



Pathological and virological analysis of concurrent disease of chicken anemia virus infection and infectious bronchitis in Japanese native chicks

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ABSTRACT. A concurrent infection of chicken anemia virus (CAV) and infectious bronchitis virus (IBV) was detected in Japanese native chicks in 2017, in which a high mortality rate (97.7%) was recorded in a small flock of 130 chicks exhibiting poor growth. Histological examination revealed that the affected chicks exhibited two different pathological entities: one was severe hematopoietic and lymphocytic depletion with abnormally large cells containing intranuclear inclusion bodies of CAV, whereas the other was renal tubular necrosis due to IBV infection. Immunohistochemistry detected CAV antigens in the bone marrow, liver, and spleen as well as IBV antigens in the kidneys, trachea, and air sacs. CAV was isolated from the liver sample of the chicks, and the isolated strain was designated as CAV/Japan/HS1/17. A phylogenetic analysis of the CAV VP1 gene revealed that CAV/Japan/HS1/17 is genetically similar to Chinese strains collected from 2014 to 2016. An experimental infection was performed using CAV/Japan/HS1/17 and specific-pathogen-free chicks to determine the pathogenicity of CAV/Japan/HS1/17. The isolate caused 100% anemia and 70% mortality to chicks inoculated at one day old, 80% of chicks inoculated at seven days old also developed anemia, and 10% died from CAV infection. These results suggest that the unusually high mortality in Japanese native chicks can be attributed to dual infection with both CAV and IBV. The results of the experimental infection suggest that CAV/Japan/HS1/17 has a pathogenic potential to specific-pathogen-free chicks and a relatively higher pathogenicity than previous Japanese CAV strains.

KEY WORDS: chickens anemia virus, infectious bronchitis virus, Japanese native chicken, pathology, virology

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Chicken anemia virus (CAV) infection or chicken infectious anemia is a viral disease of chickens clinically characterized by anemia and immunosuppression [19, 21, 25]. CAV is a non-enveloped, single-stranded DNA virus belonging to the genus *Gyrovirus* in the family *Anelloviridae* [16]. It was first detected in Japan in the 1970s [42]. Subsequently, CAV culturing became possible using MDCC-MSB1 cells derived from a lymphoma of Marek's disease [36]. CAV is ubiquitous in the environment and highly resistant to heat and various chemical agents [8, 20, 38, 42].

The transmission of CAV among chickens occurs along both vertical and horizontal routes [13, 40, 42]. Chickens of all ages are susceptible to CAV [19]. In the field, the clinical disease generally occurs in chicks around two weeks of age without a maternal antibody against CAV [4, 5, 9–11, 43]. Vaccination of the parent flock before laying eggs is important in preventing vertical transmission of the virus and in giving the maternal antibody to chicks [5, 43]. CAV infection in older chickens can be subclinical [13, 26, 32, 42], but the virus can persist within the infected body for a long period of time [1, 3, 14, 21, 44].

CAV primarily infects the hematopoietic progenitor cells of the bone marrow and lymphocytes of the thymus and spleen [14, 27, 29, 30, 42]. However, the replication site of CAV in chickens remains to be fully elucidated because CAV can be detected by isolation, immunohistochemistry (IHC), or other methods in tissues aside from bone marrow and lymphoid organs

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[1, 4, 27, 44]. Therefore, more information regarding its viral distribution in infected birds is necessary in order to determine the pathogenesis of CAV infection.

A clinical outbreak of CAV infection is currently rare in Japan because of vaccination of commercial poultry. However, in 2017, we encountered a concurrent infection of CAV and infectious bronchitis virus (IBV) in a flock of Japanese native chicks. Here, we will report the pathological and virological findings of the outbreak as well as certain characteristics of an isolated CAV.

MATERIALS AND METHODS

Field case samples

Samples were collected from three dead Japanese native chicks (Nos. 1–3) during an outbreak in the Hiroshima prefecture of Japan in 2017. Pathological, virological, and bacteriological analyses were performed for the diagnosis. A detailed case history is outlined in the results section.

Histopathology

The tissues of the three chicks were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 2–3 μm . The paraffin sections were stained by hematoxylin and eosin (HE) and Gram's method for histological evaluation.

IHC

IHC was performed to detect avian viruses. The primary antibodies used were as follows: mouse monoclonal antibodies to CAV (clone MoCAV/E6, 1:8,000 dilution, laboratory-made) [33], IBV (clone IB95, 1:5,000 dilution, Hytest Corp., Turku, Finland), infectious bursal disease virus (IBDV) (clone IBDV105, 1:2,000 dilution, Hytest Corp.), type A influenza virus (AIV) (clone GA2B, 1:500 dilution, Abcam Corp, Cambridge, UK), and Newcastle disease virus (NDV) (clone 22C483, 1:12,800 dilution, laboratory-made) [35] and rabbit immune serum against fowl adenovirus (FAV) (1:5,000 dilution, laboratory-made) [22].

The antigen retrieval methods used in IHC were enzyme digestion using 0.1% actinase-E (Kaken Pharmaceutical Corp., Tokyo, Japan) solution at 35°C for 10 min to detect NDV and FAV and heating using a microwave oven at 500 W for 15 min to detect the other viruses. A 5% skim milk solution was used as a blocking agent. The primary antibodies were applied overnight at 4°C, and a Histofine Simple Stain MAX PO kit (Nichirei Biosciences Inc., Tokyo, Japan) was used as the secondary antibody. The slides were counterstained with Mayer's hematoxylin. The positive reaction was visualized using an AEC Substrate Kit (Nichirei Biosciences Inc.). Positive control sections for each virus were prepared from past stored samples. The tissues of normal specific-pathogen-free (SPF) chickens were used as the uninfected control.

Bacteriology

The brain, trachea, lung, heart, liver, kidney, and intestine of the three chicks were tested for bacterial isolation using DHL agar plates under aerobic conditions at 37°C for 24 hr and 5% sheep blood agar plates under anaerobic conditions at 37°C for 48 hr.

Virus isolation

MDCC-MSB1 cells were used to isolate CAV from a 10% liver homogenate of the three chicks. The MDCC-MSB1 cells were cultured at 39°C in an RPMI-1640 medium, supplemented with 10% fetal bovine serum, 10% Daigo's GF21 growth factor (Wako Pure Chemical Corp., Osaka, Japan) and antibiotics. The cells were inoculated with the supernatant of the liver homogenates and passaged every 2–3 days until cytopathic effects were observed, as previously described [33, 36]. An indirect immunofluorescence assay using chicken antiserum against the CAV A2 strain was performed to confirm the presence of CAV according to the method used by Yuasa *et al.* [41]. Embryonating chicken eggs and primary chicken kidney cells were also used for virus isolation from the brain, trachea, lung, heart, liver, kidney, and intestine.

Nucleotide sequencing of the CAV gene

DNA was extracted from the liver homogenate of the chicks via a High Pure PCR Template Preparation Kit (Roche Corp., Berlin, Germany). PCR was performed to amplify the full-length protein-coding sequence of the CAV VP1 gene using a primer set, namely, CQ1F/CQ1R [45], in addition to two pairs of primers designed in this study, namely, 1247F/1956R and CA42F/CA37R (the sequence of the primers is available upon request).

PCR amplification was carried out using GoTaq Green Master Mix (Promega Corp., Madison, WI, USA) via the following cycling profile: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation, and then annealing and extension at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min and 30 sec, respectively. The PCR products were purified using a GENECLAN II Kit (MP Biomedicals, Solon, OH, USA). The purified DNA samples were used as templates for nucleotide sequencing.

Nucleotide sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The obtained nucleotide sequences were analyzed using GENETYX Ver.10 software (GENETYX Corp., Tokyo, Japan) and then compared with the sequences of other CAV strains using the Basic Local Alignment Search Tool in the National Center for Biotechnology Information database. The sequenced data were aligned using the ClustalW [31] and BioEdit software. The phylogenetic tree of the CAV VP1 gene was constructed using the maximum likelihood method and bootstrap analysis (1,000 replicates) via the MEGA 6.0 software [28].

Experimental infection of isolated CAV

The pathogenicity of the CAV isolated from the chick was determined by experimental infection. A total of 23 Line-M SPF chicks (*Gallus gallus domesticus*) were purchased from Nippon Institute for Biological Science (Hokuto, Japan) for the experiment. We principally followed the experimental conditions described by Yuasa *et al.* [39]. The chicks (Nos. 4–26) were randomly assigned into three groups in three separate isolators: Group A, one-day-old inoculation (n=10, Nos. 4–13); Group B, seven-day-old inoculation (n=10, Nos. 14–23); and Group C, uninoculated control (n=3, Nos. 24–26). The inoculum of CAV for experimental infection was prepared from the supernatant of the fifth passage of MDCC-MSB1 cells used for virus isolation from Japanese native chicks. Prior to inoculation, the inoculum of CAV was treated at 56°C for 30 min to inactivate possibly contaminated Marek's disease virus in the MDCC-MSB1 cells [2, 8]. The chicks in Groups A and B were intramuscularly inoculated with 0.1 ml of inoculum containing 10^{5.5} 50% tissue culture infectious doses of the isolated virus via the thigh muscle. The inoculated chicks were provided feed and water *ad libitum* and observed for 20 days. Anemia was checked on Days 14 and 20 post-inoculation (pi) by a blood test and defined by hematocrit (Ht) values of 27 or less. On Day 20 pi, the body weight of the chicks was measured, and then, all chicks were euthanized via an overdose intravenous injection of pentobarbital sodium (100 mg/kg body weight). All euthanized chicks and birds that died during the experiment were necropsied for pathological evaluation and IHC to detect CAV antigens. CAV re-isolation was performed from the livers collected at necropsy. All experimental procedures involving birds were approved by the Ethics Committee of the National Institute of Animal Health, Japan (authorization number 18-015).

RESULTS

Case history

Concurrent infection of CAV and IBV occurred in a Japanese native chicken farm in the Hiroshima prefecture. In May 2017, a flock of 130 birds of day-old chicks (*Gallus gallus domesticus*) for meat production were introduced to the farm from another prefecture. The chicks were raised in hand-made cages. The farm-made feed for the chicks was composed of raw rice (50%), rice bran (30%), fish meal (10%), crushed soybeans, and oyster shells. Many chicks started exhibiting anorexia, diarrhea, and lethargy on the fourth day after introduction. The daily mortality of the flock sharply increased approximately 14 days after introduction. A total of 127 chicks (97.7%) died within 20 days after birds were introduced to the farm. Three chicks (Nos. 1–3) found dead 15 days after introduction were necropsied by veterinarians, and tissue samples were collected for diagnosis. The chicks in the flock had not been vaccinated for any infectious disease before or after hatching. The anemic status of the flock was also unknown.

Epidemiological investigation revealed that the parent flock of the chicks had a record of vaccination against NDV, IBDV, IBV, avian reovirus, fowlpox virus, and Marek's disease virus but not against CAV. The serum of the parent flock was not available, so the immune status of the parents against CAV was unknown.

Necropsy findings

All three chicks were severely emaciated with very poor growth. Their body weight was 39–43 g, less than one-third of the standard body weight at their age. The bursa of Fabricius (BF) and thymus went unnoticed, likely because of their significantly atrophied size. Hemorrhage in the skin or muscle was not observed.

Histological findings

Major histological lesions associated with CAV were observed in the bone marrow, liver, and spleen. The bone marrow of all chicks exhibited severe depletion of all hematopoietic lineages. The hematopoietic tissue had been replaced by loose connective tissue, with remaining hematopoietic cells and erythrocytes (Fig. 1A). Rarely, abnormally large cells with a pale bizarre nucleus and small amount of cytoplasm were scattered individually in both the loose connective tissue and vascular space of the bone marrow. The large cells in the vasculature were sometimes attached to the capillary walls (Fig. 1B). Some large cells contained singular or a few small eosinophilic intranuclear inclusions, suggestive of CAV infection.

In the liver of two chicks (Nos. 1 and 3), rare foci of hepatocellular necrosis with large cells containing intranuclear inclusions were discovered (Fig. 1D). The large cells were also observed in the foci of the extramedullary hematopoiesis (Fig. 1E) and lymphoid nodules located near the hepatic triad. On occasion, large cells were individually present in the hepatic sinusoid. Many Kupffer cells were enlarged and often contained cellular debris. Fibrin thrombi and small colonies of Gram-negative bacteria were also occasionally observed in the hepatic sinusoid of chicks Nos. 2 and 3.

The lymphoid tissue in the spleen of all chicks was diffusely atrophied, and a few large cells with intranuclear inclusions were scattered throughout the spleen (Fig. 1G). Focal fibrinoid necrosis of the splenic ellipsoids was observed in chick No. 3, and some foci were accompanied by Gram-negative bacterial colonies. For histopathological evaluation, the thymus sample was collected only from chick No. 2, and both the thymic cortex and medulla were diffusely atrophied because of lymphocyte depletion.

Large cells with intranuclear inclusions were observed only rarely in the connective tissue of the heart and kidney as well as in the lymphoid follicles of the duodenum and cecal tonsil.

Histological changes associated with IBV were found in the kidney (Fig. 1H). Focal renal tubular necrosis with occasional heterophilic infiltrations and cellular casts in the lumen were found. Mild to moderate inflammatory infiltrations and hyperplastic epithelium with loss of cilia were observed in the trachea. The air sacs appeared normal.

Other minor lesions were a small number of roundworms, coccidia, and cryptosporidium parasites in the small intestine.

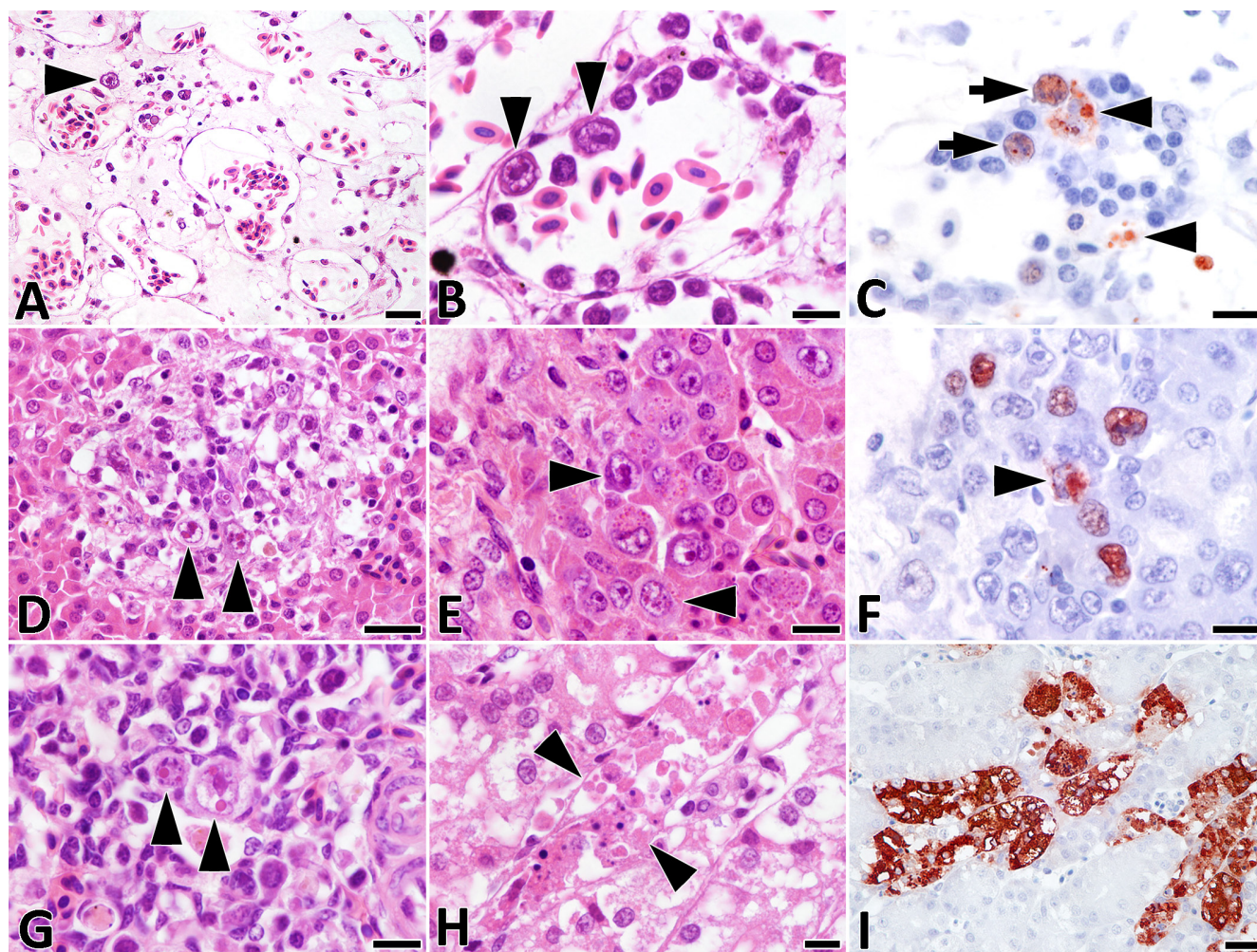


Fig. 1. Histopathology of Japanese native chicks died at 15 days old. A: Bone marrow of chick No. 3. Hematopoietic depletion with a large cell in the extravascular space (arrowhead), hematoxylin and eosin (HE). Bar=20 μ m. B: Bone marrow of chick No. 3. Large cells with small intranuclear inclusions in the vascular space (arrowheads). HE. Bar=10 μ m. C: Bone marrow of chick No. 3. Chicken anemia virus (CAV) antigens detected in the nucleus of large cells (arrows) and in the cytoplasm of an unidentified cell (arrowheads). Some small intranuclear dot-like structures strongly positive for CAV antigens (arrows) might be consistent with intranuclear inclusions. Immunohistochemistry (IHC). Bar=10 μ m. D: Liver of chick No. 1. Hepatic necrosis characterized by loss of hepatocytes, inflammatory cells and large cells with a pale nucleus containing intranuclear inclusions (arrowheads). HE. Bar=20 μ m. E: Liver of chick No. 1. Large cells with intranuclear inclusions (arrowheads) observed in the foci of extramedullary hematopoiesis. A large cell (the lowest arrowhead) has multiple intranuclear inclusions and cytoplasmic granules resembling those of immature granulocytes. HE. Bar=10 μ m. F: Liver in the same location as Fig. 1E. Intranuclear and intracytoplasmic (arrowhead) CAV antigens detected in some cells of the extramedullary hematopoiesis. IHC. Bar=10 μ m. G: Spleen of chick No. 1. Large cells with intranuclear inclusions (arrowheads). HE. Bar=10 μ m. H: Kidney of chick No. 1. Epithelial necrosis of the distal tubule. HE. Bar=10 μ m. I: Kidney of chick No. 1. Infectious bronchitis virus antigens detected in the renal tubular cells. IHC. Bar=20 μ m.

IHC findings

IHC detected CAV and IBV antigens in chicks Nos. 1 and 3. Owing to postmortem tissue damage, chick No. 2 was excluded from the evaluation. The IHC for IBDV, AIV, NDV, and FAV was negative in the examined chicks.

CAV antigens were detected primarily in the bone marrow, liver, and spleen. Large cells observed in the bone marrow (Fig. 1C), liver (Fig. 1E) and other organs exhibited finely granular, weak to strong positive staining of CAV antigens in the nucleus. However, some of the large cells and most intranuclear inclusions were negative for IHC in the present study for unknown reasons.

Another staining pattern of the CAV antigens was cytoplasmic. Small to large round aggregations of IHC-positive products were found in the cytoplasm of unidentified cells in the bone marrow (Fig. 1C), liver (Fig. 1E) and spleen. There were rare unidentified cells with one to two very small cytoplasmic granules of CAV antigens in the hepatic sinusoid, spleen and the stroma of the heart, BF, and kidney.

IHC detected abundant IBV antigens in the renal tubular epithelial cells, with or without necrotic changes throughout the kidney (Fig. 1I), and in the epithelium of the trachea and air sacs. On the odd occasion, IBV-antigen-positive cells were also found in the epithelial cells of the immature oviduct and pulmonary bronchus.

Bacteriology

Escherichia coli was isolated from the intestine of all chicks (10^6 – 10^9 cfu/g) and the liver of chick No. 2 (10^4 cfu/g).

Virus isolation

CAV was isolated from the liver of all chicks. Cell death was clearly observed in MDCC-MSB1 cells after 3 passages. CAV antigens were detected via an indirect immunofluorescence assay in MDCC-MSB1 cells exhibiting cytopathogenic effects. The isolated CAV strain was designated as CAV/Japan/HS1/17 and then used for phylogenetic analysis and experimental infection.

IBV was isolated from the respiratory tissue of all chicks and the kidney of chicks Nos. 1 and 3. FAV was detected in various organs of the three chicks: the lung and small intestine of chick No. 1; the brain, lung, and heart of chick No. 2; and the trachea of chick No. 3.

Phylogenetic analysis of the isolated CAV

The genetic sequence (1,350 bp) of the CAV VP1 gene was prepared from CAV/Japan/HS1/17 (accession number MK624991) and other CAV strains. CAV/Japan/HS1/17 is phylogenetically similar to Chinese strains collected in 2014–2016 and positioned in the largest group designated as Genotype III in Fig. 2.

Experimental infection

The clinical disease was reproduced in SPF chicks via inoculation with isolated CAV/Japan/HS1/17 (Table 1). The prevalence of anemia and mortality was higher in Group A than in Group B.

Poor growth of all chicks in Group A was evident by approximately 10 days after inoculation. From Day 13 pi, all birds in Group A started exhibiting clinical signs, including anorexia, lethargy, drowsiness, depression, ruffled feathers, drooped wings, pale combs, and recumbency. Facial swelling was noted in three birds (Nos. 9, 10, and 13) (Fig. 3A). Mortality in Group A occurred in 7 chicks (Nos. 4, 6, 7, 9–11, and 13). One chick (No. 4) died on Day 13 pi. Others died from Days 15–17 pi. The birds' clinical signs gradually began to resolve from Day 18 pi.

Many chicks in Group B exhibited mild to moderate poor growth and anemia. Four birds exhibited mild to severe clinical signs that were similar to those in Group A from Days 16–18 pi, of which one bird (No. 14) with facial swelling died on Day 17 pi.

CAV was re-isolated from the liver collected at necropsy in 90% of the birds in Group A and 60% in Group B.

Gross pathological change was apparent in the thymus and bone marrow of birds exhibiting clinical signs. The bone marrow of the

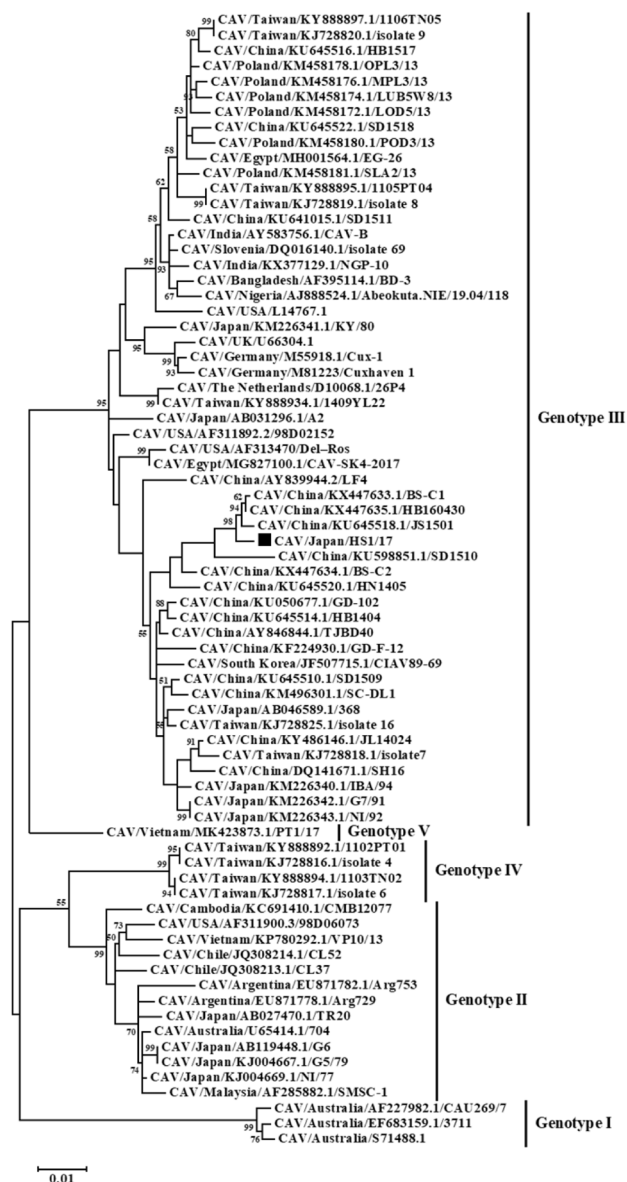


Fig. 2. Phylogenetic tree of the chicken anemia virus VP1 gene sequence (1,350 bp). Numbers at each branch indicate bootstrap values $\geq 50\%$. Five major genotypes were identified and designated as Genotypes I–V. A strain CAV/Japan/HS1/17 isolated in the present study is marked with closed square in the largest group designated as Genotype III.

Table 1. Clinical data of specific-pathogen-free chicks inoculated intramuscularly with CAV/Japan/HS1/17^{a)}

Clinical data	Group		
	A	B	C
Age at inoculation	1-day-old	7-day-old	Uninoculated
Number of birds	10	10	3
Mortality (dpi)	70% (13–17 dpi)	10% (17dpi)	0%
Anemia (Ht $\leq 27\%$)	100%	80%	0%
Average body weight ^{b)}	164.2 g	299.0 g	279.0 g ^{c)} , 374.5 g ^{d)}

a) dpi, day postinoculation; Ht, hematocrit value. b) Body weight was measured on 20 dpi. c) Data for comparison to Group A. d) Data for comparison to Group B.

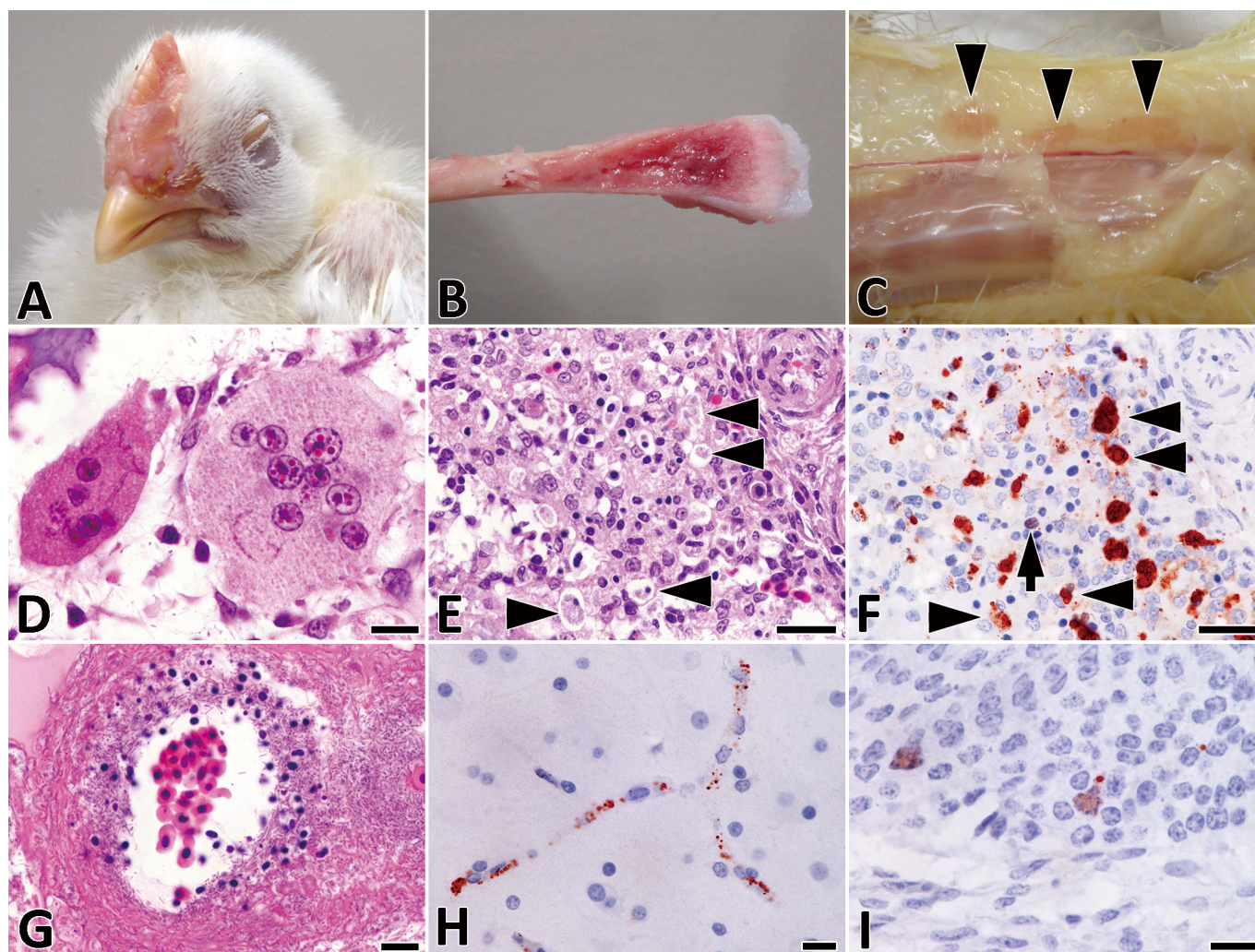


Fig. 3. The pathological pictures of specific-pathogen-free chicks experimentally inoculated with CAV/Japan/HS1/17 in Group A (one-day-old inoculation). A: Swollen head and comb in chick No. 9 died on Day 16 postinoculation (pi). B: Pale bone marrow of the tibiotarsus in chick No. 9 died on Day 16 pi. C: Atrophic thymic lobes (arrowheads) in chick No. 5 euthanized on Day 20 pi. D: Bone marrow of chick No. 9 died on Day 16 pi. Two osteoclasts containing eosinophilic intranuclear inclusions, hematoxylin and eosin (HE). Bar=10 μ m. E: Thymic cortex of chick No. 6 died on Day 15 pi. Lymphoid depletion with remaining stromal cells and foamy cells (arrowheads). HE. Bar=20 μ m. F: Thymic cortex in the same position as Fig. 3E. Abundant granular to clumpy chicken anemia virus (CAV) antigens (detected in the cytoplasm of stromal cells and foamy cells (arrowheads). An arrow shows the intranuclear CAV antigen. Immunohistochemistry (IHC). Bar=20 μ m. G: Swollen facial skin of chick No. 10 died on Day 17 pi. Necrotic vasculitis with edema and many bacterial rods in the subcutaneous tissue. HE. Bar=10 μ m. H: Brain of chick No. 6 died on Day 15 pi. Granular staining of CAV antigens along the blood capillary. IHC. Bar=10 μ m. I: Feather of chick No. 6 died on Day 15 pi. Intranuclear and intracytoplasmic CAV antigens in the feather epidermis. IHC. Bar=10 μ m.

femur and tibiotarsus was diffusely pale (Fig. 3B). The thymi were pale and severely atrophied (Fig. 3C), and the spleen and BF were mildly to moderately small. Subcutaneous edema was found in the birds at the site of facial swelling.

Histologically, hematopoietic depletion and rare scattered large cells with intranuclear inclusions were observed in the bone marrow of all chicks in Group A. The bone marrow of chicks euthanized on Day 20 pi contained some foci of regenerating hematopoietic cells embedded in the adipose tissue. In one chick (No. 9) of Group A, many osteoclasts contained intranuclear inclusions (Fig. 3D).

Lymphoid atrophy of the thymus was consistently severe, and many stromal cells, occasional foamy cells, and remaining lymphocytes were present throughout (Fig. 3E). The presence of large cells with intranuclear inclusions was not confirmed in the thymus. The spleen, BF, and cecal tonsil exhibited mild to moderate lymphoid atrophy, often accompanied by cellular debris. Large cells with intranuclear inclusions were rarely observed in the spleen and cecal tonsil.

Small foci of fibrinoid necrosis with bacterial colonies and fibrin thrombi were found in the lung, liver, and spleen of 50% of the chicks in Group A, and these findings were considered a secondary infection. The skin of chicks with facial swelling exhibited strong perivascular edema with bacterial colonies and fibrin thrombi (Fig. 3G). Many Kupffer cells were enlarged in the hepatic sinusoid, but necrotic foci were not observed in the liver of the experimental chicks.

The histological changes in Group B were consistently milder than those in Group A, except for the bird (No. 14) that died on Day 17 pi. Large cells with intranuclear inclusions were observed far more rarely than in Group A.

According to IHC, CAV antigens were consistently and most abundantly detected in the thymus, especially in the thymic cortex of severely diseased birds. Most CAV-antigen-positive reactions in the thymus were observed as small to large granules in the cytoplasm of stromal cells and foamy cells (Fig. 3F), and intranuclear CAV antigens were rare.

CAV-antigen-positive cells were also found in the bone marrow and spleen. Many positive cells were observed as granules in the cytoplasm of unidentified cells, except for large cells, which exhibited mild to strong positive staining in the nucleus. Osteoclasts with intranuclear inclusions in chick No. 9 were negative in the IHC.

Interestingly, small granular CAV-positive products were observed along the blood capillaries in the brain and spinal cord (Fig. 3H). The cytoplasm of small neurons contained CAV antigens in one bird (No. 6) of Group A.

Other IHC findings included rare CAV antigens in the feather epidermal cells (Fig. 3I) and leukocytes in the meningeal blood vessels. Faint granular CAV-positive staining was observed upon occasion in the cytoplasm of unidentified cells in a variety of tissues, including the hepatic sinusoid, the interstitium of the lung, BF, heart, pancreas, kidney, feather follicles, and ovary, and the lymphoid follicles of the proventriculus and intestine.

DISCUSSION

Our analysis revealed that the clinical disease in Japanese native chicks presenting with an unusually high mortality could be attributed to a concurrent infection of CAV and IBV. The mortality due to CAV infection in the field case was generally less than 30% [4, 5, 7, 9–11, 25]. However, CAV can induce immunosuppression and poor growth in infected birds, leading to increased susceptibility against various pathogens [21, 25]. In addition to CAV infection, the IBV infection in the flock, as well as a secondary bacterial infection in some chicks, was considered a primary trigger of the high mortality in the present case. As IBV transmission is principally horizontal [17], the Japanese native chicks were likely horizontally infected with IBV at the farm. An early onset of clinical signs that developed four days after introduction of the day-old Japanese native chicks suggests that some clinical signs such as diarrhea in the flock might be associated with infection by IBV or other pathogens. Findings of cryptosporidiosis, bacterial infection, and very poor growth were suggestive of a severe immunocompromised status of the affected chicks. Although FAV was isolated from the chicks, histological lesions associated with adenovirus were not observed, suggesting that FAV was hardly involved in the clinical disease.

The lack of vaccination against CAV to the parent flock of the Japanese native chicks suggests that vertical transmission of CAV was a possible source of infection to the chicks, although the CAV infection status of the parent flock was unknown. The peak mortality period of the Japanese native chicks, which was observed on 14–20 days after introduction, was consistent with the clinical course of CAV vertical infection in the past outbreak [9]. In experimental settings, CAV infection generally requires 8–10 days for clinical signs to develop in chicks inoculated at one day old, and mortality usually begins 12–14 days after inoculation [12, 29, 42], as observed in our experiment. Untreated chicks co-housed with CAV inoculated birds rarely develop clinical signs or anemia [24, 42], suggesting that horizontal infection of CAV is not largely responsible for the clinical disease of CAV infection in the field. Vaccination of breeder chickens against CAV is popular for Japanese commercial poultry, so field outbreaks of CAV infection are currently rare. By contrast, the present case underscores the importance of proper vaccination and management in small-scale poultry farming such as that of Japanese native chickens and backyard poultry.

Generally, the pathogenicity of CAV strains does not vary substantially [25]. However, minor differences of the pathogenicity of CAV have been reported in experimental infection studies using SPF chickens [39]. The pathogenicity of CAV/Japan/HS1/17 was evaluated by experimental infection using one-day-old and seven-day-old SPF chicks, following the protocol created by Yuasa *et al.* [39]. Typical CAV infection was reproduced in inoculated chicks, demonstrating the pathogenic potential of CAV/Japan/HS1/17. In addition, the high prevalence of anemia and mortality observed in the seven-day-old inoculation group was an interesting result. Specifically, the mortality of chicks inoculated at seven days old with CAV virus has not been recorded in past experimental infection studies [20, 39]. Our experimental results suggest that CAV/Japan/HS1/17 has a pathogenicity comparable to or stronger than that of the CAV A2 strain, i.e., the most virulent strain among 11 Japanese CAV isolates evaluated in the past [39].

Previous studies have revealed that CAV primarily infects the hematopoietic progenitor cells of the bone marrow and lymphocytes of the thymus and spleen [14, 27, 29, 30, 42]. However, the replication site of CAV in other organs remains controversial [3, 4, 15, 23, 44]. In the present study, we successfully detected CAV antigens on paraffin sections of both field and experimental cases via IHC. Our IHC results suggest that CAV can be present in a variety of organs of infected chickens. The CAV antigen distribution in the bone marrow and lymphoid organs in the present study was similar to that reported in the past [14, 18, 27]. However, some IHC findings were unique to the present analysis. First, it is interesting that CAV antigens were detected in the cells of the extramedullary hematopoiesis in the liver of the field cases. Local lymphoid follicles, unidentified cells in the hepatic sinusoid, and blood have been reported as possible viral locations in the liver [27, 34], an organ commonly used for the isolation of CAV [11, 20, 37]. Our IHC results suggest that CAV can replicate in the foci of the extramedullary hematopoiesis, indicating the tropism of CAV to the hematopoietic tissue. Second, our IHC results suggest that CAV can replicate in the vascular endothelium of the brain and spinal cord. Curiously, this IHC finding regarding the endothelial cells was observed only in experimental birds and confined to the endothelium of the central nervous system. To our knowledge, CAV detection in endothelial cells by IHC has not been reported in previous studies [4, 7, 14, 18, 27]. The presence of CAV antigens in the brain has only

been described in choroid plexus cells, ependymal glial cells, and unidentified cells in the past [4, 27]. Finally, although the CAV antigens detected in the feather epidermis were rare, our finding in the present study could support CAV replication and possible virus shedding associated with chicken feathers [6]. The pathological significance of osteoclasts containing intranuclear inclusions is unknown due to the negative result for IHC in the present study.

At times, we found it difficult to histologically identify the cell type of CAV-antigen-positive cells based on the cell morphology and location, as pointed out by Smyth *et al.* [27]. Specifically, the type of cells that contained cytoplasmic granular staining of the CAV antigens remains inconclusive, although they were rarely detected in the interstitium of a variety of organs. Similarly, the origin of the abnormally large cells with CAV inclusions in tissues other than the bone marrow is still unclear. A detailed IHC analysis using a variety of cell identification makers is necessary in order to confirm the target cells of CAV in infected chickens.

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REFERENCES

1. Brentano, L., Lazzarin, S., Bassi, S. S., Klein, T. A. P. and Schat, K. A. 2005. Detection of chicken anemia virus in the gonads and in the progeny of broiler breeder hens with high neutralizing antibody titers. *Vet. Microbiol.* **105**: 65–72. [Medline] [CrossRef]
2. Calnek, B. W. and Adlinder, H. K. 1971. Some characteristics of cell-free preparations of Marek's disease virus. *Avian Dis.* **15**: 508–517. [Medline] [CrossRef]
3. Cardona, C. J., Oswald, W. B. and Schat, K. A. 2000. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. *J. Gen. Virol.* **81**: 2067–2075. [Medline] [CrossRef]
4. Castaño, P., Benavides, J., Lee, M. S., Fernández, M., Fuertes, M., Royo, M., Fernández, J. M., Pérez, V. and Ferreras, M. C. 2019. Tissue tropism of chicken anaemia virus in naturally infected broiler chickens. *J. Comp. Pathol.* **167**: 32–40. [Medline] [CrossRef]
5. Chettle, N. J., Eddy, R. K., Wyeth, P. J. and Lister, S. A. 1989. An outbreak of disease due to chicken anaemia agent in broiler chickens in England. *Vet. Rec.* **124**: 211–215. [Medline] [CrossRef]
6. Davidson, I., Artzi, N., Shkoda, I., Lublin, A., Loeb, E. and Schat, K. A. 2008. The contribution of feathers in the spread of chicken anemia virus. *Virus Res.* **132**: 152–159. [Medline] [CrossRef]
7. Dobos-Kovács, M., Varga, I., Békési, L., Drén, C. N., Németh, I. and Farkas, T. 1994. Concurrent cryptosporidiosis and chicken anaemia virus infection in broiler chickens. *Avian Pathol.* **23**: 365–368. [Medline] [CrossRef]
8. Engström, B. E. 1988. Blue wing disease of chickens: isolation of avian reovirus and chicken anaemia agent. *Avian Pathol.* **17**: 23–32. [Medline] [CrossRef]
9. Engström, B. E. and Luthman, M. 1984. Blue wing disease of chickens: signs, pathology and natural transmission. *Avian Pathol.* **13**: 1–12. [Medline] [CrossRef]
10. Goodwin, M. A., Brown, J., Miller, S. L., Smeltzer, M. A., Steffens, W. L. and Waltman, W. D. 1989. Infectious anemia caused by a parvovirus-like virus in Georgia broilers. *Avian Dis.* **33**: 438–445. [Medline] [CrossRef]
11. Goryo, M., Shibata, Y., Suwa, T., Umemura, T. and Itakura, C. 1987. Outbreak of anemia associated with chicken anemia agent in young chicks. *Nippon Juigaku Zasshi* **49**: 867–873. [Medline] [CrossRef]
12. Goryo, M., Suwa, T., Umemura, T., Itakura, C. and Yamashiro, S. 1989. Histopathology of chicks inoculated with chicken anaemia agent (MSB1-TK5803 strain). *Avian Pathol.* **18**: 73–89. [Medline] [CrossRef]
13. Hoop, R. K. 1992. Persistence and vertical transmission of chicken anaemia agent in experimentally infected laying hens. *Avian Pathol.* **21**: 493–501. [Medline] [CrossRef]
14. Hoop, R. K. and Reece, R. L. 1991. The use of immunofluorescence and immunoperoxidase staining in studying the pathogenesis of chicken anaemia agent in experimentally infected chickens. *Avian Pathol.* **20**: 349–355. [Medline] [CrossRef]
15. Imai, K., Mase, M., Tsukamoto, K., Hihara, H. and Yuasa, N. 1999. Persistent infection with chicken anaemia virus and some effects of highly virulent infectious bursal disease virus infection on its persistency. *Res. Vet. Sci.* **67**: 233–238. [Medline] [CrossRef]
16. International Committee on Taxonomy of Viruses. <https://talk.ictvonline.org/taxonomy/> [accessed on February 2, 2020].
17. Jackwood, M. W. and de Wit, S. 2013. Infectious bronchitis. pp. 139–159. In: *Diseases of Poultry*, 13th ed. (Swayne, D. E., Glisson, J. R., McDougald, L. R., Nolan, L. K., Suarez, D. L. and Nair, V. eds), Wiley Blackwell publishing, Ames.
18. Kuscu, B. and Gürel, A. 2008. Lesions in the thymus and bone marrow in chicks with experimentally induced chicken infectious anemia disease. *J. Vet. Sci.* **9**: 15–23. [Medline] [CrossRef]
19. McNulty, M. S. 1991. Chicken anaemia agent: a review. *Avian Pathol.* **20**: 187–203. [Medline] [CrossRef]
20. McNulty, M. S., Connor, T. J., McNeilly, F., McLoughlin, M. F. and Kirkpatrick, K. S. 1990. Preliminary characterisation of isolates of chicken anaemia agent from the United Kingdom. *Avian Pathol.* **19**: 67–73. [Medline] [CrossRef]
21. Miller, M. M. and Schat, K. A. 2004. Chicken infectious anemia virus: an example of the ultimate host-parasite relationship. *Avian Dis.* **48**: 734–745. [Medline] [CrossRef]
22. Nakamura, K., Mase, M., Yamamoto, Y., Takizawa, K., Kabeya, M., Wakuda, T., Matsuda, M., Chikuba, T., Yamamoto, Y., Ohyama, T., Takahashi, K., Sato, N., Akiyama, N., Honma, H. and Imai, K. 2011. Inclusion body hepatitis caused by fowl adenovirus in broiler chickens in Japan, 2009–2010. *Avian Dis.* **55**: 719–723. [Medline] [CrossRef]
23. Novak, R. and Ragland, W. L. 1997. *In situ* hybridization for detection of chicken anaemia virus in peripheral blood smears. *Mol. Cell. Probes* **11**: 135–141. [Medline] [CrossRef]
24. Rosenberger, J. K. and Cloud, S. S. 1989. The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian Dis.* **33**: 753–759. [Medline] [CrossRef]
25. Schat, K. A. and van Santen, V. L. 2013. Chicken infectious anemia virus and other Circovirus infections. pp. 247–288. In: *Diseases of Poultry*, 13th ed. (Swayne, D. E., Glisson, J. R., McDougald, L. R., Nolan, L. K., Suarez, D. L. and Nair, V. eds.), Wiley Blackwell publishing, Ames.
26. Smyth, J. A., Moffett, D. A., Connor, T. J. and McNulty, M. S. 2006. Chicken anaemia virus inoculated by the oral route causes lymphocyte depletion in the thymus in 3-week-old and 6-week-old chickens. *Avian Pathol.* **35**: 254–259. [Medline] [CrossRef]
27. Smyth, J. A., Moffett, D. A., McNulty, M. S., Todd, D. and Mackie, D. P. 1993. A sequential histopathologic and immunocytochemical study of

- chicken anemia virus infection at one day of age. *Avian Dis.* **37**: 324–338. [[Medline](#)] [[CrossRef](#)]
28. 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](#)]
 29. Taniguchi, T., Yuasa, N., Maeda, M. and Horiuchi, T. 1982. Hematopathological changes in dead and moribund chicks induced by chicken anemia agent. *Natl. Inst. Anim. Health Q. (Tokyo)* **22**: 61–69. [[Medline](#)]
 30. Taniguchi, T., Yuasa, N., Maeda, M. and Horiuchi, T. 1983. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. *Natl. Inst. Anim. Health Q. (Tokyo)* **23**: 1–12. [[Medline](#)]
 31. 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](#)]
 32. Toro, H., Ramírez, A. M. and Larenas, J. 1997. Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. *Avian Pathol.* **26**: 485–499. [[Medline](#)] [[CrossRef](#)]
 33. Trinh, D. Q., Ogawa, H., Bui, V. N., Baatartsogt, T., Kizito, M. K., Yamaguchi, S. and Imai, K. 2015. Characterization of mAbs to chicken anemia virus and epitope mapping on its viral protein, VP1. *J. Gen. Virol.* **96**: 1086–1097. [[Medline](#)] [[CrossRef](#)]
 34. Wani, M. Y., Dhama, K. and Malik, Y. S. 2016. Impact of virus load on immunocytological and histopathological parameters during clinical chicken anemia virus (CAV) infection in poultry. *Microb. Pathog.* **96**: 42–51. [[Medline](#)] [[CrossRef](#)]
 35. Yamamoto, Y., Shimizu, S., Hirota, J., Kurokawa, A. and Mase, M. 2019. Development of a monoclonal antibody-based immunohistochemical method for detecting Newcastle disease virus. *Jpn. Agric. Res. Q.* **53**: 135–141. [[CrossRef](#)]
 36. 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](#)]
 37. Yuasa, N. 1990. Diagnosis of chicken anemia agent infection. *Natl. Inst. Anim. Health Q. (Tokyo)* **24**: 219–223.
 38. Yuasa, N. 1992. Effect of chemicals on the infectivity of chicken anaemia virus. *Avian Pathol.* **21**: 315–319. [[Medline](#)] [[CrossRef](#)]
 39. Yuasa, N. and Imai, K. 1986. Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). *Avian Pathol.* **15**: 639–645. [[Medline](#)] [[CrossRef](#)]
 40. Yuasa, N. and Yoshida, I. 1983. Experimental egg transmission of chicken anemia agent. *Natl. Inst. Anim. Health Q. (Tokyo)* **23**: 99–100. [[Medline](#)]
 41. Yuasa, N., Imai, K. and Tezuka, H. 1985. Survey of antibody against chicken anaemia agent (CAA) by an indirect immunofluorescent antibody technique in breeder flocks in Japan. *Avian Pathol.* **14**: 521–530. [[Medline](#)] [[CrossRef](#)]
 42. 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](#)]
 43. Yuasa, N., Noguchi, T., Furuta, K. and Yoshida, I. 1980. Maternal antibody and its effect on the susceptibility of chicks to chicken anemia agent. *Avian Dis.* **24**: 197–201. [[CrossRef](#)]
 44. Yuasa, N., Taniguchi, T., Imada, T. and Hihara, H. 1983. Distribution of chicken anemia agent (CAA) and detection of neutralizing antibody in chicks experimentally inoculated with CAA. *Natl. Inst. Anim. Health Q. (Tokyo)* **23**: 78–81. [[Medline](#)]
 45. 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](#)]