



Investigation on the prevalence of *chicken anaemia virus* in poultry flocks of Kerala, India[#]

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Abstract

Chicken anaemia virus (CAV) is the aetiological agent of chicken infectious anaemia (CIA), an immunosuppressive disease, which brings a huge economic burden to the poultry industry globally. Concurrent infections and vaccination failures further aggravate the impact of the disease on the sector. The epidemiology of the disease and virulence of the circulating strains is to be known while formulating prevention strategies for any infectious disease. So far, there are no reports regarding the presence of CAV among poultry flocks in Kerala. In this scenario, the present study contemplated the investigation of the chicken anaemia virus using ELISA and PCR techniques. Indirect ELISA revealed the presence of CAV antibodies in 80 samples out of the 92 serum samples screened. Among the 100 samples collected from suspected cases, 29 were found to be positive for CAV infection with detection primers targeting the VP2 gene. This implies widespread distribution of the virus in Kerala.

Keywords: *Chicken anaemia virus, Chicken infectious anaemia, ELISA, PCR*

Chicken infectious anaemia (CIA), a viral infection of poultry caused by the *Chicken anaemia virus* (CAV) has gained attention over the years due to its ubiquitous nature and immunopathological effects. The virus is non-enveloped with a circular single-stranded negative sense DNA genome with 3 overlapping genes, *VP1*, *VP2* and *VP3* and belongs to the genus *Gyrovirus* of the *Anelloviridae* family. The capsid protein *VP1* along with the scaffolding protein *VP2* forms the immunogenic part of the virus, whereas, *VP3*, also known as apoptin can induce apoptosis in infected cells. Affected birds come mostly under the age group below 4 weeks and are presented with ill thrift, severe

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anaemia, pectoral haemorrhage, weight loss and severe mortality (Karimi *et al.*, 2009). Virus has been reported to be present in all organs, but initial establishment in lymphoid organs and its outcome is responsible for severe pathological changes like depletion in blood cell counts, precursor T cells in thymus, mature T cells in spleen and degeneration of lymphoid organs (Todd, 2000; Wani *et al.*, 2015). Since its first identification in Japan (Yuasa *et al.*, 1979), the virus has been isolated from different parts of the world and serological evidence for the presence of the virus in poultry flocks of the United States in the later 1950s is also available (Toro *et al.*, 2006). Despite any history of clinical signs or confirmed cases, these viruses have shown high seroprevalence in studies conducted in various parts of the world, even in SPF flocks (Owoade, 2004; Hadimli *et al.*, 2008; Chu, 2010).

In India, the disease was reported first in Tamil Nadu in the year 1994 (Venugopalan *et al.*, 1994) and outbreaks were reported thereafter from different parts of India (Natesan *et al.*, 2006; Wani *et al.*, 2013; Sreekala *et al.*, 2019; Ganar *et al.*, 2017). The seroprevalence study conducted by Bhatt *et al.* (2011) in samples collected from three states revealed that 86.88 per cent of samples were positive for CAV antibodies. Presence of the virus in the reproductive tracts of seronegative birds and possible vertical transmission along with the resistant nature of the virus and lack of preventive measures could make this emerging disease a possible threat to the Indian poultry industry (Cardona *et al.*, 2000; Miller and Schat, 2004; Wani *et al.*, 2013). Even though several epidemiological studies were conducted in various parts of India no incidents of the presence of the virus in Kerala have been reported so far, despite being a fast-growing sector in the state. The present study was conducted to assess the prevalence of the

disease in Kerala employing serological and molecular techniques.

Materials and methods

Serum sample collection

A total of 92 serum samples were collected from 19 different poultry farms situated in different parts of Kerala, which includes commercial broiler and layer farms, breeder flocks and backyard flocks during the period February to April 2022 (Table 1). The birds represent different age groups ranging from 3 weeks to 14 months and suggestive symptoms of CIA were not reported from any of the flocks. None of the flocks under study were vaccinated against CIA. Blood (2 mL) was collected from the wing vein of randomly selected birds aseptically and serum was separated and stored under -20° C till use.

Tissue samples collection

A total of 100 pooled tissue samples (liver, spleen, thymus, bone marrow and bursa of Fabricius) were collected from recently dead/ ailing birds with symptoms and lesions suggestive of CIA and preserved in Phosphate buffered saline (PBS) at -20 °C. The birds/ carcass was presented to departments of Veterinary Pathology and Veterinary Microbiology for disease investigation and represent age groups ranging from 3 days to 4 months with no history of vaccination against CIA.

Indirect enzyme-linked immunosorbent assay

All the 92 samples under investigation were tested for the presence of antibodies against CAV using a commercial indirect ELISA kit (AsurDx™ CAV Antibody test kit, Catalogue No.: 10033-02, Biostone Animal Health, Dallas)

Table 1. Details of serum samples collected

Type of chicken	No. of Farms Screened	No. of birds tested
Broiler	3	11
Layer	4	14
Breeder	5	53
Backyard Flocks	7	14
Total	19	92

using the manufacturer's protocol and the absorbance is measured at 630 nm (iMark™ Microplate Absorbance Reader, Bio-Rad, USA) as suggested. The controls and samples were added in duplicates and the mean was taken for analysis.

Polymerase chain reaction

Total DNA was extracted from the homogenised tissue samples using a commercial DNA extraction kit (QIAamp DNA mini kit, Catalogue No.: 51304, Qiagen Ltd., Qiagen House, Fleming Way, Crawley, West Sussex) following the manufacturer's protocol. Triturated tissue (≥ 25 mg) samples were lysed and ethanol precipitation of nucleic acids was carried out followed by elution in 100 μ L of elution buffer provided in the kit. The commercial vaccine (Chicken infectious anaemia inactivated oil adjuvant vaccine, Ventri Biological Pvt. Ltd., Maharashtra (VH/CAP/02 strain)) available was used as the source of positive control DNA. The oil portion was removed using Isopropyl Myristate (Sigma-Aldrich, USA) as per the method followed by Yang *et al.* (2021) prior to DNA extraction using the kit method. One negative control without template DNA was included to monitor any extraneous DNA contamination. The concentration and purity of the DNA were measured using NanoDrop 2000C (Thermo Scientific). The samples showing DNA concentration above 200 ng/ μ L and mean ratio of optical density (OD) at 260/230 nm between 2.0 and 2.2 and at 260/280 nm between 1.8 and 2 were chosen for PCR. The extracted DNA samples were stored at -20°C till use.

Oligonucleotide primers targeting the VP2 gene of CAV genome (F: 5'ATG CAC GGG AAC GGC GGA C 3' and VP2 R: 5' TCA CAC TAT ACG TAC CGG GG 3') corresponds to an amplicon of size 671 bp were commercially synthesised (Sigma Aldrich, India) in lyophilised form.

The polymerase chain reaction was carried out in a 12.5 μ L reaction mixture which consists of 6.25 μ L PCR master mix (Emerald Amp GT PCR master mix, Catalogue No.: RR310A, Takara), 1 μ L each of primers (10 pM/ μ L concentration), 1.25 μ L nuclease-free water and 3 μ L of template DNA. The amplification

was carried under the following conditions in a thermal cycler (MJ Mini Bio-Rad thermal cycler, USA): Initial denaturation for 5 min. at 95°C followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 64.5°C for 30 sec and extension at 72°C for 1 min. with a final extension at 72°C for 10 min. The PCR product was then analysed by submarine electrophoresis in two per cent agarose gel using ethidium bromide staining in the gel documentation system. Representative positive amplicons were sequenced by Sanger's method for confirmation.

Results and discussion

A total of 92 serum samples collected from 19 poultry farms across the state of Kerala have been tested for the presence of IgG antibodies against CAV infection. Screening of sera using a commercial indirect ELISA kit revealed a high prevalence of CAV infection among the flocks under study. Among the 92 samples screened for CAV antibodies 80 (86.95 per cent) were found to be positive while the other 12 samples (13.04 per cent) were negative. All the samples from backyard rearing flocks were positive for CAV antibodies. Among the samples collected from organised farms, the highest seropositivity was recorded among the broilers (Fig. 1). Similar studies conducted among the poultry flocks in northern states of India also revealed a high prevalence of 86.88 per cent (Bhatt, *et al.*, 2011). The commercial layer flocks in southern states of India like Telangana, Karnataka and Tamil Nadu had shown a prevalence rate above 50 per cent in a study conducted by Baksi *et al.* (2016). Several Asian and African countries had also reported a high seroprevalence of CAV in apparently healthy flocks (Hadimli *et al.*, 2008; Oluwayelu, 2010). This implies a widespread distribution of the virus around the world, even in the absence of clinical conditions. This also indicates that the virus is a severe threat to the poultry industry and eradication seems to be a difficult task. The ability of the disease to precipitate the effects of the other infectious disease also makes the disease undiagnosed which further complicates the disease monitoring (Dhama *et al.*, 2008; Gowthaman, 2012; Krishan *et al.*, 2015; Yao *et al.*, 2019).

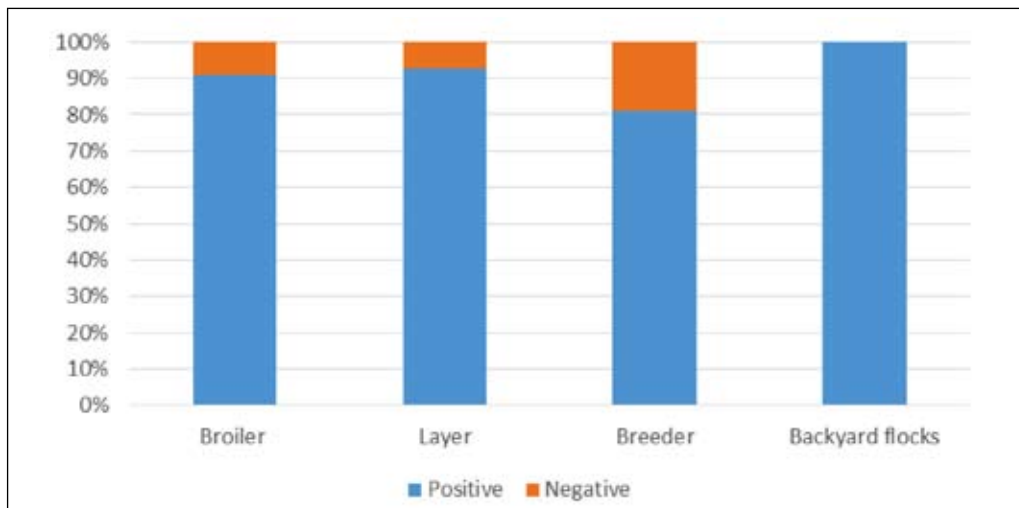


Fig. 1. Seroprevalence of CAV antibodies in flocks of Kerala

Polymerase chain reaction (PCR) has the advantage over other diagnostic methods for being a rapid yet sensitive technique, especially in the case of fastidious viral agents like CAV (Manoharan *et al.*, 2012). In the present study PCR detection of 100 suspected tissue samples revealed that 29 samples were positive for CAV DNA (Fig. 2). Representative amplicons were sequenced and nucleotide blast analysis confirmed 100 per cent sequence similarity with existing CAV genomes in GenBank. The sequences were submitted to GenBank (Accession nos. OP776186, OQ032556, OQ061157, OQ061159). Wani *et al.* (2013) conducted a study on the molecular detection of CAV in suspected tissue samples

collected from 12 states of India during 2007-2012 and found a high incidence of 73.3 per cent, among which, 80.3 per cent of the cases involved chicks below 3 weeks old. A prevalence of 78.3 per cent was recorded by Krishan *et al.* (2015) among the poultry flocks in north India. Considering these findings, the endemic spread of the virus is raising concern over the future of the poultry industry. Proper monitoring of immunosuppressive disease agents and prevention strategies are to be promptly implemented.

Conclusion

CIA is an emerging threat to the poultry sector of the country. Even though commercial vaccines against CAV are available in India, regular active immunisation of the flocks is rare and none in Kerala so far, which means the seroconversion possibly arises from a natural infection with the virus. The birds screened using ELISA were also apparently healthy and had no history of the clinical disease. The chances of infection with CAV being subclinical and the presence of concurrent infectious diseases could be a reason behind the disease being undetected so far. Considering the high seroprevalence of the disease and reported disease outbreaks from different regions of the country, it is high time we implement better prevention and control strategies against the disease, including vaccination.

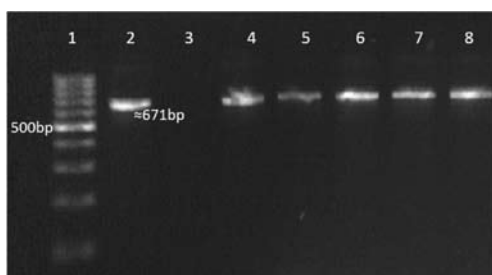


Fig. 2. Representative figure of agarose gel electrophoresis of PCR amplified products of VP2 gene from tissue DNA (671 bp)

Lane 1 : 100 bp DNA marker
 Lane 2 : Positive control
 Lane 3 : Negative control
 Lane 4-8 : Tissue samples

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Conflict of interest

The authors declare that they have no conflict of interest.

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