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Research Article

Molecular detection of concurrent infections of chicken infectious anaemia and Newcastle disease in poultry flocks in Andhra Pradesh

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Abstract

Chicken Infectious Anaemia (CIA) is a globally significant viral disease affecting the poultry industry, caused by the Chicken Infectious Anaemia Virus (CIAV). The study focused on detecting and characterizing CIAV in commercial poultry farms across Andhra Pradesh, India, using molecular and serological techniques. Tissue samples including liver, thymus, bone marrow and spleen were collected from 75 birds across 22 farms and analysed for CIAV using PCR targeting the VP2 gene. The findings revealed a high prevalence of the virus with 72.7% (16/22) of farms testing positive. Notably, the detection rate was higher in layer farms (84.6%) compared to broiler farms (55.5%). Phylogenetic analysis of the sequenced VP2 gene isolates (CIAV-NZ, CIAV-C, CIAV-K and CIAV-M) showed a high degree of similarity (95.3%-100%) with previously reported Indian and international strains, suggesting strong genetic conservation of the VP2 gene. Additionally, concurrent Newcastle Disease Virus (NDV) infection was detected in four CIAV-positive flocks, confirming the immunosuppressive effects of CIAV that predispose birds to secondary infections. These findings highlight the significant economic and health effects of CIAV in commercial poultry farming. The study stresses the need for strong disease monitoring, strict biosecurity measures and effective vaccination to prevent CIAV-related immunosuppression and co-infections in poultry.

Keywords: Chicken Infectious Anaemia Virus, Newcastle Disease Virus, Phylogenetic analysis, Polymerase Chain Reaction

Highlights

- CIAV detected in 72.7% of poultry farms, higher in layers (84.6%) than broilers (55.5%)
- VP2 gene sequences clustered with Indian and Asian CIAV strains, showing high genetic conservation
- NDV co-infection confirmed in CIAV-positive flocks, amplifying disease severity

INTRODUCTION

Chicken infectious anaemia (CIA) is an emerging viral disease of significant economic impact on the poultry industry (Zhang *et al*., 2012). The disease is caused by the chicken infectious anaemia virus (CIAV), a small DNA virus from the family *Circoviridae* and belongs to the genus *Gyrovirus*. CIAV is widespread and has caused severe outbreaks in young chicken flocks worldwide. The virus was first discovered in Japan in 1979 (Yuasa *et al*., 1979), while serological evidence suggests its presence in the USA as early as 1959 (Toro *et al*., 2006). The CIAV genome encodes three proteins: VP1, VP2 and VP3. VP1, a structural protein and VP2, a scaffolding protein, together form the viral capsid and serve as the epitope for neutralizing antibodies against CIAV. The third protein, VP3 commonly known as "apoptin" due to its ability to induce apoptosis in susceptible cells and plays a key role in the pathological changes associated with the disease (Schat, 2009). Significant economic losses result from poor growth, increased mortality, carcass condemnation and the cost of antibiotics to control secondary bacterial infections. While the average mortality rate is 5-10%, it can rise as high as 60% (Chowdhury *et al*., 2017). The disease can be transmitted both vertically and horizontally leading to either clinical or subclinical infections. The virus pathogenesis begins with its attachment and entry into haemocytoblasts in the bone marrow and lymphoblasts in the thymus, culminating in the manifestation of signs and symptoms (Noteborn, 2004). Other lesions include a pale, discoloured and enlarged liver and spleen, along with areas of congestion and hemorrhages in other visceral organs (Wani *et al*., 2014). CIAV

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primarily targets the cellular immune system, sparing the humoral system as B cells are not susceptible to the infection.

This study aims to diagnose CIAV in poultry flocks in Andhra Pradesh using PCR targeting the VP2 gene, conduct a phylogenetic analysis of field strains based on VP2 gene sequences and investigate concurrent NDV infection in CIAV-infected flocks.

MATERIALS AND METHODS

Sample collection: Samples were collected from different districts of Andhra Pradesh from commercial poultry layer flocks and broiler flocks, exhibiting clinical symptoms of anaemia, poor performance, lethargy, pallor comb and wattles. The postmortem findings that included were regressed thymus, pale bone marrow, hemorrhages on the internal organs, an atrophied spleen and high mortality rates. Tissue samples including liver, thymus, bone marrow and spleen from 75 birds across 22 different flocks (13-layer flocks and 9 broiler flocks) were aseptically collected. The samples were collected in phosphate-buffered saline, transported on ice and stored in a -20°C freezer.

Viral DNA isolation and amplification of VP2 Gene by PCR: Tissue samples from each farm were pooled individually and DNA was extracted using DNAZOL® Reagent following the manufacturer's instructions. The extracted DNA was then used as a template for PCR analysis targeting the VP2 gene. To amplify the gene oligonucleotide primers CIAV VP2-F (5'- AGCGCACATACCGGTCGGCAGT -3') and CIAV VP2-R (5'- AGGGGTTCGGCAGCCTCACACTAT -3') (Hiremath *et al*., 2013) were used. The PCR reaction was carried out in a buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer and 1.0 Unit of Taq polymerase with a total reaction volume of 25 µL. PCR conditions included with an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation, annealing and extension at 94°C, 64°C and 72°C respectively each for 1 minute. The process concluded with a final extension at 72°C for 10 minutes. The amplified PCR products were resolved on a 1.5% agarose gel and visualized using a Bio-Rad gel documentation system.

Nucleotide sequencing and phylogenetic analysis: The PCR products from four field isolates - CIAV-NZ, CIAV-C, CIAV-K and CIAV- Mthat tested positive for the VP2 gene were sequenced at Barcode Biosciences Private Limited in Bangalore, Karnataka, India. Nucleotide sequences were manually edited in BioEdit software by aligning them with reference CIAV strain sequences obtained from GenBank. Phylogenetic analysis was conducted using MEGA 11 and ClustalW, with a Neighbor-Joining tree generated using 1000 bootstrap replicates.

Detection of concurrent Newcastle disease infection in birds infected with chicken infectious anaemia virus (CIAV): Tissues such asspleen, liver, proventriculus, caecal tonsils and trachea were collected during necropsy from the farms under suspicion of Newcastle disease in CIAV infected flocks. The samples were homogenized with PBS and the homogenized samples were then clarified by centrifugation at 10,000 g for 10 minutes at 4°C and filtered using 0.2 µm syringe filters. The resulting filtrate was supplemented with antibiotic-antimycotic solution (containing 10,000 U Penicillin, 10 mg Streptomycin and 25 µg Amphotericin B per mL in 0.9% normal saline) and stored at -80°C until further use for virus isolation.

The processed samples were inoculated into 9-11 day old embryonated chicken eggs via the allantoic route and incubated at 37°C in an egg incubator. The embryos were periodically examined using an egg candler in a dark room to assess their viability. Embryos that died after 24 hours or within a maximum of 120 hours were harvested. Viral RNA was extracted from harvested amnio-allantoic fluid using the TRIsoln method. The first strand cDNA from the viral RNA was synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit with random hexamers following the manufacturer's instructions.

The detection of NDV was confirmed through RT-PCR by amplifying the FPCS gene with the primers NDV FPCS-F (5'-GCAGCTGCAGGGATTGTGGT -3') and NDV FPCS-R (5'-TCTTTGAGCAGGAGGA TGTTG -3') (Nanthakumar *et al*., 2000). The PCR reaction was carried out in a buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer and 1.0 Unit of Taq polymerase with a total reaction volume of 25 µL. PCR conditions included with an initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The process concluded with a final extension at 72°C for 10 minutes. The amplified PCR products were resolved on a 1.5% agarose gel and visualized using a gel documentation system (Bio-Rad).

RESULTS

Detection of CIAV by PCR: Out of the 22 poultry farms tested in this study, 72.7% (16 farms) were positive for the VP2 gene of CIAV when analysed using VP2 F and VP2 R primers, yielding a 713 bp amplicon confirmed on agarose gel (Fig. 1). Among these, the detection rate was higher in layer farms (84.6%, 11/13) compared to broiler farms (55.5%, 5/9), indicating a greater prevalence of CIAV in layers.

Phylogenetic analysis of CIAV VP2 gene sequences: The positive samples for the VP2 gene identified as CIAV-NZ, CIAV-C, CIAV-K and CIAV-M, were sequenced and submitted to GenBank, the accession numbers obtained were PQ059472, PQ059473, PQ059474 and PQ059475, respectively.

In phylogenetic analysis of VP2 gene CIAV-C, CIAV-K and CIAV-M clustered with Indian strains (TANUVAS, Nagpur, Gujarat) along with the strains from China, Japan, Malaysia and the UK including the

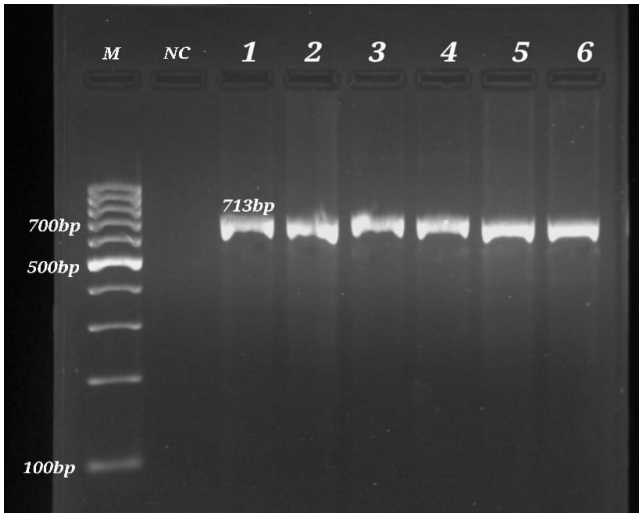

Fig. 1. Gel showing amplicon of CIAV VP2 gene (713 bp). Lane M: 100 bp DNA ladder, Lane NC: Negative control, Lane 1: Positive control and Lane 2-6: Samples positive for CIAV.

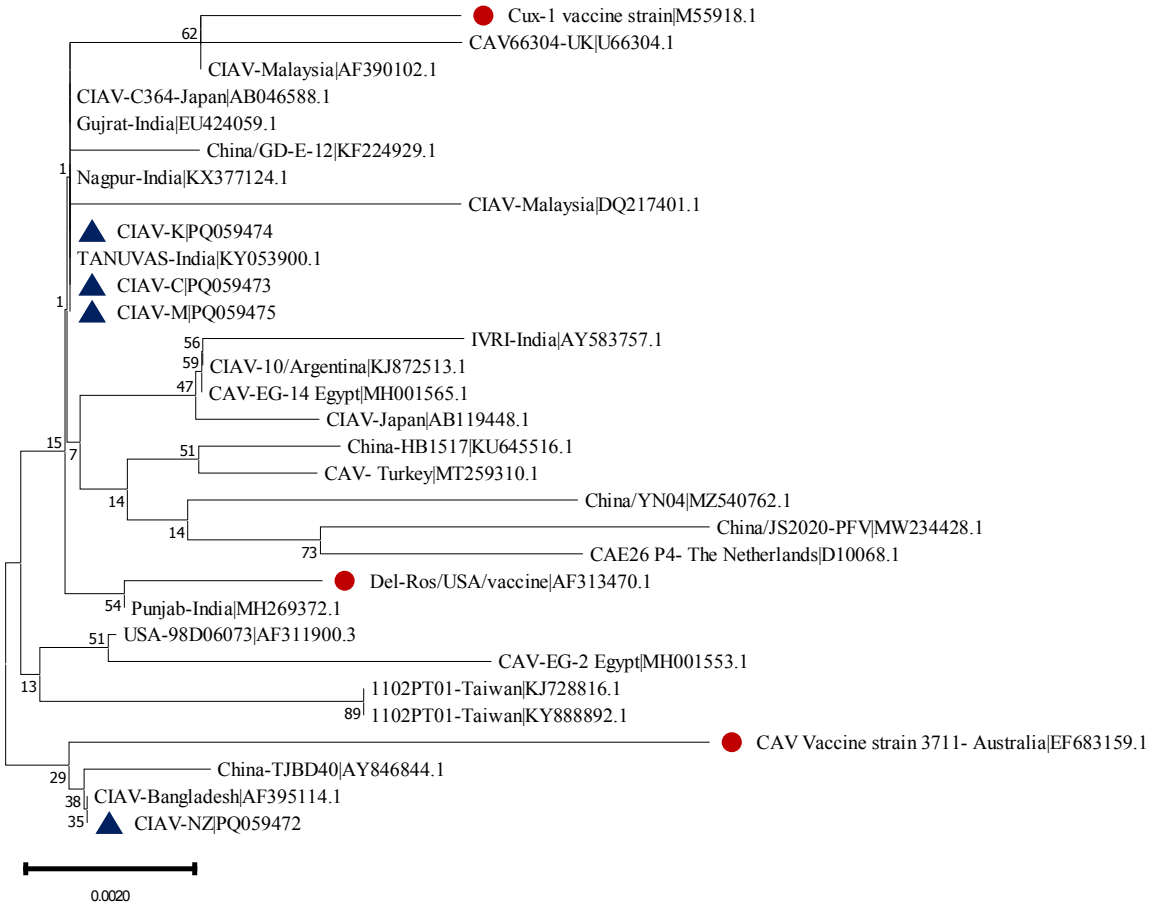

Fig. 2. Phylogenetic tree based on multiple sequence alignment of VP2 gene nucleotide sequences by Neighbour Joining Method.

Cux-1 vaccine strain (Fig. 2). CIAV-NZ clustered with strains from Bangladesh and China.

Detection of concurrent Newcastle disease infection in birds infected with chicken infectious anaemia virus (CIAV): In this current study four flocks were tested positive for 356 bp partial fusion gene of Newcastle Disease Virus (NDV) indicating a concurrent infection with CIAV (Fig. 3). The immunosuppression caused by CIAV likely rendered the birds more vulnerable to NDV infection.

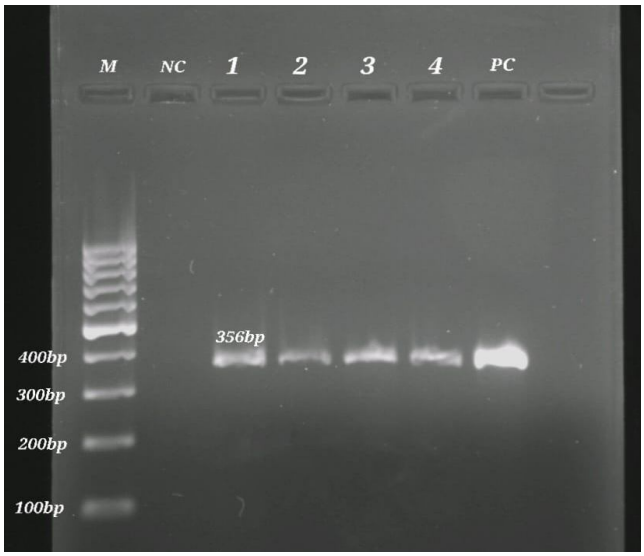

Fig. 3. Gel Documentation of the amplicon of NDV-FPCS gene (356 bp) from CIAV positive field strains. Lane M: 100 bp DNA ladder, Lane NC: Negative control, Lane 1-4: Samples positive for NDV-FPCS gene and Lane PC: Positive control.

DISCUSSION

Poultry farming is a rapidly growing industry in India, playing a crucial role in the country's economic development and enhancing nutritional standards. The current study indicates that the CIAV strains circulating in Andhra Pradesh have a high potential for transmission and possess motifs characteristic of highly virulent viruses, suggesting significant pathogenicity. Detection of VP2 gene by PCR aligns with previous studies Eltahir *et al*. (2011) successfully amplified the VP1 and VP2 genes of CIAV from 47 spleen samples, obtaining specific products of 1390 bp and 713 bp, respectively. Similarly, Gholami-Ahangaran *et al*. (2013) detected the VP2 gene of CIAV in thymus and blood samples from chickens, ostriches and turkeys, confirming the presence of a 713 bp amplicon. These studies collectively reinforce the reliability of the VP2 gene as a molecular target for CIAV detection and highlight its widespread occurrence in commercial poultry, particularly in layer farms. Additionally, Andrabi *et al*. (2021) reported CIAV detection in 26% of poultry farm samples from Punjab, with a similar trend of higher prevalence in layer farms (84.6%) than in broiler farms (55.5%). This disparity may be attributed to the longer lifespan of layer birds, which increases their susceptibility to horizontal transmission and repeated exposure to the virus. Furthermore, the common use of multi-age systems in layer farms facilitates ongoing viral circulation, unlike the all-in/all-out management practiced in broiler farms, which helps limit viral persistence.

Upon BLAST analysis of the VP2 gene nucleotide sequence, the local CIAV sample CIAV-NZ showed the highest identity (100%) with the reference strain IVRI-India-AY583757.1 and the lowest identity (95.3%) with Punjab-India-MH269372.1. Similarly, the isolates CIAV-C, CIAV-K and CIAV-M showed the highest similarity (100%) with strains from Gujrat-India-EU424059.1, TANUVAS-India-KY053900.1, Nagpur-India-KX377124.1 and CIAV-C364-Japan-AB046588.1, while exhibiting the lowest identity with Punjab-India-MH269372.1. Andrabi *et al*. (2021) similarly noted 100% similarity between their local CIAV samples and strains from Gujarat, TANUVAS and Japan, further supporting the conserved nature of the VP2 gene in Asian CIAV isolates.

The phylogenetic analysis of VP2 gene sequences aligns with Hiremath *et al*. (2013), who reported high VP2 gene sequence identities (98.8-99.8%). The current findings were consistent with the conservation of VP2 across different geographic locations, as these isolates shared clades with strains from India and several other countries, including China and Malaysia. Andrabi *et al*. (2021) found 100% similarity between Indian strains (e.g., TANUVAS, Gujarat) and those from Japan, similar to this study shared clades between CIAV isolates and strains from India and other regions. Abdelhalim *et al*. (2021) and Hosseini *et al*. (2021) did not focus directly on the VP2 gene but noted genetic diversity in field isolates, which is in consistent with the current findings where CIAV-NZ clustered with international strains and other isolates formed clades with local Indian strains. Li *et al*. (2023) classified Asian strains into genotype A which aligns with present study CIAV-NZ grouping with strains from China and Bangladesh emphasizing geographic similarities in VP2.

The detection of concurrent NDV infection in CIAV-infected flocks aligns with several studies, including those by Dhama *et al*. (2008), Hadimli *et al*.

(2008), and Adedeji *et al*. (2016) have demonstrated that CIAV has strong immunosuppressive effects which make birds more susceptible to co-infections. This was consistent with findings from Gowthaman *et al*. (2017) who reported that 37% of CIAV-positive commercial poultry flocks also tested positive for NDV, highlighting the propensity for CIAV-infected birds to develop secondary viral infections like NDV. The amplification of the fusion gene in these samples highlights the significance of NDV in CIAV-infected birds, further supporting the known relationship between CIAV and viral co-infections. Similarly, Yao *et al*. (2019) and Li *et al*. (2023) documented high rates of viral coinfections, including Marek's Disease Virus (MDV), Reticuloendotheliosis Virus (REV), Avian Leucosis Virus (ALV) and Fowl Adenovirus in CIAV-positive flocks.

The findings in the study align with the conservation of the VP2 gene across various geographical regions, as these isolates cluster with strains from India, China, Malaysia and other countries. Additionally, the detection of NDV by RT-PCR in CIAV-positive flocks supports previous studies highlighting CIAV's role in promoting co-infections. The identification of a 356 bp fusion gene in these flocks underscores the strong interaction between CIAV and NDV, which may contribute to more severe disease outcomes in poultry. This information is crucial for formulating effective disease control strategies and management practices to minimize economic losses and improve vaccine efficacy against CIA in commercial poultry.

Conflict of interest: The authors declare that there is no conflict of interest.

Data availability statement: This study was a part of the author's research work conducted as part of their M.V.Sc. thesis, which was submitted to Sri Venkateswara Veterinary University (SVVU), Tirupati. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author's contribution: PKK: Involved with the investigation, analysis and writing-original draft preparation for the research article; RPRN, SAR: Involved in conceptualization and study design, corrected and approved the final manuscript.

Ethical Approval: Not needed

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