RESEARCH ARTICLE

Molecular Characterization of *Chicken Anaemia Virus* in Layers

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Abstract

Chicken Anaemia Virus (CAV) is one of the emerging pathogens of the poultry with its genome containing three pathologically important gene *viz.VP1, VP2 and VP3*. In the present study, molecular detection and histopathological examination was carried out on 25 pooled tissue samples collected from birds suspected of *CAV* from different districts of Maharashtra. From 25 pooled samples, 20 samples were found positive for the VP3 gene with an amplicon size of 376 bp. Histopathological examination revealed the loss of stem cells in the bone marrow and lymphocytes and RBCs in the white and red pulp of spleen. Liver and kidney showed degenerative changes. The *VP3* gene confirmed tissue samples were used for chicken embryo inoculation. For virus inoculation, 07 days old embryonated chicken eggs were inoculated with viral inoculum via yolk sac route. The inoculated eggs were incubated at 37^{0}C for 10 days. The eggs were harvested after 10 days and confirmed with PCR which revealed *VP3* positive results.

Keywords: *Chicken Anaemia Virus*, Chicken embryo inoculation, Molecular detection.

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Introduction

Indian poultry is one of the huge assets for the economy, but it has always faced some problems in the way of growth, including infectious diseases especially viral diseases, which cause huge mortality and morbidity, leading to production losses. (Kumar, 2007; Shah *et al*., 2013; Ullah *et al*., 2013). *Chicken anaemia virus (CAV)* is one of the major emerging viral diseases of economic importance in the poultry industry (Zhang *et al.,* 2017)

The disease is caused by, *Chicken anaemia virus (CAV)* of genus *Gyrovirus* of the family *Anelloviridae* (Wu *et al.,* 2019), which was first isolated by Yuasa *et a*l. (1979). *CAV* is a disease occurring mostly in young chicks, and it is characterized by severe anemia, generalized lymphoid atrophy, reduced weight gain, and immunosuppression, leading to vaccination failures. Transmission of the disease can occur in both vertical and horizontal ways. (Schat , 2009 and Dhama *et al*., 2008).

Chicken anaemia virus (CAV) is smallest avian DNA virus, non-enveloped with icosahedral symmetry and circular, negative-sense, single-stranded DNA virus. It has 2.3 kb DNA containing three open reading frames (ORFs) encoding for three proteins *VP1, VP2 and VP3* (Fenfen *et al.,* 2018). The *VP1* plays a major role in the growth and spread of virus. *VP2* plays a minor role in apoptosis. *VP3* is an important protein for pathogenesis. It causes apoptosis in the host cells; hence, its alternative name is apoptin and causes generalized lymphoid atrophy and anemia, leading to mortality in infected chicken. Its size is 13 kD (Noteborn *et al.,* 2004; Natesan *et al*., 2006**).** *CAV* is responsible for worldwide economic losses. The present study was planned to diagnose the *CAV* using PCR with targeting *VP3* gene and histopathological examination of the tissues collected from the suspected birds and viral isolation using chicken embryo inoculation.

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Materials and Methods

Sample Collection

For this study, 25 pooled tissue samples (4–5 same organs collected from the same place and pooled) were collected from the *CAV* suspected outbreaks, occurred at different poultry farms from different regions of Maharashtra. The samples included thymus, spleen, liver, and bone marrow which were collected during post mortem of suspected birds showing gross lesions of atrophied thymus and bursa of Fabricius, pale to yellowish bone marrow, hemorrhages on the internal organs, and having a history of anorexia, signs of anemia, depression and stunted growth. The suspected birds were between the age group of 8-9 weeks. Samples were collected from the Department of Pathology,

KNPCVS, Shirwal and OMEGA laboratories Lonand, Satara, Maharashtra; in sterile containers containing 50% Glycerine Phosphate Buffer saline (GPBS), which were carried to the laboratory along with icepacks and were stored at -20°C refrigerator till further processing.

Molecular Detection of Virus

DNA was extracted following the Phenol-Chloroform-Isoamyl alcohol method (Sambrook *et al.* 1989) from the collected tissue samples, further used as a DNA template for PCR reaction targeting the *VP3* gene. PCR was performed by preparing a final reaction volume of 25 µL in 0.2 mL thin-walled PCR tubes consisting of 12.5 µL master mix supplied with Taq DNA, $MgCl_2$ and dNTPs; 1 µL each of forward and reverse primers, 4 µL template DNA, and 6.5 µL nuclease-free water. The cycling condition for PCR reaction was; Initial denaturation 94°C for 4 minutes, Denaturation 94°C for 1-minute, 58°C for 1 min and extension 72°C for 1-minute along with final extension of 72°C for 8 minutes. The primer sequence used was *VP3 F* 5′-ATGAACGCTCTCCAAGAAG-3′ and *VP3 R* 5′-ACTTACAGTCTTATACACCTT-3′ (Hiremath *et al.,* 2013). The results of PCR reaction were recorded with the help of electrophoresis, which was further visualized under the trans-illuminator.

Isolation of Virus

For isolation of virus, chicken embryo inoculation procedure was followed. Viral suspension was prepared from the tissue homogenate of PCR positive pooled samples. For this three representative pooled samples were selected. The prepared homogenate was transferred into the sterile eppendorf tubes and then centrifuged at 1008 x *g* for 15 minutes; the supernatant was removed and filtered through 0.22 µm syringe filter. It was further treated with antibiotics (Penicillin 1000 units and Streptomycin 1000 µg/mL). Prepared inoculums were checked for bacterial contamination by streaking on the nutrient agar. The inoculums were stored at -20^{0}C till further use (Mutinda *et al.,* 2015, Simeonov *et al.,* 2014).

For inoculation, old embryonated eggs were procured from Bhagavan hatcheries, Lonand, Satara. The eggs were initially candled to check the viability of the embryo, and then the position of the air sac and the embryo was marked. Eggs were wiped with alcohol to sterilize the surface, and 0.2 mL of viral inoculum was injected into the yolk sac using a sterile syringe and needle. After inoculation for 7-8 days, the eggs were kept into the incubator at 37°C for 10 days. To provide uniform heat, egg positioning was changed every 3-4 days. Candling was done twice a day to observe the viability of the embryo. After ten days, eggs were removed from the incubator and kept in the refrigerator for 8–12 hours before harvesting. Organs like the liver, bursa, spleen, and bone marrow were collected in the sterile collection tube containing 50% GPBS. Polymerase Chain Reaction amplification was done following similar procedures as mentioned above.

Histopathology

During the post-mortem examination of *CAV* suspected birds, tissue samples of liver, spleen, kidney and bone marrow were collected separately in the 10% formalin for histopathological examination following standard method in practice for staining with Hematoxylin (H) and Eosin (E).

Results and Discussion

PCR results showed that out of 25 pooled samples 20 (80%) samples were positive for *CAV* (Image 1). According to Douglas *et al.* (1995) *VP3* gene is detected during the early course of disease and can be used for confirmatory diagnosis. Zhang *et al.* (2017) opined that *VP3* can be used as a potential drug to treat cancer. They further stated about the role of *VP3* in attacking the tumor cells selectively and not the healthy cells making it important for further research.

During virus isolation out of 10 eggs, 03 were found dead on the next day of incubation during candling which were discarded and 02 eggs were found dead on the third day of inoculation. Post-mortem examination of these 02 dead eggs showed rupture of yolk sac and stunted growth of embryos. After 10 days of incubation, the remaining 05 eggs were harvested, which did not show any gross pathological changes, except for one embryo. Fatty changes were observed in liver, which was visible by color change to yellowish. Tissues from all five embryos were collected separately and processed for detection of *VP3* gene. Out of 05 embryos, two embryo tissue samples showed positive results for *VP3* amplification (Image 2), suggesting viral replication. All this suggests that, viral replication took place in those two inoculated eggs. Simeonov *et al.* (2014) couldn't find any gross pathological changes except for the petechiae on the tissues. Peters *et al.* (2007) found similar results for PCR amplification of samples collected from inoculated eggs, with which they confirmed the viral replication in the inoculated eggs.

Histopathological examination of Bone marrow revealed loss of erythroid and myeloid series cells, increased adipose tissue (Image 3), and depletion of lymphocytes in

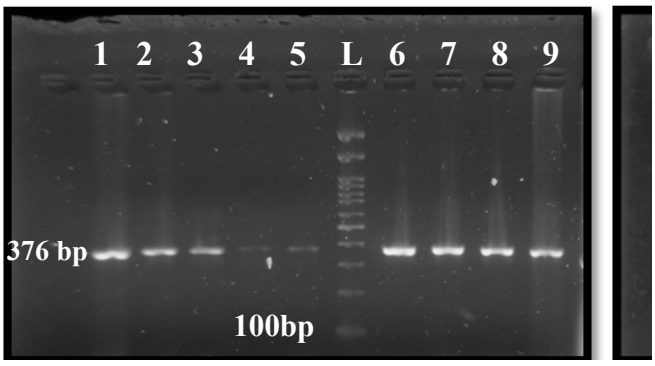

Image 1: Agarose gel electrophoresis showing *VP3* amplicon 376 bp
Lane 1, 2, 3, 4, 5 and 6, 7, 8, 9 : Positive L: ladder

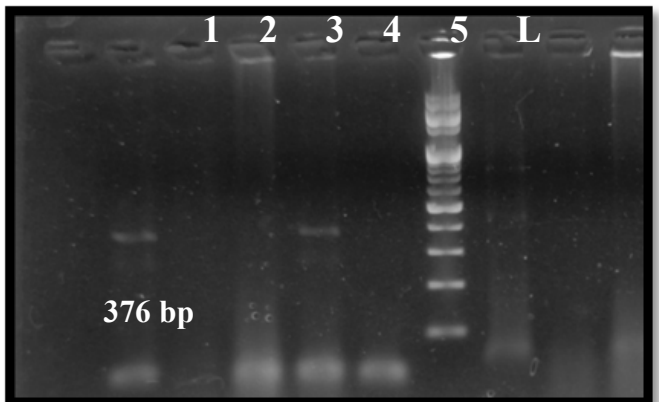

Image No.2: Agarose gel electrophoresis VP3 amplicon for embryo tissue (376 bp) Lanes 1 and 4: Positive results L- ladder (100)

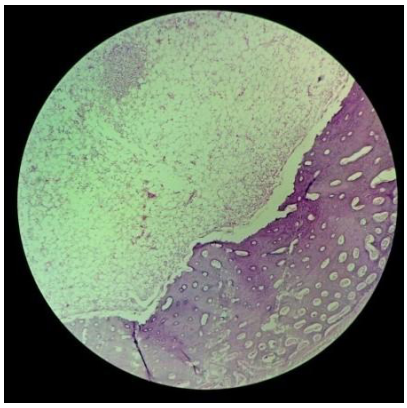

Image 3: Histopathology of Bone marrow (Loss of stem cells and replacement of Bone marrow with adipose tissue)

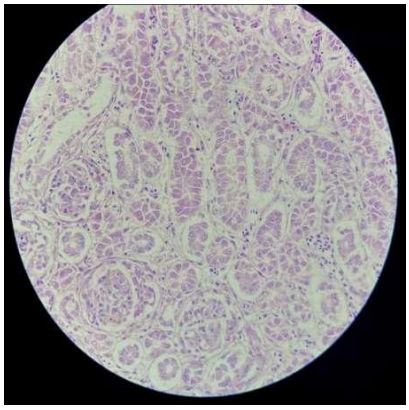

Image 4: Histopathology of Kidney (severe congestion, distorted tubules loss of tubular epithelium)

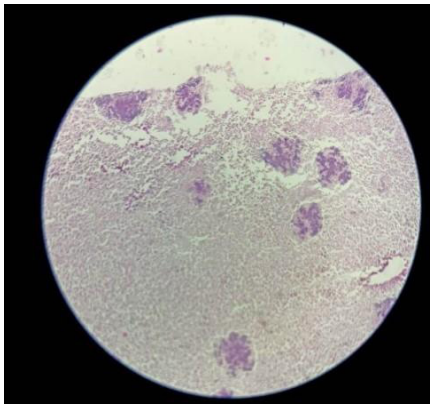

Image 5: Histopathology of Spleen. (Depletion of RBC's and Lymphocytes from red and white pulp)

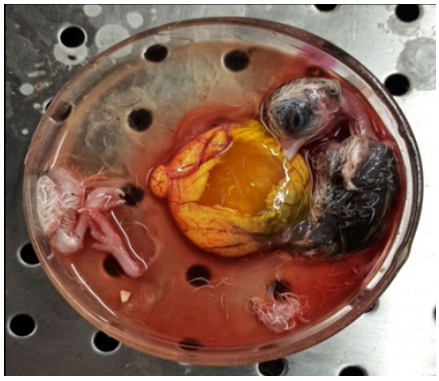

Image 6: Harvested embryo along with yolk sac

splenic follicles (Image 5). Liver tissue showed multifocal degenerative changes along with the presence of necrotic foci. Hepatocytes showed vacuolation in the cytoplasm with loss of nucleus. Kidney tissue showed tubular degeneration and focal necrosis of the tubular epithelium. In the cortical region focal interstitial hemorrhages along with congested blood vessels were observed (Image 4). Haridy *et al.*(2012) and Castino *et al.* (2019) observed similar histopathological changes in *CAV*-affected flocks. Hegazy *et al.* (2010) observed lymphoid depletion in spleen tissue and, marked hypoplasia in the bone marrow tissue. In liver tissue they observed cellular swelling along with apoptosis with marked apoptic bodies. Our results corroborated with Hegazy *et al.* (2010). Histopathological examination indicated the presence of *CAV*

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