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NOTE

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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

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ABSTRACT. Three strains of chicken anemia virus (CAV) were detected in 11 to 14-weeks old chickens, showing depression, wasting, and increased mortality, from three farms in eastern Japan. Another strain was detected in 12-weeks old chickens from one farm without clinical signs. Bacterial infections were suggested in three farms with clinical signs and its involvement in the occurrence of the diseases might be suspected. Sequence analysis of the VP1, VP2, and VP3 genes of four CAV strains revealed that the three from farms with clinical signs belonged to genotype A2, whereas that from the apparently-normal farm belonged to A3. This may be a rare case report about the diseases suspected of the involvement of the CAV infection in older birds.

KEY WORDS: chicken anemia virus, east Japan, gangrenous dermatitis, phylogenetic tree, replacement pullet

Chicken anemia virus (CAV) was first isolated in Japan by Yuasa *et al*. [17]. The virus belongs to the genus *Gyrovirus* under the family *Anneloviridae* [12]. The virion is small [7], non-enveloped and contains a single-stranded negative sense DNA genome [2, 11]. This virus is considered to be a ubiquitous poultry pathogen present worldwide [9]. Generally, it is understood that this virus affects mainly young birds up to 3-weeks old [9]. Affected birds which have no maternal antibodies show syndrome with anemia, anorexia, depression, immunosuppression, and increased mortality. This can lead to complications because of opportunistic infections [10, 11]. When birds older than 3 weeks are infected, the infection is usually subclinical [8, 11], however, clinical disease in birds older than 3 weeks has been reported in Israel [3], and Bulgaria [14]. These clinical cases might have occurred in Japanese pullets also. However, these cases may have been misdiagnosed or unrecognized as CAV infections. The diagnosis might have leaned towards other diseases which show similar symptom/syndrome, such as infectious bursal disease (IBD) [1]. This study was conducted to confirm CAV infections in late stage of replacement pullet flocks from eastern Japan. The four strains, described in this report, were classified and compared with local and foreign CAV strains by genetic analysis.

The target for this study was four pullet-farms in eastern Japan. Two of them were located in Fukushima prefecture, one in Ibaraki prefecture and one other farm was in Chiba prefecture (Table 1). Farms A, B, and D had multiple houses and each house contained one or more flocks. Farm C had one flock in one house. The parent-flocks of farms A, B, and C came from the same line of White Leghorn from the same hatchery, and different parent stock. Sampled birds from farm D were brown layers, these came from a hatchery different from farms A, B, and C. The farms employed standard management and rearing procedures. Vaccinations included Marek's disease virus (MDV) Rispens CVI 988 strain and herpes virus of Turkey FC126 strain, IBD virus (IBDV) K strain, fowl pox virus Beaudette strain, infectious laryngotracheitis virus SPL strain, and egg drop syndrome-76 virus. Vaccinations against *Salmonella* Enteritidis infection and infectious coryza Types A and C were also performed. CAV 26P4 strain live vaccine was given to parent flocks only. All flocks, parent included, employed the same management and vaccination programs except for the *Salmonella* Enteritidis vaccine.

Depression, wasting, and increased mortality of chickens were observed in three of these farms. In farm A, there were 7 houses; 1 to 6 had one flock per house and 7 had two flocks. The clinical signs were observed in 2014 in houses 5 and 6, and sometimes observed in house 5 in 2018. In this report, the case of house 5 in 2014 was described. In farm B, there were 6 houses and each

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house had one flock. The clinical signs were first observed in 2013 in house 5, however, these cases were not examined thoroughly. Similar cases were observed again in a new flock in house 5 in 2015; which was described here. The signs were observed only during 2016 in farm C. The investigations were done at the time when the mortality was increased (Table 1). The birds from farm D did not show signs similar with the other 3 farms, and the birds were taken for comparison purposes only.

In the investigation, the daily report of each flock was requested, and the accumulated mortality for the 3-week period, including sampling date in each sampled house was calculated (Table 1). The accumulated mortality of the apparently normal flocks was 0% to 0.04% in farms, including farms B and D, in Fukushima prefecture where we had periodical health examination. In contrast, the accumulated mortalities of farms A and B were 0.24% and 0.2% during 3 weeks. On the other hand, accumulated mortality of farm D was 0.03%. In farm C, the accumulated mortality was noticeably increased (3.45%) as compared to farms A and B. Figure 1 showed the weekly mortality of the sampled house of each farm and the spike in the mortality rate of farm C can be seen clearly.

Samples collected from these four farms underwent necropsy. In the flocks of farms A, B, and C, the gross findings were exemplified by subcutaneous edema, gangrenous dermatitis, hemorrhage in muscle and/or proventricular membrane, splenomegaly, pale spleen, pale kidney, swollen kidney, and pale liver. Seemingly, sample birds on farms B and C had similar gross finding as farm A, however, there were slight differences. The fatty and pale features were confirmed in bone marrow in some of the birds with severe symptoms from farms A, B, and C. Characteristic eyelid and facial swelling were observed in the flock of farm B only. In addition, slight nephritis and hepatitis were confirmed in some birds from farm C. Based on the necropsy observations, birds from farm D were apparently healthy.

Birds from farms A, B, and C were checked for bacteria with some organs. As shown in Table 2, *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli* was isolated from the dermal tissue, parenchymal organs, and eyelid of the birds from farms A and B. Additionally, *Pseudomonas* spp. was isolated from some organ samples of birds from farm C.

Viral infection was suspected based on symptom and necropsy observations. CAV infection shows gross findings such as pale or fatty bone marrow, pale parenchyma organs, and gangrenous dermatitis. Fatty bone marrow is accepted as a characteristic finding of CAV infection. IBDV infection in the field can lead to an increased mortality, especially in young birds. Although no bursal hemorrhaging was observed, increased mortality was observed in farms A, B, and C. Infectious bronchitis virus (IBV) is known to cause green or white diarrhea, but it may show kidney swelling as well. Kidney swelling was observed in the birds from farm B.

Considering these observations, sampled portions of each organ collected were tested by PCR or reverse transcriptase PCR (RT-PCR) against CAV, MDV, and IBV using previously described methods [4–6] (Table 3). For PCR and RT-PCR, pooled parenchyma

Table 1. Informations of the sampled farms

Farm	Location	Population of sampled house	Hatching date	No. of bird samples	Date collected	Age (wk)	Mortality of sampled house	
							Sampling date	3-week period[a)] (including sampling day)
A	Ibaraki prefecture	84,542	2014/8/19	8	2014/11/25	14	0.002%	0.24%
B	Fukushima prefecture	42,189	2014/10/24	8	2015/1/15	11	0.002%	0.20%
C	Chiba prefecture	38,728	2016/8/11	4	2016/11/12	12	0.105%	3.45%
D	Fukushima prefecture	9,200	2016/10/21	4	2017/1/12	12	0%	0.03%

a) Accumulated mortality.

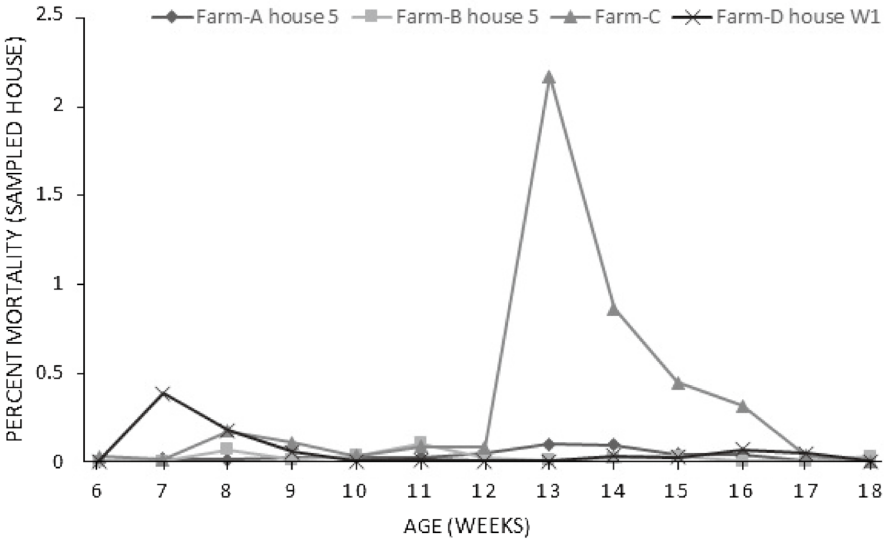

Fig. 1. Weekly mortality of sampled house in farms A, B, C, and D.

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Table 2. Bacterial isolation of sampled birds

Farm	Organ	Bacterial Isolation			
		Staphylococcus spp.	*Streptococcus* spp.	*Escherichia coli*	*Pseudomonas* spp.
A	Heart	5/8[a)]	0/8	3/8	0/8
	Liver	8/8	0/8	8/8	0/8
	Spleen	8/8	0/8	8/8	0/8
	Kidney	8/8	1/8	8/8	0/8
	Dermal lesions	8/8	0/8	8/8	0/8
B	Heart	5/8	0/8	3/8	0/8
	Liver	8/8	0/8	7/8	0/8
	Spleen	8/8	0/8	7/8	0/8
	Kidney	8/8	1/8	7/8	0/8
	Dermal lesions	8/8	0/8	7/8	0/8
	Infraorbital exudates	8/8	0/8	7/8	0/8
C	Heart	1/4	0/4	2/4	0/4
	Liver	1/4	0/4	4/4	0/4
	Spleen	0/4	0/4	4/4	0/4
	Kidney	0/4	0/4	4/4	0/4
	Lung	0/4	0/4	2/4	2/4
	Trachea	2/4	0/4	3/4	2/4
	Dermal lesions	0/4	0/4	4/4	0/4

a) Positive/Tested.

Table 3. Primers and PCR conditions used for viral genome detection and sequencing

Assay	Target virus	Primer			Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles	Expected size (bp)	Reference
PCR	Chicken Anemia Virus	CAV-1	Screening/ sequencing	GCAGTAGGTATACGCAAGGC	94°C	94°C	60°C,	72°C	72°C	35	186	[4]
		CAV-2		CTGAACACCGTTGATGGTC	2 min	30 sec	30 sec	1 min	7 min			
	Chicken Anemia Virus	VP1F	Sequencing	AGCCGSCCCCGAACCGCAAGAA	94°C	94°C	60°C	72°C	72°C	34	1,390	[4]
		VP1R		TCAGGGCTGCGTCCCCCAGTACA	4 min	1 min	1 min	1 min	15 min			
	Chicken Anemia Virus	VP2F	Sequencing	GCGCACATACCGGTCGGCAGT	94°C	94°C	63°C	72°C	72°C	34	731	[4]
		VP2R		GGGGTTCGGCAGCCTCACACTAT	4 min	1 min	1 min	1 min	5 min			
	Marek's Disease Virus	Oligo1 5′	Screening	TGCGATGAAAGTGCTATGGAGG	94°C	94°C	55°C	72°C	72°C	35	132	[13]
		Oligo2 3′		CGCGAAAGAGTATCCCTAAGAG	1 min	1 min	1 min	3 min	5 min			
	Marek's Disease Virus	Meq 5′	Screening	GGCACGGTACAGGTGTAAAGAG	94°C	94°C	56°C	72°C	72°C	34	1,080	[15]
		Meq 3′		GCATAGACGATGTGCTGCTGAG	5 min	1 min	1 min	1.5 min	10 min			
RT-PCR	Infectious Bursal Disease Virus	V1	Screening	CCAGAGTCTACACCATAA	94°C	94°C	57°C	72°C	72°C	34	472	[6]
		V2		CCTGTTGCCACTCTTTCGTA	4 min	30 sec	30 sec	40 sec	5 min			
	Infectious Bronchitis virus	S1	Screening	AGGAATGGTAAGTTRCTRGTWAGAG	94°C	94°C	55°C	74°C,	74°C,	34	670	[5]
		S2		GCGCAGTACCRTTRAYAAAATAAGC	2 min	30 sec	30 sec	30 sec	5 min			

organs and thymus of sampled bird from farm A were used. Sampled organs from farms B and C were used separately, and pooled spleen, thymus and bone marrow from farm D were used. In farm C, lymphoma-like lesions in the liver of some birds were present, thus, MDV-PCRs [13–15] were performed additionally. DNA and RNA were extracted from the selected organs (Table 4) using the QIAamp DNA Mini Kit and Viral RNA Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. CAV screening by PCR was performed using primers for the VP3 gene [4] (Table 3). RNA was reverse transcribed to cDNA by using random hexamers and Primescript Reverse Transcriptase (Takara Bio Inc., Kusatsu, Japan). RT-PCR for IBV and IBDV was performed on samples from farms A, B, and C using previously described methods [5, 6]. All reactions were performed using KOD Dash (Toyobo, Osaka, Japan), and PCR products were analyzed using 1.2% agarose gel electrophoresis (Fig. 2 and Table 4).

All of the tested birds from farms A and B yielded positive results against CAV by PCR assay. On the other hand, positive birds

Table 4. PCR results of sampled birds from each farm

Farm	Samples	PCR				
		Chicken anemia virus	Infectious bronchitis virus	Infectious bursal disease virus	Marek's disease virus[d]	
					All types	Pathogenic
A[b]	Pooled parenchyma organs	8/8[a]	0/8	0/8	Not tested	
	Thymus	8/8	0/8	0/8		
B[c]	Heart	4/4	0/4	0/4		
	Liver	4/4	0/4	0/4		
	Spleen	4/4	0/4	0/4		
	Kidney	4/4	0/4	0/4		
	Thymus	4/4	0/4	0/4	Not tested	
	Lung	4/4	0/4	0/4		
	Payer's patch	4/4	0/4	0/4		
	Bursa	3/4	0/4	0/4		
	Caecal tonsils	4/4	0/4	0/4		
	Bone marrow	4/4	0/4	0/4		
C	Spleen	1/4	0/4	0/4		
	Thymus	2/4	0/4	0/4	3/4	0/4
	Bone marrow	2/4	0/4	0/4		
D	Pooled spleen, thymus and bone marrow		0/4	0/4		
		3/4	0/4	0/4	Not tested	
			0/4	0/4		

a) Positive/Tested. b) In farm A, bird-8 with weak positive result. c) In farm B, only 4 birds with the most significant necropsy findings were selected for PCR. d) Two sets of MDV-PCR primers were used; one specific to pathogenic strains only, and one able to amplify all strains including non-pathogenic vaccine.

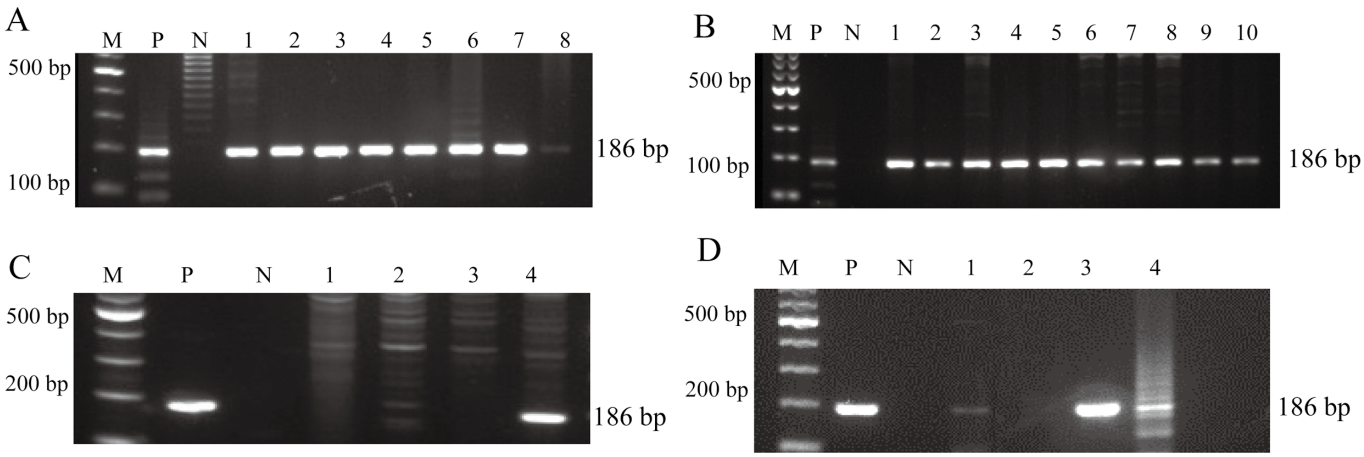

Fig. 2. Detection of chicken anemia virus (CAV) genes. M: 100-bp ladder; P: Vaccine (positive control); N: negative control. A: CAV detection in pooled organ samples from farm A. 1-8: bird 1-8, respectively (bird 8 weak-positive result). B: CAV detection in a single bird sample (designated bird 1) from farm B. 1: bone marrow; 2: thymus; 3: cecal tonsils; 4: bursa; 5: Payer's patch; 6: lung; 7: kidney; 8: spleen; 9: liver; 10: heart. C: CAV detection in spleen samples from farm C. 1-4: bird 1-4, respectively. D: CAV detection in pooled organ samples from farm D. 1-4: bird 1-4, respectively.

from farms C and D were 2/4 (50%) and 3/4 (75%), respectively. All samples were negative for IBV and IBDV. Pooled organ samples from farm C were MDV-positive by PCR. However, against pathogenic MDV, the result was negative (Table 4).

A second set of PCRs was performed on CAV-positive samples. For farm A, CAV-positive thymuses from all 8 birds were pooled. For farm B, wherein only 4 birds with the most lesions were screened, the thymuses were pooled. For farm C, CAV-positive thymus, spleen, and bone marrow were pooled. For farm D, thymus, spleen, and bone marrow from 2 CAV-positive birds were pooled (Table 4). Previously described methods were used for sequencing of the VP1, VP2, and VP3 genes [4]. PCR products were analyzed by 1.2% agarose gel electrophoresis and purified from the gel using QIAquick Gel Extraction Kit (Qiagen). The nucleotide sequences were determined using the Big Dye terminator cycle-sequencing kit version 3.1 and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). DNA products were sequenced in both directions. Phylogenetic analysis, sequence assembly, and editing were performed using Codon Code Aligner (v.3.7.1, CodonCode Corporation, Centerville, MA, USA) and ClustalX (v. 2.1, Conway Institute UCD. Dublin, Ireland). Identity and homology confirmation were performed using NCBI BLAST. Phylogenetic and molecular evolutionary analyses were based on the VP1, VP2, and VP3 gene sequences using MEGA 6. Phylogenic analysis of nucleic acid sequences was performed using the neighbor-joining method, and the Kimura 2-parameter model. Relevant VP1, VP2, and VP3 sequences, available in GenBank, were used for comparison. Multiple sequence alignment was performed on the field isolates using Clustal Omega (European Molecular Biology Laboratory-European Bioinformatics Institute, Cambridgeshire, UK).

The strains detected were named Japan/Ibaraki/Sashima/2014 for farm A-strain; Japan/Fukushima/Tamura/2015 for farm B-strain; Japan/Chiba/Sousa/2016 for farm C-strain; and Japan/Fukushima/Date/2017 for farm D-strain. All have been submitted to GenBank with pending accession numbers. As shown in Table 5, the nucleotide sequences of these 4 strains are closely related to each other.

The phylogenetic tree showed that the viruses from farms A, B, and C were under genotype A2; together with the vaccine strain 26P4 (D10068.1) which is available in Japan (Fig. 3). Also included in the genotype A2 were the Japanese isolates CAA82-2 (D31965.1) and A2 (AB031296), and the vaccine strain Cuxhaven-1 (M8 1223.1). The virus from farm D was under genotype A3 with the Japanese isolate AH9410 (AB046590). Other Japanese isolates G6 (AB119448) and TR20 (AB027470) in genotype D2. The strains in this study were not so much clustered with other Japanese strains and they appear closer to Chinese isolates. The strains from farms A and B were the closest to LN15170 (KY486155.1) with 99.43% and 99.55% homology, respectively. The one from farm C was the closest to JL15120 (KY486149.1) at 99.75%. The strain from farm D was closely related to Chinese isolate GX1804 (MK484615.1) with 99.62% homology.

Many CAV strains in China belong to genotypes A2 and A3 [4]. Strains in genotype A1, B, and D1 are mostly from China. On the other hand, two strains in genotype C are from Australia; and those in genotype D2 are from India, Malaysia and Japan. The number of registered Japanese CAV strains in Genbank is not many, and these were only registered between 1999 and 2001. More recent CAV sequencing data in Japan should be collected, and compared with each other, for better understanding of the virus characteristics and epidemiology of cases.

The three strains belonging to A2 were detected in pullets with clinical signs, while one belonging to A3 from apparently normal birds. Previously, Yuasa and Imai reported that there is no great difference in pathogenicity among CAA strains isolated in Japan [16]. Among the CAA strains they used, A2 belonged to genotype A2 and G6 belonged to genotype D2 (Fig. 3). To speculate the differences of pathogenicity among four present strains was not possible from the genetic analysis in this study. Bacterial examination suggests the occurrence of staphylococcal infection in farms A and B. Also *E. coli* was isolated from many samples of farms A, B, and C. The involvement of these bacterial infections in the occurrence of the diseases might be suspected. However, bacterial examination for farm D was not performed and the exact relationship between bacterial and viral infections was unknown. More minute investigations are necessary to clarify the cause of different clinical signs.

Clinical CAV disease in later stages of growing seems to be uncommon, however, it does occur and has been described previously [3, 14]. When clinical infection does occur, diagnosis may be difficult because the symptoms are similar to other diseases; such as IBD [1]. Additionally, other opportunistic pathogens are able to cause complications [9]. It may be meaningful to know the prevalence of CAV in older flocks.

In farms A and B, the similar clinical signs were observed periodically even in new flocks. Though we could not clarify whether the pathogen was same or not, careful observations will be necessary for these farms.

In conclusion, these results indicated the possibility that CAV and bacterial coinfection occurred in late-stage replacement pullets. It is most likely that the combined effects of coinfection led to the clinical signs. On the other hand, CAV was also detected in asymptomatic birds in other farm. The three virus strains from the diseased chickens and one from the normal chicken belonged to

Table 5. Nucleotide sequence identity of the field strains based on the VP1, VP2, and VP3 gene sequences

Strain	Nucleotide sequence identity (%)			
	Japan/Fukushima/ Date/2017	Japan/Fukushima/ Tamura/2015	Japan/Ibaraki/ Sashima/2014	Japan/Chiba/ Sousa/2016
Japan/Fukushima/Date/2017	100.00	97.58	97.45	97.83
Japan/Fukushima/Tamura/ 2015	97.58	100.00	99.30	99.43
Japan/Ibaraki/Sashima/2014	97.45	99.30	100.00	99.49
Japan/Chiba/Sousa/2016	97.83	99.43	99.49	100.00

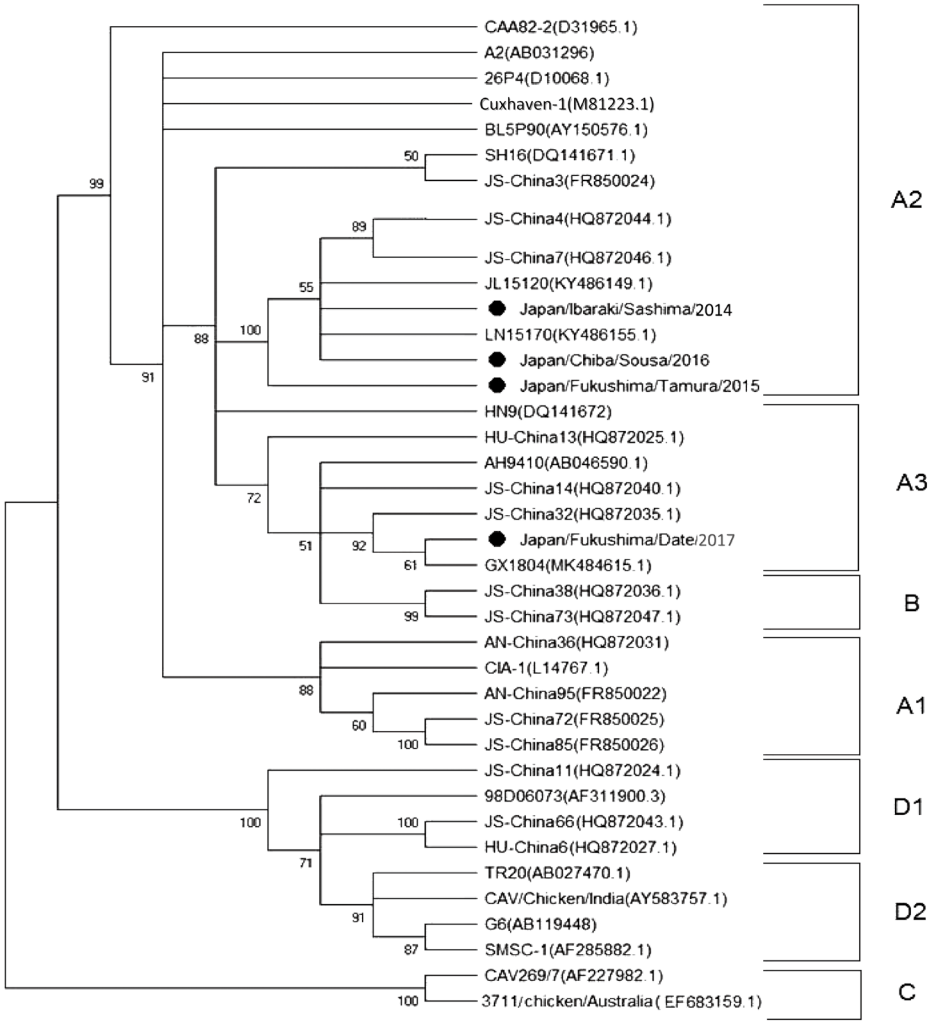

Fig. 3. Phylogenetic analysis of VP1, VP2, and VP3 genes of chicken anemia virus strains. Bootstrap values (1,000 replications) were indicated in each tree.

different genotypes. However, the relationship between the difference of genotype and clinical disease is unknown.

This may be a rare case report about the diseases suspected of the involvement of the CAV infection in older birds. This study may provide additional information regarding the CAV infections in Japan.

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