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

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Abstract
We detected three chicken astrovirus strains in 4-day-old broiler chickens with high mortality and visceral gout and one strain from 150-day-old hens without clinical symptoms in Saga prefecture, Japan. Phylogenetic analysis based on ORF2 amino acid sequences revealed that the strains from the visceral gout cases belonged to subgroup Bi, and the strain from hens without clinical symptoms belonged to subgroup Aiii. Our study showed that CAstV had infiltrated into Saga prefecture, Japan. This is the first report of CAstV in Japan.

Chicken astrovirus (CAstV) belongs to the family Astroviridae, genus Avastrovirus. CAstV is a small round virus that is 28 to 30 nm in diameter with no envelope, and it contains a single-stranded positive-sense RNA genome. It is genetically closely related to avian nephritis virus (ANV), but these viruses 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 is associated with diarrhea, visceral gout, RSS, white chick syndrome (WCS) [2–4], malabsorption, and stunting [5], resulting in economic losses. Interestingly, chickens may gain resistance to CAstV with age [5].

CAstV strains are classified into seven subgroups, Ai, Aii, Aiii, Bi, Bii, Biii, and Biv, based on the amino acid sequence of a capsid protein encoded by ORF2. The strains belonging to subgroup Biii cause high mortality and visceral gout in specific-pathogen-free chickens [2], and those belonging to subgroup Biv cause RSS and WCS [3–5]. However, little is known about the relationship between the subgroups and the pathogenicity of the virus.

CAstV has been reported 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 on multiple farms from 2004 to 2008 [7]. CAstV strains of subgroups Ai, Aii, Aiii, Bi, and Bii were isolated in the UK [5]. In India, CAstV infection was found in 41.7% of chickens, with high mortality, from 2011 to 2012. [2]. In China, 82.5% of 1-day-old chickens with mild stunting were found to be infected with CAstV from 2017 to 2019 [6]. Therefore, there is concern that CAstV may spread around the world.

The transmission route of CAstV remains unclear, but chickens may become infected with CAstV via feces or by vertical transmission [5]. Since 2014, chickens have been imported from the UK, France, the Netherlands, the USA, Canada, and New Zealand to Japan, and it is possible that some were infected with CAstV. The purpose of this study was to detect CAstV and genetically characterize the strains present in Japan.

In this study, we used 4-day-old commercial broiler chickens with high mortality and visceral gout and 111- to 660-day-old hens without clinical symptoms. The broiler chickens and hens were negative for avian influenza virus (AIV), Newcastle disease virus, infectious bronchitis virus, and ANV. The broiler chickens were raised on the same farm (farm A), and the hens were raised on three different farms (farms B, C, and D) during 2020 to 2021. Farms B, C, and D were chosen randomly from farms that raised more than 1,000 hens, all of which were in Saga
There was no epidemiological relationship among the four farms. The intestinal tracts and the kidneys were collected from four dead broiler chickens with visceral gout from farm A. The kidney tissue samples were pooled. From farms B, C and D, 10 hens were chosen randomly from a flock, and cloaca swabs were collected each month and pooled. The intestinal tracts and the kidney pool were homogenized mechanically in Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) using a bead-beating method. The cloaca swab pools were suspended in 10 ml of phosphate-buffered saline (PBS). The emulsions and suspensions were centrifuged at 10,000 rpm for 10 min, and the supernatants were passed through a 0.22-μm filter.

The filtered emulsions and suspensions were inoculated into 9- to 11-day-old embryonated eggs by the allantoic cavity route. The eggs were incubated for 7 days at 37 °C, and the allantoic fluid was collected. Three blind passages were carried out. PBS was used as a negative control.

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

Reverse transcription polymerase chain reaction (RT-PCR) targeting the ORF1 region was performed as a screening step as described previously [7], using the primers CAstV-for (188-KCAT GGC TYC ACC GYAADCA-207, positions relative to the genome sequence of strain FP3 [accession no. JN582328]) and CAstV-rev (697-CGG TCC TTC AAC CTC GTC GCA-2136), 1446R (1446-GTG TAT GCC TGC GAT GTT GAG-1426), and 2140R (2140-CCA TTGCGACGAGGTGAAG-2121). The other primer group consisted of primers 1475F (1475-ACG GTG TAT GTG TTT TCA GGT TAT -2289). The primer group used depended on the strain being sequenced. Cycle sequencing was performed, and the resulting sequences were combined to assemble the ORF2 sequence.

To sequence the ORF2 region, we used the primer-walking method. Two primer groups were designed to cover the ORF2 region. One primer group consisted of the primers 1475F (1475-ACG GTG TAT GTG TTT TCA GGT TAT -2289), 2116F (2116-GGT AAC GCC TGC GAT GTT GAG-1426), and 1446R (1446-GTG TAT GCC TGC GAT GTT GAG-1426), and 2140R (2140-CCA TTGCGACGAGGTGAAG-2121). The other primer group consisted of primers 1266F (1266-CGCTTGGGATGAAAC ACTGAC-1285), 1968F (1968-ACT ACA CAA CCA GGG CCA), 1562R (1562-TCACTGACCAGTTGGAGGAGT-1541) and 2312R (2312-TGTGTCATATCTTCTTTGCGTGTT-2289). The primer group used depended on the strain being sequenced. Cycle sequencing was performed, and the resulting sequences were combined to assemble the ORF2 sequence.

The amino acid sequences were deduced using MEGA-X software [9], and an amino acid sequence alignment was generated using the Clustal W program [10]. Molecular phylogenetic analysis was performed using the strains shown in Table 1, whose sequences were downloaded from the GenBank database. A phylogenetic tree was created by the neighbor-joining method with 1,000 bootstrap replicates. 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 ORF1 was positive when using allantoic fluid from eggs injected with intestinal tract samples from the three broiler chickens with visceral gout on farm A (SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020) and the cloaca swab suspensions from 150-day-old hens without clinical symptoms on farm B, which were collected in October 2020 (SG4/C/2020).

The kidney pool from four chickens and the intestinal tract from one chicken on farm A and the cloaca swabs from...
farms C and D were negative for ORF1 by RT-PCR. The negative control was also negative for ORF1.

The nucleotide sequence of the ORF2 gene determined using primers 1475F, 2116F, 1446R, and 2140R for SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020 and primers 1266F, 1968F, 1562R, and 2312R for SG4/C/2020. The partial ORF2 sequences from SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020 were 2,202 nt in length, encoding 734 amino acids, whereas that of SG4/C/2020 was 2,148 nt in length, encoding 716 amino acids. The nucleotide sequences of SG1/Int/2020, SG2/Int/2020, and SG3/Int/2020 were 99.9% to 100% identical to each other. SG1/Int/2020 showed 90.1% identity to the FP3 strain, and SG4/C/2020 showed 89.9% identity to the VF08-46 strain. The nucleotide sequence of the four Japanese strains were deposited in the GenBank database with the accession numbers OL321958-OL321961.

A phylogenetic tree was created using the amino acid sequence deduced from the ORF2 sequence (Fig. 1). CAstV strains were divided into groups A and B. Group A was divided into subgroups Ai, Aii, and Aiii. Group B was divided into subgroups Bi, Bii, Biii, and Biv. All bootstrap values were 100. 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.

| Strain name      | Country   | Sampling year | Subgroup | Accession no. |
|------------------|-----------|---------------|----------|---------------|
| CAstV FP3        | UK        | 1984          | Bi       | JN582328      |
| CAstV 612        | South Africa | 1994         | Ai       | JN582317      |
| CAstV P22-18.8.00| Netherlands | 2004         | Ai       | JN582318      |
| CAstV 11522      | UK        | 2004          | Bi       | JN582305      |
| CAstV-11672      | UK        | 2004          | Bi       | HQ3305065     |
| CAstV 05V150/152/154 | Germany  | 2007          | Bi       | JN582312      |
| CAstV VF06-7/8   | UK        | 2006          | Bi       | JN582311      |
| CAstV VF06-1/4   | UK        | 2006          | Bi       | JN582309      |
| CAstV VF06-7/5   | UK        | 2006          | Bi       | JN582310      |
| CAstV VF06-1/1   | UK        | 2006          | Bi       | JN582307      |
| CAstV VF06-1/2   | UK        | 2006          | Bi       | JN582308      |
| CAstV VF06-7/3   | UK        | 2006          | Bi       | JN582313      |
| CAstV GA2011     | USA       | 2007          | Bi       | JF414802      |
| CAstV VF07-4/2   | UK        | 2007          | Bi       | JN582314      |
| CAstV VF08-29    | UK        | 2008          | Bi       | JN582315      |
| CAstV VF08-3     | UK        | 2008          | Bi       | JN582316      |
| CAstV VF08-56    | UK        | 2008          | Ai       | JN582319      |
| CAstV VF08-60    | UK        | 2008          | Ai       | JN582320      |
| CAstV VF08-46    | UK        | 2008          | Aiii     | JN582321      |
| CAstV VF08-65    | UK        | 2008          | Aii      | JN582322      |
| CAstV VF08-54    | UK        | 2008          | Aii      | JN582323      |
| CAstV VF08-18/7  | UK        | 2008          | Aii      | JN582324      |
| CAstV VF08-36    | UK        | 2008          | Aii      | JN582325      |
| CAstV VF08-48    | UK        | 2008          | Aii      | JN582326      |
| CAstV PDRC/573/West Zone | India  | 2011 | Bi | JX4945861 |
| CAstV PDRC/200/East Zone | India  | 2011 | Bi | JX4945853 |
| CAstV CAstV/Poland/G059/2014 | Poland | 2014 | Aiii | KT886453 |
| CAstV/CA-AB/Chicken/14-1235a/14 | Canada | 2014 | Biv | MT789774 |
| CAstV CAstV/INDIA/ANAND/2016 | India  | 2016 | Bi | KY038163 |
| CAstV CkP5       | USA       | 2016          | Biv      | KX397576      |
| CAstV CAstV/CHN/GDYHTJ718-6/2018 | China  | 2018 | Bi | MN725026 |
| CAstV SG1/Int/2020 | Japan   | 2020         | Bi       | OL321958      |
| CAstV SG2/Int/2020 | Japan   | 2020         | Bi       | OL321959      |
| CAstV SG3/Int/2020 | Japan   | 2020         | Bi       | OL321960      |
| CAstV SG4/C/2020 | Japan   | 2020         | Aiii     | OL321961      |
| ANV-1            | China     | 2009          | -        | HM029238      |
Multiple inter-tubular tophi and mild interstitial lymphocyte infiltration were observed in the kidneys of two out of four chickens examined (Fig. 2). We isolated CAstV (SG2/Int/2020) from one of these two chickens.

We detected four CAstV strains belonging to subgroups Aiii and Bi from chickens in Saga prefecture, Japan. This is the first report of CAstV in Japan. CAstV strains were detected on two farms (farms A and B). There was no traffic between these farms, which are several tens of kilometers apart geographically. Therefore, two CAstV subgroups were found to have infiltrated into chicken populations in Saga prefecture. In contrast, at least five subgroups have been found in the UK [5]. Since CAstV is a relatively new virus that was discovered only recently, it had not yet been

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**Fig. 1** Phylogenetic tree based on the amino acid sequence of the capsid protein encoded by the ORF2 gene. The tree was constructed by the neighbor-joining method with 1000 bootstrap replicates using the strains listed in Table 1. The isolates from this study (●) were classified into subgroups Aiii and Bi.
investigated in Japan. A survey of more chicken flocks in Japan might lead the detection of other subgroups of CAstV.

Subgroup Bi was detected in three chickens with visceral gout. No problems with feeding management, such as a lack of drinking water, were reported by the farmers. Histopathological examination revealed the presence of gouty nodules in the renal tubules in two of the four chickens examined. Subgroup Biii infections have been reported to cause such lesions [2]. Although we did not find other factors that might have caused the lesions, it is unknown whether CAstV actually was the cause of visceral gout. On the other hand, CAstV was not isolated from one of the two chickens with histological kidney lesions. In that chicken, it appeared that the virus did not replicate in the intestinal tract. More experiments are necessary to examine the pathogenicity and replication of subgroup Bi in the intestinal tract.

Subgroup Aiii was isolated from 150-day-old hens without clinical symptoms. Although it is possible that subgroup Aiii is not pathogenic, it is also possible that the hens had gained resistance to CAstV due to their age. Further studies are needed to clarify this issue.

We conclude that CAstV has infiltrated into Saga prefecture, Japan, and that some strains may be associated with visceral gout. Information about CAstV in Japan will be important to the poultry industry.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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