Asian Journal of Microbiology and Biotechnology

Volume 9, Issue 2, Page 115-120, 2024; Article no.AJMAB.12453
ISSN: 2456-8341

Molecular Detection and Isolation of Chicken Astrovirus from Commercial Chicken Flocks of Kerala, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/ajmab/2024/v9i28921

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.ikprress.org/review-history/12453

Short Communication

Received: 15/08/2024
Accepted: 23/10/2024
Published: 28/10/2024

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Cite as: *Sarma, Suprativ, Rajasekhar R, Chintu Ravishankar, Sumod K, Pradeep M, Anoopraj R, Sandhiya K, Madhanraj N, Vishnu P, and Abhijith S. 2024. "Molecular Detection and Isolation of Chicken Astrovirus from Commercial Chicken Flocks of Kerala, India". Asian Journal of Microbiology and Biotechnology 9 (2):115-20. https://doi.org/10.56557/ajmab/2024/v9i28921.*

ABSTRACT

Reduced productivity due to enteric infections in chickens is a very common challenge in the poultry industry of India. Various studies associated Chicken astrovirus (CAstV) as a common pathogen in commercial flocks exhibiting poor weight gain, stunted growth and visceral gout. However, no published report documented the detection of the virus from Kerala so far. Hence, this study was done to investigate the presence of CAstV in 50 broiler flocks from different parts of Kerala with clinical signs such as retarded growth, diarrhoea, and a mortality of 5 to 10 %. Out of the 50, five flocks were found positive for the virus by using the Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) method with the primers targeting the partial ORF1b gene of the viral nucleic acid. The viral presence was confirmed by isolation using embryonated chicken eggs (ECE), which produced notable alteration to the infected embryos. Nucleotide sequencing of the isolates revealed a high identity of up to 100 % with the other CAstV sequences retrieved from the NCBI database. The study confirms the first report of molecular detection and isolation of CAstV from the state of Kerala.

Keywords: Chicken astrovirus; molecular detection; enteric infection; isolation; Kerala.

1. INTRODUCTION

The commercial poultry industry is one of the fastest-growing segments of the agricultural sector in India. Population and dietary shifts have increased the demand for poultry meat and eggs. The poultry sector in Kerala has experienced substantial expansion, playing a vital role in the state's economy while satisfying the rising demand from consumers. Despite this progress, disease challenges, especially enteric infections continue to be a major obstacle to achieving maximum productivity in the sector.

Astroviruses are generally associated with various enteric and nephritic challenges in the poultry industry [1]. Chicken astrovirus (CAstV) from the family *Astroviridae* is a positive-sense single-stranded RNA virus without an envelope [2]. The genome of CAstV is ~7.5 kb in length [3] and consists of three open reading frames (ORF) viz. ORF1a, ORF1b, and ORF2 encode for serine protease, RNA-dependent RNA polymerase (RdRp) and viral capsid protein respectively, along with 5' and 3' untranslated regions [4]. The ORF1b gene has a significant conserved region in the genome which is usually opted for amplification and molecular detection of the virus [5]. The virus primarily transmits horizontally through faeco-oral routes and contaminated fomites. Young chicks are more susceptible to clinical infections with the virus mainly multiplying in the gastrointestinal tract [6]. Frequent clinical manifestations observed are malabsorption-related disorders such as diarrhoea and runting and stunting syndrome (RSS) [7,8]. The association of CAstV with nephritic conditions has also been documented in several gout outbreaks among commercial broiler chickens in India [9]. Reports of dead-in-shell chicks having hatchability problems along with 'white chick hatchery disease' are also recorded [10,11].

Although several investigations have been done to isolate and study various enteric viral pathogens, only a few reports about avian astroviruses from India can be found. No published reports currently exist regarding the presence of CAstV in Kerala. Therefore, this study aims to detect and isolate the virus from various commercial flocks exhibiting enteric symptoms accompanied by mortality.

2. METHODOLOGY

2.1 Sample Collection

Cloacal swabs from live birds, along with intestinal contents and samples from the kidney, liver, thymus, and spleen collected post-mortem, were obtained from various flocks exhibiting symptoms of stunted growth, diarrhoea, or enteritis. These samples were collected in sterile containers with cell culture media and transported on ice.

2.2 RNA Extraction

The total RNA from the samples were extracted by conventional method using TRIzol reagent (Ambion, USA) according to manufacturer's instructions and stored at – 80 ℃ until further processing.

2.3 Amplification by RT-PCR

Viral RNAs extracted from the samples were utilized for reverse transcription to synthesize complementary DNA (cDNA) using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol. To the PCR tube kept on ice, 8 µL of total RNA and 1 µL (0.2 µg/µL) of random hexamer were added. Then, the volume to 12 µL by adding nuclease-free water (NFW). The mixture was incubated at 65 ºC for 5 min. The above mixture was cooled down to 25 ºC and 4 µL of 5X reaction buffer, 1 µL (20 U/µL) Ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix and 1 µL (200 U/µL) RevertAid H minus M-MuLV reverse transcriptase enzyme were added. Then the mixture was incubated at 25 ºC for 5 min followed by 60 min at 42 ºC. The reaction was terminated by heating at 70 ºC for 5 min. The cDNA was stored at –20 ºC for further use.

The detection of CAstV was carried out by amplification of a ~362 bp segment of the ORF1b region of the Polymerase gene, using a specific primer set outlined by Day et al. [12]. The primer sequence details include: Forward primer: **5'-**GAYCARCGAATGCGRAGRTTG - **3'** and Reverse primer: **5'-**TCAGTGGAAGTGGGK ARTCTA- **3'.**

The PCR conditions incorporated in the study for a 25 µL reaction mixture are taking of 12.5 µL 2X EmeraldAmp GT PCR master mix (2X) (Takara), 1 µL each of forward and reverse primers (10 pmol), 2 µL of cDNA and rest NFW to make up the volume. The cycling conditions were 94°C for 5 min (initial denaturation), 35 cycles of 94°C for 30 sec (denaturation), 53°C for 30 sec (annealing) and 72°C for 45 sec (polymerisation) followed by a single cycle at 72°C for 3 min (final extension). No template control was included in each run. After the gel run visualisation of the PCR product was carried out using 1.5 % agarose gel electrophoresis.

2.4 Virus Isolation

Samples confirmed positive for CAstV through RT-PCR analysis were chosen for viral isolation. Tissue homogenate from infected birds was filtered through a 0.22 µm filter and used as the inoculum. Embryonated chicken eggs, 9 to 10 days old, were candled and selected for inoculation via the allantoic route. The eggs were incubated at 37.5 ºC and monitored regularly for mortality up to 96 hours post-infection. The eggs were kept for overnight chilling at 4 ºC before harvesting.

2.5 Sequencing

To confirm the identity of the obtained amplicon, the PCR product of positive samples, along with the corresponding primers, was sent to GenSpec Pvt. Ltd., Kochi for sequencing.

3. RESULT AND DISCUSSION

3.1 Molecular Detection

Out of the 50 samples analysed, five (10%) tested positive for CAstV. In these positive samples, a ~362 bp segment of the CAstV Polymerase gene was successfully amplified using RT-PCR (Fig. 1). This shows a relatively lower positive percentage of the virus than the previous findings from India which recorded 80.2%, 38.75% and 20.52% positivity assessing flocks from various regions of the country [9,13,14]. However, prior research in India primarily concentrated on identifying CAstV in flocks affected by gout and enteritis [9,13,14,15,16]. The present CAstV isolates are primarily linked to stunted growth and decreased productivity, which is consistent with multiple studies that have demonstrated the virus' role in these issues globally [7,17,18].

3.2 Sequencing

The nucleotide sequences obtained from the amplicons positive for the virus were submitted to GenBank under the accession numbers PP741993, PP741994, PP741995, PP741996 and PQ408913. The BLAST analysis of the partial ORF1b gene (321 bp) from the current CAstV isolates, corresponding to nucleotide positions 790 to 1110 of the full-length ORF1b gene (reference isolate: MN725026), demonstrated 96.24% to 98.72% nucleotide similarity with Indian isolates. While comparing current isolates with foreign isolates, the highest identity was recorded with Chinese isolates, ranging from 94.98% to 95.49%. However, previous studies from India revealed more similarity to Poland, USA and Brazil isolates [14,15].

3.3 Amino Acid Analysis

The amino acid sequence of the partial ORF1b gene from the current isolates, comprising 107

amino acids (spanning positions 264 to 370 of the full-length ORF1b sequence, reference isolate MN725026), was compared with closely related Indian and foreign sequences. The current isolates exhibited 97.2% to 100% similarity with Indian isolates and 97.1% to 99% homology with foreign isolates. Notably, in isolate PP741994, a unique non-synonymous mutation was identified at position 360, where Lysine (K) was substituted with Asparagine (N). Although the polymerase gene is highly conserved, previous reports from India also recorded few mutations, which can be due to natural selection and better virus adaptation [13].

3.4 Virus Isolation

After three passages, prominent alterations were recorded in the inoculated embryos. All the embryos exhibited stunting, while some of them had haemorrhages over the body. Hepatic necrosis was another common finding in the embryos (Figs. 2 and 3). No mortality was observed till 96 hours PI. The presence of the virus was confirmed by RT-PCR. Similar alterations along with kidney lesions in the ECE after inoculation with RSS or gout-affected samples have been recorded previously [9,11,13].

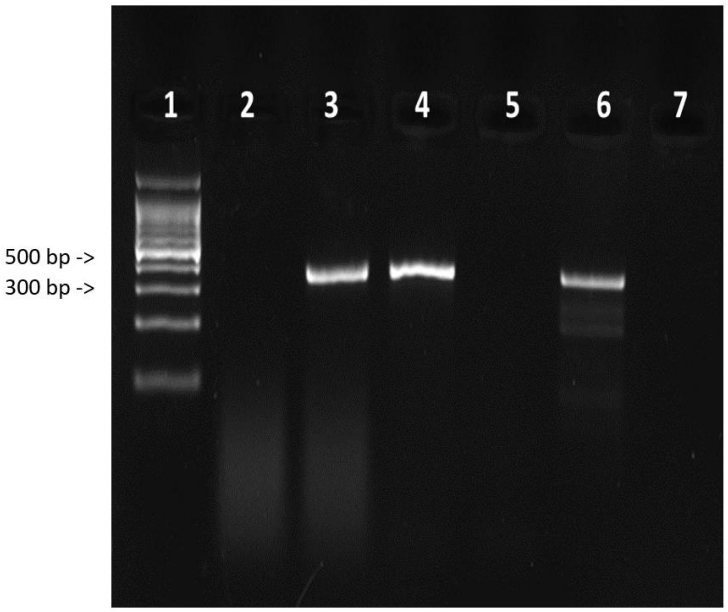

Fig. 1. PCR amplification showing amplicon size of ~362 bp, specific for ORF1b gene of CAstV (Lanes: 1: DNA marker (100bp); 2: Negative control; 3,4 and 5: Positive isolates)

Fig. 2. Stunting and haemorrhages observed in embryos inoculated with CAstV

Fig. 3. CAstV inoculated embryo showing hepatic lesions after 3rd passage 96 h PI

4. CONCLUSION

The current study confirms the presence of CAstV for the first time in Kerala through molecular detection and isolation using embryonated chicken eggs. This also finds RT-PCR as a reliable method for diagnosis of the virus from clinical samples targeting the ORF1b gene. However, the presence of the virus in 10 % of the commercial flocks showing enteric symptoms cannot fully justify CAstV as the sole cause of such symptoms and mortality. The current findings can be useful for future characterisation and study of the pathogenesis of the virus, along with its prevention and control.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

IRB approval for the research work: KVASU/DAR/A3/2457/2023(1) Dated 23/12/ 2023, of the Director of Academics and Research, KVASU, Pookode, Wayanad, Kerala.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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