SHORT COMMUNICATION

Molecular Characterization and Phylogenetic Analysis of Chicken Infectious Anaemia Virus in Commercial Poultry Flocks in Andhra Pradesh

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Abstract

Chicken infectious anaemia (CIA) is a significant economic concern in the poultry industry, particularly among young chicks. It is characterized by inadequate weight gain, anaemia, immunosuppression, and production loss. The study aimed to identify chicken anaemia viruses (CAVs) in suspected commercial poultry flocks and molecular characterization of the VP1 gene of circulating CAVs, along with phylogenetic analysis of the VP1 gene. Throughout the study, samples were obtained from 47 birds in nine commercial poultry flocks during necropsy. Tissue samples including the thymus, bone marrow, liver, and spleen, were collected in phosphate buffer saline (PBS) and subjected to PCR targeting VP1 gene, resulting in the amplicon size of 454 bp. Among 47 samples 40 were found positive for the VP1 gene. The DNA from four different farms, tested positive, was sent for sequencing of the VP1 gene. Phylogenetic analysis based on nucleotide sequence alignment revealed that CAV isolates from Nuzividu region clustered with isolates from China and the remaining three CAV isolates did not form any group with the already known genotypes. Furthermore, to understand the spread of the disease and help to develop effective control and prevention strategies, the detailed information about the specific circulating CAV strains is needed.

Key words: Chicken anaemia virus, Chicken infectious anaemia, Phylogenetic analysis, Polymerase chain reaction.

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Introduction

Chicken infectious anaemia (CIA) is an economically important viral disease of poultry, first reported in 1979 in Japan (Dhama *et al*., 2008), which causes considerable health problems in poultry flocks worldwide (Praveen *et al*., 2008). Chicken infectious anaemia is caused by the chicken infectious anaemia virus (CIAV). This virus is one of the smallest avian viruses, with a circular single-stranded DNA genome that is 2.3 kb in size. It belongs to the Gyrovirus genus within the Circoviridae family. CIAV acts as a potent immunosuppressive agent, primarily affecting young chicks. Symptoms include poor weight gain, severe anaemia, bone marrow aplasia, generalized lymphoid atrophy of all haematopoietic and lymphoid organs, and intramuscular and subcutaneous haemorrhages (Schat, 2003). The disease spreads both horizontally and vertically. CIAV targets the thymus, preventing T lymphocyte maturation and compromising the response of cell-mediated immunity. Lymphocyte depletion-induced immunosuppression raises the susceptibility to diverse bacterial and viral infections and diminishes the effectiveness of vaccine responses and production performance (Ganar *et al*., 2017).

It is the smallest non-enveloped avian virus (25 nm), with icosahedral symmetry and a single-stranded, negative sense circular DNA genome. It contains three main open reading frames (ORFs) that code for VP1, VP2, and VP3 proteins. VP1 is a major capsid protein, it plays an important role in the growth and spread of the virus. VP2 is a non-structural protein that helps in scaffolding during virus assembly and VP3 (apoptin) is a non-structural protein that is mainly involved in the induction of apoptosis and responsible for the disease pathogenesis. A tentative diagnosis of CIAV infection can usually be made based on clinical signs, flock history and gross pathological findings in affected birds. The diagnosis can be confirmed by isolating and identifying the virus (Praveen *et al*., 2008). The present study was aimed at PCR-based diagnosis of CIAV from clinical cases in the commercial

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poultry flocks in Andhra Pradesh by targeting VP1 gene and phylogenetic analysis of the field circulating CIAV's based on sequence analysis of VP1 gene.

Materials and Methods

Sample Collection

Samples were collected from commercial poultry layer flocks in Andhra Pradesh, aged 12-16 weeks, showing signs of anaemia, poor performance, dullness, and postmortem findings of regressed thymus, pale bone marrow, haemorrhages, atrophied spleen, and high mortality rates. The tissue samples were aseptically collected from 47 birds in nine different flocks (6 vaccinated and 3 non-vaccinated) including liver, thymus, bone marrow, and spleen in phosphate buffer saline, transported on ice to the laboratory, and stored in a -20°C refrigerator. Subsequently, all the samples were examined for the presence of CIAV-DNA using polymerase chain reaction.

Viral DNA Isolation and Amplification of VP1 Gene by PCR

Each farm's tissue samples were pooled separately and the DNA was extracted using DNAZOL® Reagent following the manufacturer's instructions. The extracted DNA was utilized as template DNA for PCR analysis targeting the VP1 gene. Oligonucleotide primer sequence CAV VP1-F 5'-AGCCGACGAACCGCAAGAA-3' and CAV VP1-R5'-AGACCCGTCCGCA ATCAACTCACC-3' (Udhayavel *et al*., 2013) was used to amplify the VP1 gene. PCR amplification was conducted in a PCR buffer, consisting of 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmoles of each primer, and 1.0 Unit of Taq polymerase, in a total reaction volume of 25 µL. The specific DNA of CAV was amplified using the primer set (CAV VP1-F and CAV VP1-R), beginning with an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation, annealing, and extension, each lasting 1 min at 94°C, 63°C, and 72°C, respectively. The final extension step was conducted at 72°C for 5 min. The amplified PCR products were separated on 1.5% agarose gel and visualized by a gel documentation system (Bio-Rad).

Nucleotide Sequencing and Phylogenetic Analysis

The PCR products of four field isolates namely CAV-NZ-BM5, CAV-K-L6, CAV-V-L1, and CAV-M-TH3, which were tested positive for the VP1 gene, were sequenced at Barcode Biosciences Private Limited in Bangalore, Karnataka, India. The nucleotide sequences were manually edited using BioEdit software by aligning them with reference sequences of CAV strains obtained from GenBank. The phylogenetic analysis was carried out using MEGA 11 and ClustalW, and a Neighbour-Joining tree was built with a bootstrap value of 1000 replicates.

Results and Discussion

Detection of CAV by PCR

The VP1 region has been highlighted in most of researches for its significance in the virulence of the virus, making it a primary target for scientific investigation. The VP1 region of the viral genome is highly variable and plays a key role in viral assembly (Sreekala *et al.,* 2020). In the present study, out of the 47 birds from nine distinct farms subjected to DNA extraction and PCR targeting VP1 gene, 40 (85%) from seven farms tested positive for CAV. PCR amplification resulted in the detection of a specific 454 bp product (Fig. 1). Hiremath *et al.* (2013) indicated that the VP1 region is highly susceptible to mutations and plays a critical role in pathogenicity. Their research focused on the VP1 gene along with two other genes. Similarly, Wani *et al.* (2013) investigated all three genes but emphasized the significance of the VP1 gene in their study.

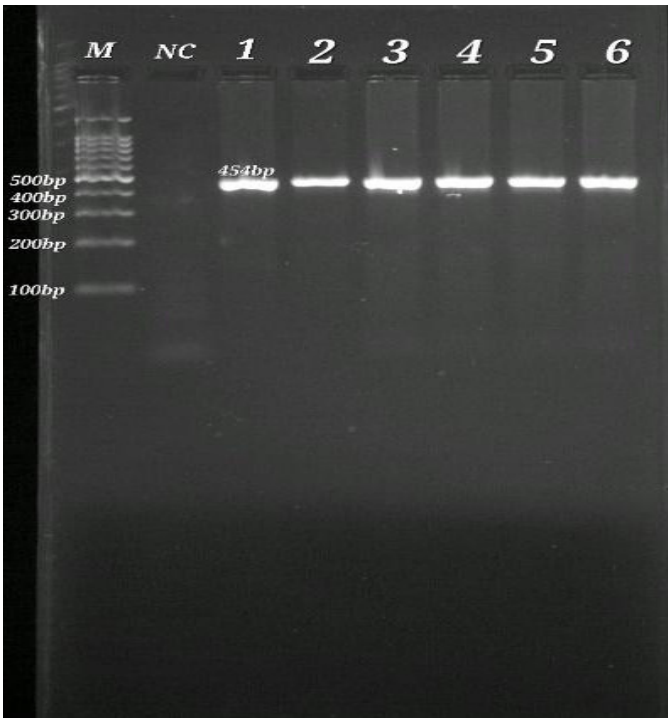

Fig. 1: Agarose gel electrophoresis of PCR-amplified VP1 (454 bp) gene products. Lane M: 100 bp DNA ladder, Lane NC: Negative control, Lane 1: Positive control, Lane 2-6: Samples positive for CAV.

Phylogenetic Analysis of CAV VP1 Gene Sequences

The nucleotide sequences of the VP1 gene (454 bp) from four samples, namely CAV-BM5-NZ, CAV-TH1-V, CAV-L6-K, and CAV-BM3-M, were submitted to the GenBank database. The accession numbers obtained were PP405627, PP471996, PP471995, and PP471997, respectively.

The phylogenetic analysis was carried out by comparing with nucleotide sequences of CAV, as well as vaccine and field strains of CAV retrieved from GenBank. The local CAV sample, CAV-BM5-NZ, exhibited the highest nucleotide identity (99%) with reference strains Nagpur-India-KX377124.1, Punjab-India-MH269371.1, and China-TJBD40-AY846844.1, and the

lowest identity (93.2%) with IVRI-India-AY583757.1 and CAV-EG-14/Egypt-MH001565.1. Similarly, the nucleotide sequence of CAV-TH1-V showed the highest identity (99.2%) with the same reference strains and the lowest identity (94.2%) with IVRI-India-AY583757.1 and CAV-EG-14/Egypt-MH001565.1. Likewise, the nucleotide sequence of CAV-L6-K and CAV-BM3-M shared the highest identity (99.2%) with the aforementioned reference strains and the lowest identity (93.7%) with IVRI-India-AY583757.1 and CAV-EG-14/Egypt-MH001565.1.

Andrabi *et al.* (2021) discovered in their research that the VP1 nucleotide sequences from the Punjab region did not group with any previously known CAV genotypes. Kulkarni *et al.* (2024) reported that the VP1 sequences of three isolates clustered with the low pathogenic C369 infectious clone CAV from Japan and the Del-Ros vaccine strain, while one isolate clustered with a strain from the UK. Phylogenetic analysis using nucleotide sequence alignment showed that CAV isolates from the Nuzividu region grouped with CAV isolates from China (China-TJBD40-AY846844.1), while the remaining three CAV isolates did not align with any known genotypes (Fig. 2). All the four isolates formed distinct clusters away from the vaccine strains (Cux-1 vaccine strain, Del-Ros/USA/vaccine, and Nobilis P4 vaccine from the Netherlands).

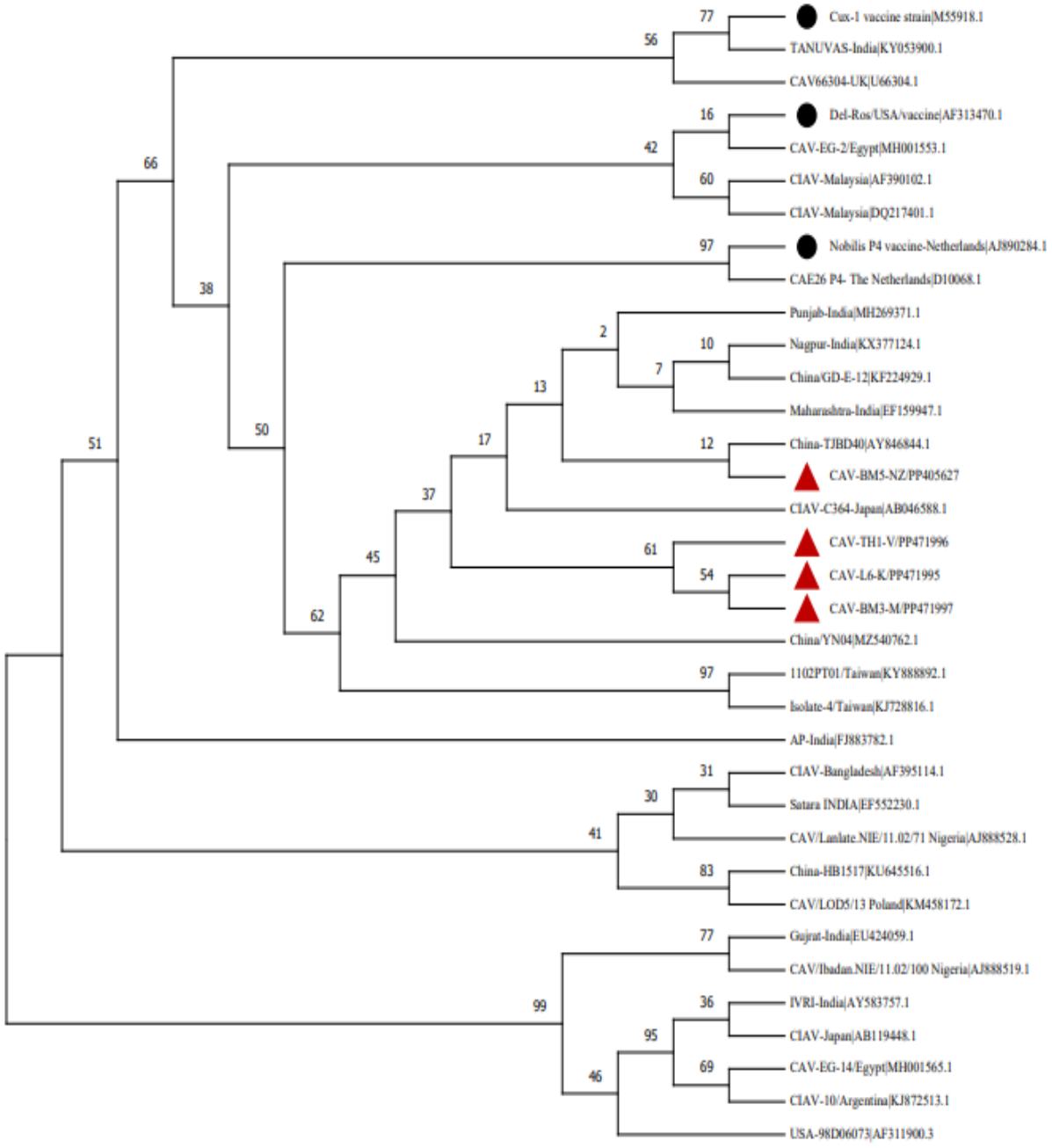

Fig. 2: Phylogenetic tree based on multiple sequence alignment of VP1 nucleotide sequences.

CAV is a significant pathogen in the poultry industry due to its economic impact, immunosuppressive characteristics, and global prevalence. The present study investigated the presence of CIAV among commercial poultry flocks in Andhra Pradesh. The tentative diagnosis was based on gross lesions, which included atrophied lymphoid organs, pale bone marrow, and gangrenous dermatitis. The confirmatory diagnosis was made using PCR. The study also found a high chicken anaemia incidence among poultry flocks in Andhra Pradesh. Out of nine farms tested, seven were positive for CIAV. The molecular detection and characterization of the CIAV genome focused on the VP1 gene and a partial genome segment (including all three ORFs) of CIAV. The genome is highly conserved along its length, except for the VP1 gene, which shows the most nucleotide variation (Todd *et al.,* 1990). Among the three CIAV genes, the hypervariable region is found in the VP1 gene, which encodes a highly immunogenic protein forming the base of the capsid in mature virus particles. The immunogenicity and virulence of the virus are influenced by the presence of specific amino acids in the VP1 gene.

Conclusion

Based on this research, it was determined that the circulating CAV in Andhra Pradesh exhibits a significant capacity for spread and comprises motifs commonly associated with virulent viruses, indicating its high pathogenicity. Moreover, phylogenetic analysis using nucleotide sequence alignment demonstrated that the indigenous CAVs did not cluster with any existing genotypes, suggesting a unique genetic composition. Hence, additional molecular epidemiological investigations are necessary to identify the genotype/sub-type and pathotypes of the CAVs prevalent in this area. This epidemiological data can help to develop effective disease control and management measures, crucial for mitigating the economic losses and vaccine ineffectiveness associated with CIA in commercial poultry flocks.

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