenBank sequences indicate the country name/accession number. MEGA 6 software and a maximum likelihood method was used to construct the phylogenetic tree (1,000 bootstrap replicates). The number at each branch point indicates the bootstrap values ≥50% in the bootstrap interior branch test. The L1531/17 and vaccine strains are marked with a circle and diamond, respectively.

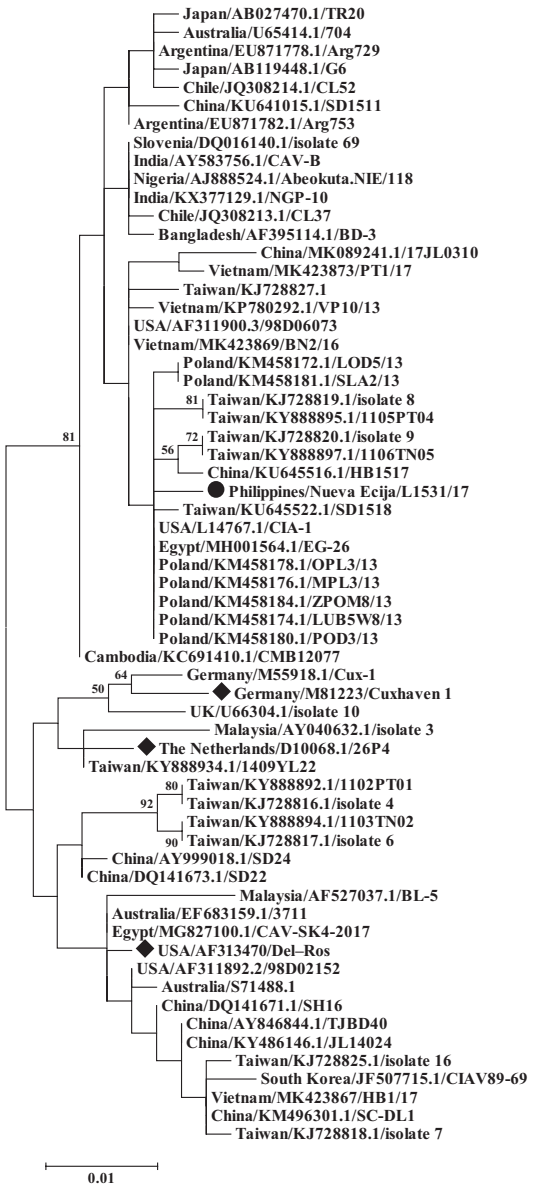


Fig. 3.
A phylogenetic tree of the VP1 protein sequence of the Filipino CAV L1531/17 strain compared with those available in GenBank. GenBank sequences are shown as the country name/accession number. MEGA 6 software and the maximum likelihood method with the Poisson correction model was used to reconstruct the phylogenetic tree (1,000 bootstrap replicates). The number at each branch point indicates bootstrap values ≥50% in the bootstrap interior branch test. The L1531/17 and vaccine strains are marked with a circle and a diamond, respectively.

1. The L1531/17 strain was closely related to CAV strains from Poland (Poland/KM458178.1/OPL3/13), China (China/KU645516.1/HB1517), and Taiwan (Taiwan/KY888897.1/1106TN05) (Fig. 2). The aa phylogenetic tree supported the L1531/17 strain located far from the vaccine strains (26P4, Cuxhaven, and Del-Ros strains) (Fig. 3).

Predicted aa in the VP1 protein of the L1531/17 strain was also compared with other strains in several lineages, and the aa motif at the 11 variable aa substitutions was found (22N-75I-97L-125I-139Q-144Q-287A-290A-370S-

Table 2. The amino acid motif in the VP1 protein of the CAV strain

Sequences	Genotypes	Amino acid position in VP1[b]												
		22	75	89	97	125	139	144	287	290	370	376	394	413
Consensus[a]		**H**	**V**	**T**	**M**	**I**	**K**	**E**	**S**	**A**	**G**	**L**	**Q**	**A**
Australia/EF683159/3711	**I**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**S**
USA/AF311900.3	**II**	**Q**	**I**	**.**	**L**	**.**	**Q**	**Q**	**A**	**.**	**S**	**.**	**.**	**.**
Netherland/D10068/26P4[c]	**III**	**.**[e]	**.**	**.**	**.**	**.**	**.**	**.**	**T**	**.**	**S**	**.**	**.**	**.**
USA/AF313470/Del–Ros	**III**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**S**
Germany/M81223/Cuxhanven 1	**III**	**.**	**.**	**.**	**.**	**.**	**.**	**D**	**A**	**.**	**S**	**.**	**.**	**.**
The Philippines/Nueva Ecija/L1531/17[d]	**III**	**N**	**I**	**.**	**L**	**.**	**Q**	**Q**	**A**	**.**	**S**	**.**	**.**	**.**
Japan/E51057/Att-CAV	**III**	**.**	**.**	**.**	**.**	**L**	**.**	**.**	**.**	**.**	**.**	**I**	**H**	**.**
Taiwan/KY888894.1/1103TN02	**IV**	**.**	**.**	**.**	**.**	**L**	**K**	**E**	**A**	**.**	**.**	**.**	**.**	**.**
Vietnam/PT1/17	**V**	**Q**	**I**	**.**	**L**	**.**	**Q**	**Q**	**.**	**.**	**.**	**I**	**.**	**.**

[a]The consensus sequence was generated with 100 CAV sequences from GenBank using GENETYX v.10 software (GENETYX Corp., Tokyo, Japan)
[b]Position of aa on VP1 of the consensus sequence
[c]Vaccine strain sequences are marked in bold
[d]The Philippines' sequence obtained in the present study is underlined
[e]Same as the consensus

376L-413A). The L1531/17 strain had T and Q at position 89 and 394 of VP1, respectively (Table 2).

For virus isolation, three homogenized samples were inoculated in MSB1 cells, followed by ten passages every three days for two cycles. The CAV strain was not successfully recovered by the two trials.

Neither CAV infection nor the CAV genome sequences have been previously reported in the Philippines. This is the first study to report the genetic characterization of the protein-coding region (1,823 bp) of the CAV genome. In this study, the genotype III strain was detected in the Philippines. The Filipino CAV L1531/17 strain was not clustered with vaccine strains. Also, the farms involved in this study had no record of having previously utilized the CAV vaccines, proposing the circulation of the field CAV strain in chickens. Since the sample number was limited in this study, further studies should be conducted to gain broader insights of CAV infection in the Philippines.

The CAV strain acquired in this study was genetically related to Chinese, Polish, and Taiwanese CAV strains, which means CAV may have been introduced from those countries to the Philippines. Approximately 2 million one-day-old broiler breeder chickens were imported into the country in 2015 and 2016. This number increased to approximately 4 million birds in the first half of 2017 [18]. Such import activities may affect the origin of CAV in the Philippines.

The aa residue at 139 and 144 in the VP1 protein may modulate virus replication in the cell culture [19]. The reduction of virus replication was observed in the NGP-10 strain, which had a 139Q-144Q motif [19]. Similarly, the L1531/17 strain with glutamine at residues 139 and 144 was not successfully isolated in MSB1 cells in the current study, which suggests that the 139Q-144Q motif in the VP1 protein may have reduced virus replication in the cell culture. Nevertheless, the good growth of the CAV strains, which have 139Q-144Q, was observed in cell culture [2,12]. The reason for this difference is still unclear.

It has been reported that the substitution of aa at residue 394 from glutamine to histidine in the VP1 protein results in less pathogenicity [25]. In contrast, cloned isolate 10 [14] and P310 [21] strains, which show a significant reduction of pathogenicity in chickens, had glutamine at aa position 394. The 75I-89T-125L-139L-144E motif in the VP1 protein

of CAV is associated with a lower pathogenicity in chickens [23], which differed from the current Filipino CAV L1531/17 strain in this study. Additional animal studies should be conducted to properly evaluate the pathogenicity of this strain.

In summary, phylogenetic and molecular analyses revealed that the current Filipino CAV L1531/17 strain belongs to genotype III, which is genetically related to CAV strains from Poland, China, and Taiwan. Furthermore, the CAV isolate differs from vaccine strains. Additional investigation should be conducted to understand CAV infection among chicken farms and the evaluation of the pathogenicity of CAV strains and also improve the control of CAV infection in chicken production in the Philippines.

Conflict of interest

The authors declare no conflict of interest.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.68.4.249

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