NOTE

Virology

Genetic characterization of chicken anemia viruses newly isolated from diseased chicks in Japan in 2020

Hieu Van DONG[1,2)], Giang Thi Huong TRAN[1,2)], Aoi KUROKAWA[3)], Yu YAMAMOTO[3)], Yohei TAKEDA[1,4)], Haruko OGAWA[1)]* and Kunitoshi IMAI[1)]

[1)]Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan
[2)]Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Trau Quy Town, Gia Lam District, Hanoi 131000, Vietnam
[3)]National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[4)]Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan

ABSTRACT. In this study, a total of nine chicken samples obtained from two broiler flocks in Oita and Tottori prefectures in 2020 were examined for Chicken anemia virus (CAV) infection. The samples were collected from clinically suspected flocks and diseased chickens. The CAV genome was detected in all nine samples tested by real-time PCR. Phylogenetic analyses and sequence comparisons of the full-length VP1 gene sequences indicated that all the Japanese CAV strains obtained in this study formed a similar cluster of genotype III and shared high nucleotide (99.62–100%) identity. The current Japanese CAV strains were closely related to Chinese CAV strains but not related to vaccine strains. One positive selection site of VP1 was detected among the Japanese CAV strains.

KEY WORDS: chicken anemia virus, genetic characterization, genotype, Japan

Chicken anemia virus (CAV) belongs to the *Gyrovirus* genus of the *Anelloviridae* family, which is a non-enveloped virus. The viral genome has circular and single-stranded DNA, consisting of three overlapping open reading frames (ORFs), ORF1, ORF2, and ORF3. The three ORFs encode the structural capsid protein VP1 and two non-structural proteins VP2 and VP3 [13]. Genetic variability has been reported in the VP1 gene sequence, whereas VP2 and VP3 genes are highly conserved among CAV strains [4, 12]. Therefore, the VP1 gene sequence has been commonly used for the genetic characterization of CAV.

CAV has been reported worldwide, causing economic losses in chicken production. CAV spreads by both horizontal and vertical transmissions [9, 13]. Vertical transmission from antibody-negative hens (or breeders) to their progeny plays a critical role in causing clinical disease in young chickens and results in increased mortality and economic losses in the field. The vertically infected chickens (or progeny) show clinical signs, including anemia, depression, muscle hemorrhage, pale bone marrow, and thymus atrophy, at 2–3 weeks of age. Horizontal transmission occurs in older chickens causing subclinical disease. In experimentally infected chickens, clinical disease is observed in chickens younger than two weeks old, while the subclinical disease is likely recorded in chickens older than two weeks [13].

The appearance of maternal antibodies derived from vaccination completely suppressed clinical disease in young chickens [20]. CAV vaccination has been applied in breeder chickens in Japan. Therefore, it is very rare to observe clinical disease caused by CAV infection in young chickens in the country. However, we previously reported on concurrent CAV and infectious bronchitis virus (IBV) in diseased chicks in Hiroshima prefecture in 2017. Severe hematopoietic and lymphocytic depletion with abnormally large cells containing intranuclear inclusion bodies were observed in bone marrow, spleen, and liver due to CAV infection, whereas renal tubular necrosis was also recorded in diseased chicks caused by IBV infection [6]. The CAV isolated from the outbreak caused 100% anemia and 70% mortality in one-day-old chicks. Further, 80% of chicks inoculated at seven days old also developed anemia, and 10% of those died from the CAV infection, suggesting that the unusually high mortality (97.7%) in the Japanese native chicks was attributable to the co-infection of CAV and IBV. In this study, we genetically characterized the CAV based on the entire protein-coding regions of the viral genome from strains newly isolated in Japan chicks exhibiting poor growth or disease symptoms.

Nine liver samples collected from young chickens in two broiler farms in Japan from February to April 2020 were used in this study. Among those, four samples were from 27-days-old chickens on a farm in Oita prefecture, where increased mortality was experienced. The other five were from 19-days-old chickens on a farm in Tottori prefecture, having increased mortality poor growth. The CAV genome was detected in all nine chickens during disease assessment performed by the local livestock hygiene service centers. Both farms had been implementing recommended vaccination program that includes CAV vaccine. Liver samples provided to our laboratory were homogenized in phosphate-buffered saline containing kanamycin (1 mg/ml), gentamycin (100 µg/ml), and amphotericin B (10 µg/ml) as a 10% homogenate. CAV isolation was performed in MDCC-MSB1 (MSB1) cells as previously described [19].

DNA was extracted from the homogenized samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR for the CAV genome was conducted as previously described [17]. The full-length protein-coding sequence (1,823 bp) of the viral genome was amplified by PCR using three pairs of primers [22]. Those PCR products were separated on agarose gels and purified by GeneClean® II Kit (MP Biomedicals, Santa Ana, CA, USA). Nucleotide sequencing was performed in duplicates using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and the Applied Biosystems 3500 Genetic Analyzer (Life Technologies). Sequence data were aligned and analyzed by the Clustal W multiple alignment tool [16] in BioEdit v.7.2.5 [5]. The homology in the nucleotide and amino acid (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). A maximum likelihood method with the Hasegawa-Kishino-Yano model of nucleotide substitutions was used to construct the phylogenetic tree based on nucleotide sequences, and a Jones-Taylor-Thornton model of aa substitutions was used to reconstruct the phylogenetic tree using MEGA6 software [14]. The protein-coding sequences of the viral genome obtained in this study have been deposited into GenBank under accession numbers MT975518 to MT975526.

Nine Japanese CAV strain sequences obtained in this study and 14 other published Japanese sequences from GenBank were analyzed by using nine algorisms supplemented in Recombination Detection Program (RDP) version Beta 4.97, including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiSan, Phyl-Pro, LARD, and 3Seq methods [8]. The algorism that showed $P<0.05$ was regarded as reliable. Elaboration of evolutionary selection profiles was performed by following the Fast Unconstrained Bayesian AppRoximation (FUBAR) method (http://www.datamonkey.org/) [10].

The CAV genome was detected in all nine liver samples by using real-time PCR. The nine CAV strains obtained were named as Japan/Oita-1(2,3,4,5)/20 or Japan/Tottori-1(2,3,4)/20. No genetic mutations were found in the VP2 and VP3 of the nine CAV strains. Phylogenetic analysis of the full-length VP1 gene sequences (1,350 bp) indicated that the nine Japanese CAV strains of this study belonged to genotype III [17]. The current CAV strains from Oita and Tottori prefectures and previous Japanese CAV strains from Hokkaido prefecture (Japan/KJ126838/HK1/13) and Hiroshima prefecture (Japan/MK624991/HS1/17) formed a single cluster. The eleven Japanese CAV strains, including the current nine and previous two strains, were closely related to a Chinese strain (China/JS-China 6). The nine CAV strains genetically differed from the CAV vaccine strain (26P4) applied in their parent stock chickens (Fig. 1). Conversely, the phylogenetic analysis based on aa sequences indicated a result slightly different from that of nucleotide sequences. Thus, the current nine CAV strains and one previous CAV strain (Japan/KJ126838/HK1/13) formed a new cluster II, which differed from previously published Japanese CAV strains belonging to clusters I, III, and IV (Fig. 2).

Deduced aa sequences of the VP1 protein of the nine current CAV strains were compared with previously published Japanese and vaccine strains. The results indicated a unique motif containing ten aa substitutions in the variable region 75V/I-97M/L-125I/L-139E/N/Q-144E/Q-287A/T-290A/P-370S/A/T-376L/I-413A/S, among the current nine CAV strains (Table 1). Based on the aa substitutions of VP1 at residues 75, 97, 139, and 144, the Japanese CAV strains were clearly divided into three clusters I, II, and III. The clusters I, II, and III retain different aa motifs, 75V-97M/L-139K-144E, 75V-97L-139N-144Q, and 75I-97L-139Q-144Q, respectively (Table 1). The Japanese CAV strains grouping based on aa sequences did not match with that on nucleotide sequences similarly to the previous reports [11, 17], and the reasons may be due to synonymous mutations. The present study confirmed differences between the classification of CAV strains by phylogenetic analysis of nucleotide sequences and molecular analysis of aa sequences.

Recombination analyses indicated no putative recombination event ($P>0.05$) was found among the current and previous Japanese CAV strains using RDP 4 software. Analyses of natural selection profiles of the Japanese CAV sequences indicated that 69 sites of VP1 protein were found to be under negative selection. However, only one positive selection was found at residue 370 of VP1 protein (Table 2).

CAV was firstly recognized in Japan in 1979 [21]. Afterward, CAV infection has been reported in many countries worldwide [9]. However, monitoring CAV infection has not been a priority in poultry disease control. In recent years, several studies on CAV infection and its disease, which affected the chicken production industry in Japan, were reported [1, 6]. Del Valle et al. [1] reported chickens showing clinical signs of diseases caused by CAV infection in distinct farms in eastern Japan (Ibaraki, Fukushima, and Chiba prefectures) from 2013 to 2016. Later, one outbreak of CAV infection was recorded in a broiler chicken farm in Hiroshima prefecture in 2019, which caused uncommon mortality in young chickens. The CAV isolate was classified as causing high mortality in specific-pathogen-free chickens [6]. This study describes two new cases of CAV infection outbreaks and the disease caused by the infection in Japan. The affected chickens showed poor growth and increased mortality in young chickens was recorded. It has been suggested that CAV infection in the field may cause disease in young chickens with under 30% mortality. It has also been noted that CAV-affected chickens encounter increased bacterial infection or enhanced pathogenicity of IBV or adenovirus because of immunosuppression caused by CAV [13]. The present results could point out the importance of monitoring CAV infection in broiler chickens to further understand the influence of the CAV strains currently circulating in the field.
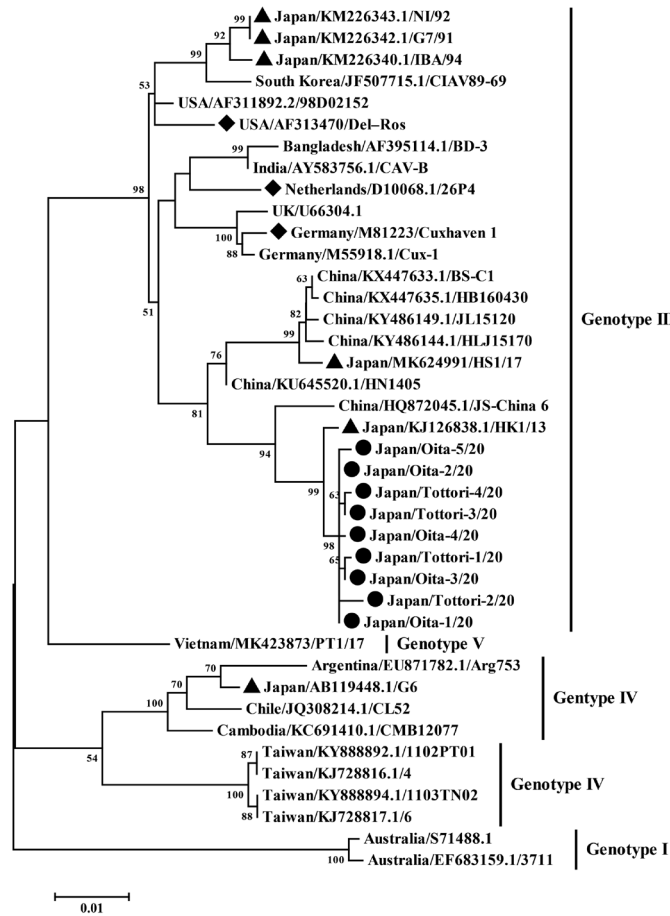
Fig. 1. Phylogenetic tree of VP1 gene sequences (1,350 bp) of the Japanese chicken anemia virus strains compared with those available in GenBank. GenBank sequences are indicated by the country name/accession number. The maximum likelihood method was used to establish the phylogenetic tree (1,000 bootstrap replicates). Number at each branch point indicates bootstrap values ≥50% in the bootstrap interior branch test. The current Japanese strains, previous Japanese strains, and vaccine strains are indicated by circles, triangles, and diamonds, respectively.
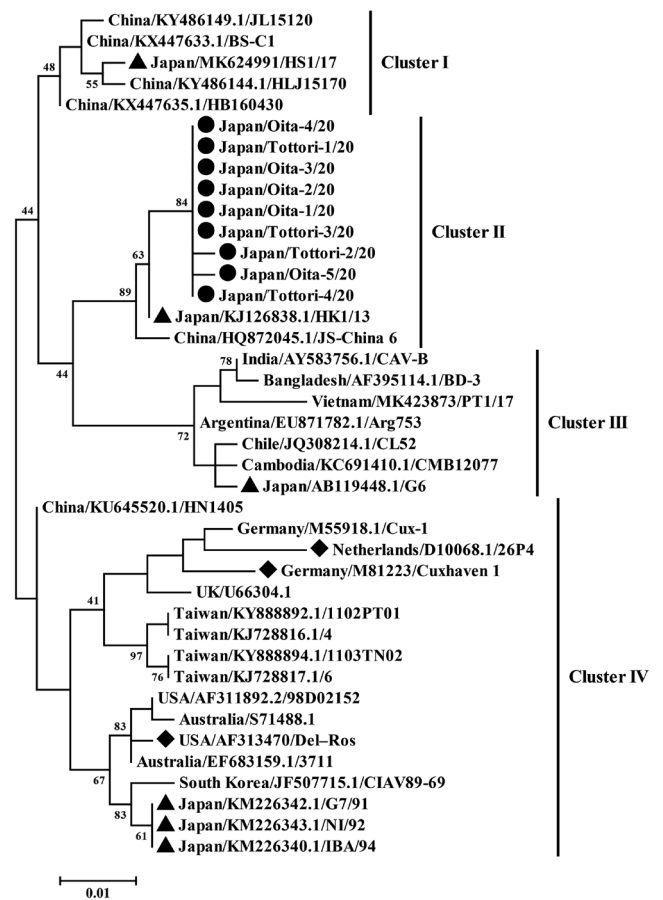
Fig. 2. Phylogenetic tree of VP1 protein sequences of Japanese chicken anemia virus strains compared with those available in GenBank. GenBank sequences are shown as the country name/accession number. The maximum likelihood method with the Jones-Taylor-Thornton model was used to establish the phylogenetic tree (1,000 bootstrap replicates). The number at each branch point indicates bootstrap values ≥50% in the bootstrap interior branch test. The current Japanese strains, previous Japanese strains, and vaccine strains are indicated by circles, triangles, and diamonds, respectively.

It was reported that the lack of a CAV vaccination program in parent flocks might result in CAV-related disease in their broiler chicken farms in Japan recently [6]. CAV is characterized as a single serotype. The presence of maternal antibodies derived from vaccination could completely suppress CAV clinical disease in young chickens [20]. According to the information provided, both flocks in Oita and Tottori prefectures experienced the CAV infection outbreak regardless of the vaccination with CAV 26P4 strain for parent stocks, indicating the maternal antibodies could not protect chicks from the CAV infection. There may be several possible reasons for the outcome, which include: low level of maternal antibody due to improper handling of vaccine or improper vaccination techniques or antibody developed by the vaccine was not effective enough against the isolated CAV strain. Additional studies are needed to understand the factors related to the CAV outbreaks.

As one of the evolutionary processes, recombination has been found in CAV in China [2, 15], Taiwan [11], Egypt [3], and Vietnam [17] based on the analyses of the complete genome or protein-coding region of the viral genome. Recombination events were commonly detected in VP1 gene sequences [2, 11, 15, 17], suggesting the frequency of recombination among CAV strains. In this study, we could not detect the evidence of recombination events among the Japanese CAV sequences. Previous studies suggested that the importance of positive Darwinian selection was regarded as a process shaping protein-coding genes evolution [7]. Wang *et al.* [18] reported that eight sites of VP1 protein were detectable to be under positive selection. The author also suggested that one selected site 287 may be linked to the virulence of CAV [18]. In the present study, only one site, 370, was an appositively selected position in the VP1 protein of the Japanese CAV strains. The role of the positive selection at 370 residues found in this study is unknown. Additional studies should be conducted to clarify this point.

In summary, we monitored CAV infection in flocks in Japan in 2020 experiencing increased mortality and poor growth, and

Table 1. Amino acid substitutions in the variable region of VP1 protein of the Japanese chicken anemia virus (CAV) strains

Sequences	Cluster	Amino acid position in VP1[b]									
		75	97	125	139	144	287	290	370	376	413
Consensus[a]		V	M	I	K	E	S	A	G	L	A
Japan/KM226342.1/G7/91	I	.[d]	.	L	.	.	.	.	.	I	S
Japan/KM226341.1/KI/80	I	.	.	.	.	.	A	.	S	.	.
Japan/KM226340.1/IBA/94	I	.	.	L	.	.	.	.	.	I	S
Japan/KM226339.1/G1/74	I	.	.	.	.	.	A	.	S	.	.
Japan/KM226338.1/AO/77	I	.	.	.	.	.	T	.	S	.	.
Japan/KM226337.1/A1/76	I	.	.	.	.	.	.	.	.	.	S
Japan/MK624991/HS1/17	I	.	L	L	.	.	T	P	.	.	.
Japan/KM226343.1/NI/92	I	.	.	L	.	.	.	.	.	I	S
Japan/Oita-1 (2,3,4,5)/20[c]	II	.	L	L	N	Q	A	P	A	.	.
Japan/Tottori-1 (2,3,4)/20	II	.	L	L	N	Q	A	P	A	.	.
Japan/KJ126838/HK1/13	II	.	L	L	N	Q	A	P	A	.	.
Japan/KJ004669.1/NI/77	III	I	L	.	Q	Q	T	P	S	.	.
Japan/KJ004667.1/G5/79	III	I	L	.	Q	Q	T	P	S	.	.
Japan/AB119448.1/G6	III	I	L	.	Q	Q	T	P	S	.	.
Japan/AB027470.1/TR20	III	I	L	.	Q	Q	T	P	S	.	.
Japan/KJ004668.1/HI/80	III	I	L	.	Q	Q	T	P	S	.	.

[a] Consensus sequence was generated with 100 CAV sequences from GenBank by the GENETYX v.10 software (GENETYX Corp., Tokyo, Japan). [b] Position of aa on VP1 protein of the consensus sequence. [c] Japanese. Sequences obtained in the present study were are underlined. [d] Same as the consensus sequence. A, alanine; E, glutamic acid; G, glycine; I, isoleucine; K, lysine; M, methionine; N, asparagine; L, leucine; V, valine; Q, glutamine; P, proline; S, serine; T, threonine.

Table 2. Positive selection in the chicken anemia virus VP1 of the the current nine Japanese and other 14 previous Japanese chicken anemia virus (CAV) strains

Site	α	β	β-α	Prob[α>β]	Prob[α<β]
370	1.31	34.35	33.04	0.00	0.99

α: indicates posterior synonymous substitution rate at a site; β: indicates posterior non-synonymous substitution rate at a site; α>β: negative selection; α<β: positive selection; α=β: neutral selection; Prob[α>β] ≥0.9: posterior probability of negative selection at a site; Prob[α<β] ≥0.9: posterior probability of positive selection at a site. One positively selective position was found at 370 position of the CAV VP1.

isolated CAVs. It is not clear how much the CAV infection was related to the diseases, however, additional studies on pathogenicity of the isolated CAV strains and further studies on CAV infection should be performed. Phylogenetic and molecular analyses revealed that the Japanese CAV strains belong to genotype III. The nine current and one previous Japanese, together with one Chinese CAV strains, formed a cluster. Molecular analysis indicated no recombination event was detected among Japanese CAV strains. However, the results obtained indicated the possible evolution of the Japanese CAV strains following positive selection.

CONFLICT OF INTERESTS. The authors declare that they have no conflict of interest.

REFERENCES

1. Del Valle, F., Camba, S., Umali, D., Shirota, K., Sasai, K., Katoh, H. and Tajima, T. 2020. The diseases suspected of the involvement of chicken anemia virus infection in 11 to 14-weeks old replacement pullets from eastern Japan: a case report. *J. Vet. Med. Sci.* **82**: 520–526. [Medline] [CrossRef]
2. Eltahir, Y. M., Qian, K., Jin, W. and Qin, A. 2011. Analysis of chicken anemia virus genome: evidence of intersubtype recombination. *Virol. J.* **8**: 512. [Medline] [CrossRef]

3. Erfan, A. M., Selim, A. A. and Naguib, M. M. 2018. Characterization of full genome sequences of chicken anemia viruses circulating in Egypt reveals distinct genetic diversity and evidence of recombination. *Virus Res.* **251**: 78–85. [Medline] [CrossRef]

4. Farkas, T., Tanaka, A., Kai, K. and Kanoe, M. 1996. Cloning and sequencing of the genome of chicken anaemia virus (CAV) TK-5803 strain and comparison with other CAV strains. *J. Vet. Med. Sci.* **58**: 681–684. [Medline] [CrossRef]

5. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**: 95–98.

6. Hosokawa, K., Imai, K., Dong, H. V., Ogawa, H., Suzutou, M., Linn, S. H., Kurokawa, A. and Yamamoto, Y. 2020. Pathological and virological analysis of concurrent disease of chicken anemia virus infection and infectious bronchitis in Japanese native chicks. *J. Vet. Med. Sci.* **82**: 422–430. [Medline] [CrossRef]

7. Jagadeeshan, S. and Singh, R. S. 2007. Rapid evolution of outer egg membrane proteins in the *Drosophila melanogaster* subgroup: a case of ecologically driven evolution of female reproductive traits. *Mol. Biol. Evol.* **24**: 929–938. [Medline] [CrossRef]

8. Martin, D. P., Murrell, B., Golden, M., Khoosal, A. and Muhire, B. 2015. RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evol.* **1**: vev003. [Medline] [CrossRef]

9. McNulty, M. S. 1991. Chicken anaemia agent: a review. *Avian Pathol.* **20**: 187–203. [Medline] [CrossRef]

10. Murrell, B., Moola, S., Mabona, A., Weighill, T., Sheward, D., Kosakovsky Pond, S. L. and Scheffler, K. 2013. FUBAR: a fast, unconstrained bayesian approximation for inferring selection. *Mol. Biol. Evol.* **30**: 1196–1205. [Medline] [CrossRef]

11. Ou, S. C., Lin, H. L., Liu, P. C., Huang, H. J., Lee, M. S., Lien, Y. Y. and Tsai, Y. L. 2018. Epidemiology and molecular characterization of chicken anaemia virus from commercial and native chickens in Taiwan. *Transbound. Emerg. Dis.* **65**: 1493–1501. [Medline] [CrossRef]

12. Renshaw, R. W., Soiné, C., Weinkle, T., O'Connell, P. H., Ohashi, K., Watson, S., Lucio, B., Harrington, S. and Schat, K. A. 1996. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *J. Virol.* **70**: 8872–8878. [Medline] [CrossRef]

13. Schat, K. A. and van Santen, V. L. 2020. Chicken infectious anemia virus and other Circovirus infections. pp 247–288. In: Diseases of Poultry, 14th ed. (Saif, A. M. F. Y. M., Glisson, J. R., McDougald, L. R., Nolan, L. K. and Swayne, D. E. eds.), John Wiley & Sons, Hoboken.

14. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [Medline] [CrossRef]

15. Tan, C., Wang, Z., Lei, X., Lu, J., Yan, Z., Qin, J., Chen, F., Xie, Q. and Lin, W. 2020. Epidemiology, molecular characterization, and recombination analysis of chicken anemia virus in Guangdong province, China. *Arch. Virol.* **165**: 1409–1417. [Medline] [CrossRef]

16. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680. [Medline] [CrossRef]

17. Van Dong, H., Tran, G. T. H., Van Nguyen, G., Dao, T. D., Bui, V. N., 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**: 643–653. [Medline] [CrossRef]

18. Wang, D., Fan, W., Han, G. Z. and He, C. Q. 2009. The selection pressure analysis of chicken anemia virus structural protein gene VP1. *Virus Genes* **38**: 259–262. [Medline] [CrossRef]

19. Yuasa, N. 1983. Propagation and infectivity titration of the Gifu-1 strain of chicken anemia agent in a cell line (MDCC-MSB1) derived from Marek's disease lymphoma. *Natl. Inst. Anim. Health Q. (Tokyo)* **23**: 13–20. [Medline]

20. Yuasa, N., Noguchi, T., Furutac, K. and Yoshida, I. 1980. Maternal antibody and its efect on the susceptibility of chicks to chicken anemia agent. *Avian Dis.* **24**: 197–201. [CrossRef]

21. Yuasa, N., Taniguchi, T. and Yoshida, I. 1979. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Dis.* **23**: 366–385. [CrossRef]

22. Zhang, X., Liu, Y., Wu, B., Sun, B., Chen, F., Ji, J., Ma, J. and Xie, Q. 2013. Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. *Sci. Rep.* **3**: 3519. [Medline] [CrossRef]