Detection and molecular phylogenetic analysis of chicken astrovirus in Saga prefecture, Japan

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Research Article

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Abstract

We detected three chicken astrovirus strains from 4-day-old broiler chickens with a high mortality rate and visceral gout, and one strain from 150-day-old hens without clinical symptoms in Saga prefecture, Japan. Phylogenetic analysis based on the ORF1 amino acid sequence revealed that the strains from the visceral gout case were classified into subgroup Bi, and the strain from chickens without clinical symptoms was classified into subgroup Aiii. Our data indicate that diseases caused by chicken astrovirus can occur in Japan, and that chicken astrovirus infection should be included in the differential diagnosis of chickens with visceral gout.

Background

Chicken astrovirus (CAstV) belongs to the family Astroviridae, genus Avastrovirus. CAstV is a small round virus of 28 to 30 nm in diameter that has no envelope and a single-stranded positive-sense RNA genome. It is genetically closely related to avian nephritis virus (ANV), which belongs to the genus Avastrovirus, but they are not serologically cross-reactive [1]. CAstV was first isolated from 5-day-old chickens with runting-stunting syndrome (RSS) and uneven growth in 2004 [1]. CAstV has been suggested to be associated with diarrhea, visceral gout, RSS, and white chick syndrome (WCS) [2-4], and it may also be involved in malabsorption and stunting in the intestinal tract [5]. Thus, CAstV can result in economic loss. Interestingly, it has also been reported that chickens may gain resistance to CAstV with age [5].

The CAstV strains reported thus far have been classified into seven subgroups, Ai, Aii, Aiii, Bi, Bii, Biii, and Biv, based on the ORF1 amino acid sequence that encodes a capsid protein. Little is known about how the subgroups are related to the pathogenicity of this virus. Strains classified into subgroup Biii have been reported to cause high mortality rates and visceral gout among specific-pathogen-free chickens [2]. It has also been suggested that strains classified into subgroup Biv can causes RSS and WCS [3-5].

CAstV has been reported worldwide, including in the United Kingdom (UK), the Netherlands, Poland, Germany, South Africa, India, the United States of America (USA), Canada, and China [1, 2, 4-6]. In the UK, CAstV was isolated from broiler chickens bred on multiple farms from 2004 to 2008 [7]. CAstV strains of subgroups Ai, Aii, Aiii, Bi, and Bii have been isolated in the UK, and the strains were found to be rich in variations [5]. In India, a survey of chickens with high mortality rates conducted in 2011 to 2012 revealed a CAstV infection rate of 41.7% [2]. In China, a CAstV infection rate of 82.5% was reported from 2017 to 2019 in a surveillance study of 1-day-old chickens with mild stunting [6]. As such, there is concern that CAstV is spreading around the world.

The transmission route of CAstV has not yet been clarified, but it is thought to be transmitted through feces or by vertical transmission [5]. Since 2014, chickens have been imported from the UK, France, the Netherlands, USA, Canada, and New Zealand, to Japan. Although CAstV may have been brought into Japan via these imported chickens and may be spreading, there have been no reports of CAstV infection in chickens in Japan. If CAstV is present in Japan, the diseases caused by CAstV may occur. To

investigate the risk of developing diseases caused by CAstV in Japan, we detected and genetically characterized CAstV strains isolated from chickens raised in Japan.

In this study, we used 4-day-old commercial broiler chickens with a high mortality rate and visceral gout that were bred on a farm (farm A), and hens that were 111-660 days old with no clinical symptoms that were bred on three farms (farm B, farm C, and farm D). The chickens were bred during 2020 to 2021. Although all of the farms were located in Saga prefecture, Japan, there was no epidemiological relationship among the four farms. The intestinal tracts and the kidneys were collected from the broiler chickens. Cloaca swabs were obtained from the hens every month. The kidney tissue samples from the chickens were pooled and homogenized mechanically in Eagle's Minimum Essential Medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in a tube using a bead-beating method. Each cloaca swab sample was suspended in 10 ml of phosphate-buffered saline (PBS), and samples from the same farm were pooled monthly. The emulsions and suspensions were centrifuged at 10,000 rpm for 10 min, and the supernatants were filtered through a 0.22 μm filter.

The filtered emulsions and suspensions were inoculated into growing chicken eggs of age 9-11 days by the allantoic cavity route. The eggs were then cultured at 37 °C for 7 days, then the allantoic fluid was collected and passaged twice. PBS alone was similarly inoculated and passaged as a negative control.

RNA was extracted from 300 μl of the allantoic cavity fluid with an automatic nucleic acid extractor (Maxwell RSC; Promega Co., Madison, WI, USA) using the Maxwell RSC Viral Total Nucleic Acid Kit (Promega Co.) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) targeting the ORF1 region was performed according to a previous report [7]. CAstV-for (5'-KCATGGCTYCACCGYAADCA-3') and CAstV-rev (5'-CGGTCCATCCCTCTACCAGATTT-3') were used as the primers. RT-PCR was performed using the One Step RT-PCR Kit (QIAGEN, Venlo, Netherlands). The RT-PCR reaction was performed with a reverse transcription step at 50 °C for 30 min, an initial denaturation step at 95 °C for 15 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min per cycle. Amplification was performed in a C1000 Touch Thermalcycler (Bio-Rad Laboratories, Hercules, CA, USA).

RT-PCR for the ORF2 region, which encodes a capsid protein, was performed for samples that were positive for the ORF1 region by RT-PCR [8]. As primers, CAstV_PRECAP (5'-TAGAGGGATGGACCGAAATATAGCAGC-3') and CAstV_POSTCAP (5'-TGCAGCTGTACCCTCGATCCTA-3') were used. RT-PCR was performed using the PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio Inc., Kusatsu, Japan). The RT-PCR reaction was performed with a reverse transcription step at 45 °C for 10 min, an initial denaturation step at 94 °C for 2 min, 35 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 15 s, and extension at 68 °C for 30 s, and a final extension step at 68 °C for 5 min. The obtained PCR product of about 2200 bp was purified using the PCR Gel Extraction Kit (QIAGEN).

For the sequencing of strains derived from the healthy chickens, cycle sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA); the samples were purified with the BigDye XTerminator Purification Kit (Thermo Fisher Scientific), and the SeqStudio Genetic Analyzer (Thermo Fisher Scientific) was used. Sequencing of the strains from the visceral gout cases was outsourced to Eurofins Genomics Co., Ltd. (Tokyo, Japan). The reagents used for sequencing of the strains derived from the healthy chickens and those from the visceral gout cases were same, and sequence analysis was performed with a 3730xl DNA Analyzer (Thermo Fisher Scientific).

The sequence of the PCR product of about 2,200 bp was analyzed using the primer walking method. A primer (F2 primer) was designed for the 3' end based on the sequence of the cycle sequence product of CAstV_PRECAP. Cycle sequencing was performed with the F2 primer, then the sequence was determined, and a primer (F3 primer) was again designed for the 3' end. Further cycle sequencing was performed with the F2 primer to determine the sequence. Then, the cycle sequencing products of CAstV_PRECAP and the F2 and F3 primers were sequenced. The same was done for CAstV_POSTCAP, and the complementary sequence was determined.

The amino acid sequence was determined based on the obtained nucleotide sequence using MEGA-X software [9]. Alignments of amino acids were generated with the Clustal W program [10]. Molecular phylogenetic analysis was performed using the strains shown in Table 1, and a phylogenetic tree was created by the neighbor-joining method with a bootstrap value of 1,000. The tree was rooted. The evolutionary distances were computed using the Poisson correction method. ANV was used as an outgroup.

The kidneys of chickens with visceral gout were fixed in 10% neutral buffered formalin and embedded in paraffin. The embedded tissues were then sliced into 4 μm sections, and the sections were stained with hematoxylin and eosin.

RT-PCR for CAstV ORF1 was positive in the allantois fluid that had been inoculated with the emulsified intestinal tract samples of the three broiler chickens with visceral gout on farm A (SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020). RT-PCR for CAstV ORF1 was positive in the allantois fluid that had been inoculated with the cloaca swab suspensions from 150-day-old layer chickens with no clinical symptoms on farm B, which were collected in October 2020 (SG4/C/2020).

The allantoic fluid samples from embryonated chicken eggs that had been inoculated with the kidney emulsions (from chickens bred on farm A) or the suspensions from the cloaca swabs (from chickens bred on farms C and D) were all negative by RT-PCR. The negative controls, i.e., the allantoic fluid samples from eggs inoculated with PBS, were also negative by RT-PCR.

The nucleotide sequence of the ORF2 region was analyzed to determine the amino acid sequence. SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020 were 2,202 nt in length, and they encoded 734 amino acids. SG4/Int/2020 was 2,148 nt in length, and it encoded 716 amino acids. The nucleotide identities ranged from 99.9% to 100% among SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020. SG1/Int/2020

showed 90.1% identity with the FP3 strain obtained in the UK, and SG4/Int/2020 showed 89.9% identity with the VF08-46 strain also obtained in the UK.

A phylogenetic tree was created using the amino acid sequence based on the ORF2 gene (Fig. 1). CAstV strains were divided into groups A and B with a bootstrap value of 100. Group A was divided into subgroups Ai, Aii, and Aiii with bootstrap values of 94, 100, and 100, respectively. Group B was divided into subgroups Bi, Bii, Biii, and Biv with bootstrap values of 100, 100, 98, and 100, respectively. SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020 were classified into subgroup Bi, which includes strains from the UK and China. SG4/C/2020 was classified into subgroup Aiii, which includes strains from the UK and Poland.

In the kidneys, multiple inter-tubular tophi and mild interstitial lymphocyte infiltration were observed (Fig. 2).

Four CAstV strains were detected from the flocks raised on farms A and B in Saga prefecture. The strains were classified into subgroups Aiii and Bi. This is the first report of the detection of CAstV in Japan. CAstV strains were detected from two farms in Saga prefecture, and the strains were classified into different subgroups. There is no traffic on these farms, and geographically, they are several tens of kilometers apart. Therefore, it was found that multiple CAstV subgroups have infiltrated into flocks of chickens raised in Saga prefecture. We investigated four farms, and found two subgroups of CAstV in Saga prefecture. In contrast, at least five subgroups have been found in the UK. Since CAstV is a relatively new virus that was discovered in recent years, it had not yet been investigated in Japan. A survey of more chicken flocks in Japan could lead to the detection of other subgroups of CAstV in Japan.

In this study, CAstV strains were detected in chickens with visceral gout as well as chickens without clinical symptoms. No chicken infectious bronchitis virus or ANV was detected (data not shown), and no problems with feeding management, such as a lack of drinking water, have been confirmed (according to the farmers). In cases with visceral gout, histopathological examination revealed the formation of gouty nodules in the renal tubules. These lesions are consistent with the findings observed in previous subgroup Bi infection experiments with specific-pathogen-free eggs [2]. It is unknown whether CAstV actually caused the lesions; other factors that may lead to the formation of the lesions were not considered in this study, and the involvement of CAstV cannot be ruled out. If the visceral gout was caused by CAstV, it is considered that not only subgroup Biii, but also subgroup Bi can cause visceral gout. Subgroup Aiii was isolated from the 150-day-old hens with no clinical symptoms. Although it is possible that subgroup Aiii is not pathogenic, it is also possible that the chickens were resistant to CAstV owing to their age, and therefore had no symptoms. There remains much uncertainty regarding the relationship between the CAstV subgroups, their pathogenicity, and age resistance; it is necessary to verify their relationships by conducting further infection experiments.

In conclusion, this study confirmed the infiltration of CAstV in Saga prefecture, Japan. The detected strains may be associated with visceral gout. In cases of visceral gout among chickens in Japan, CAstV infection should be included in the differential diagnosis.

Declarations

No funding was received for this study. The authors have no relevant financial or non-financial interests to disclose.

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Tables

Due to technical limitations, Table 1 is only available as a download in the Supplemental Files section.

Figures

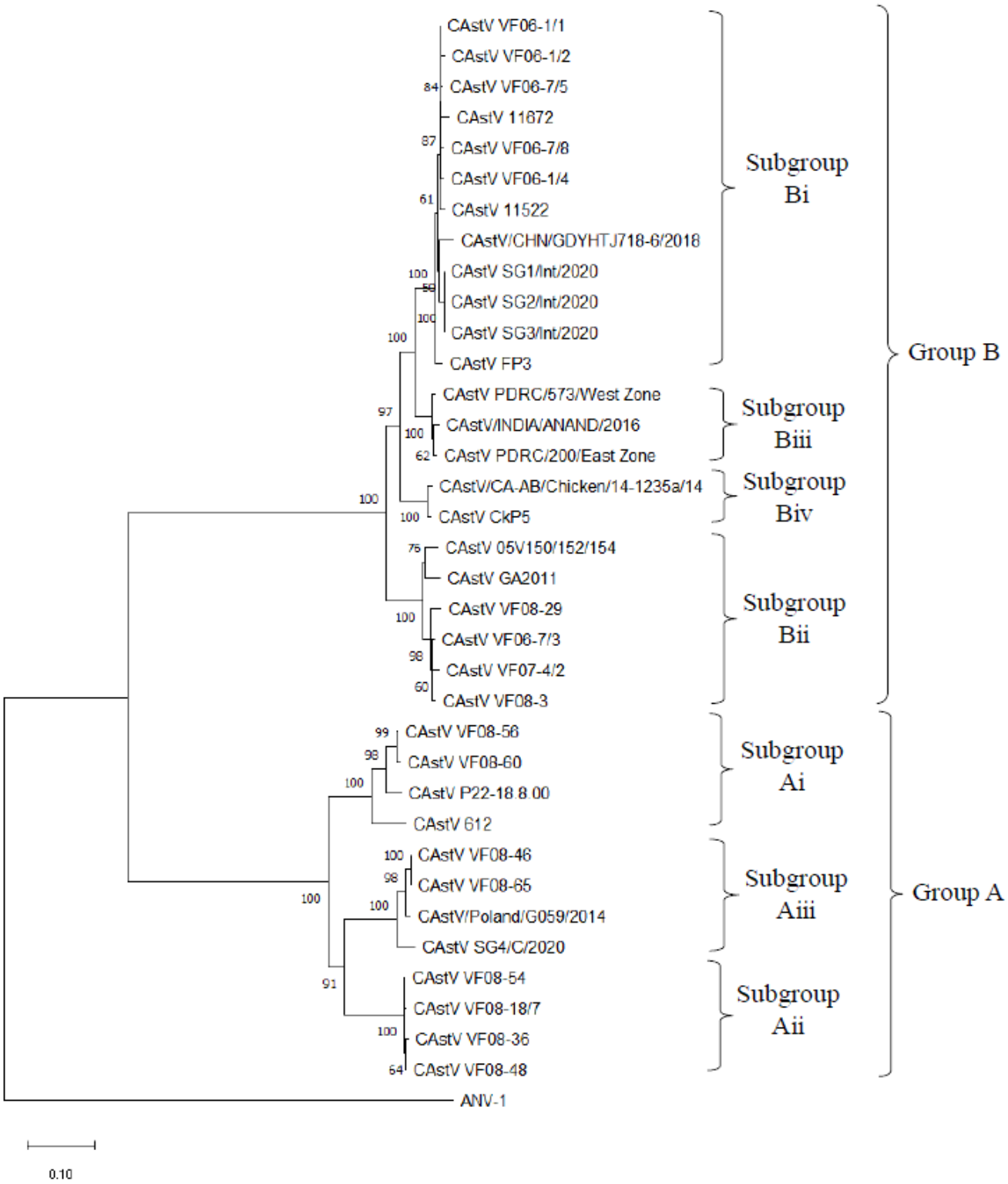

Figure 1

Phylogenetic tree based on the complete amino acid sequence of a capsid protein. The tree was constructed using the neighbor-joining method with a bootstrap value of 1000. For the sequences in the phylogenic tree, the strains in Table 1 were used.

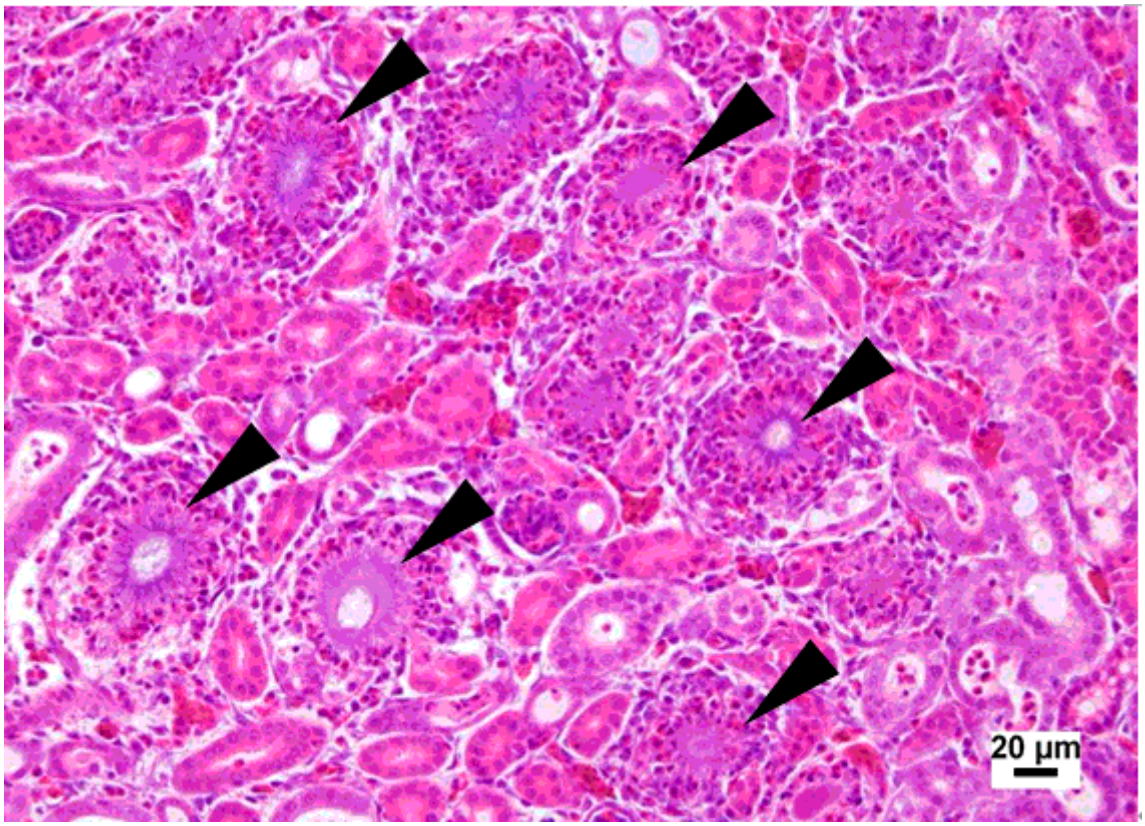

Figure 2

Histological image of a chicken kidney from a case with visceral gout showing inter-tubular tophi (arrows) and mild interstitial lymphocyte infiltration.

Supplementary Files

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- Table1.docx