Research Article

Molecular Characterization of Chicken Infectious Anemia Virus Isolated from Commercial Poultry with Respiratory Disease Complex in India

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INTRODUCTION

Chicken infectious anemia (CIA) is a highly contagious disease of young chicken, characterized by severe anemia, generalized lymphoid atrophy and increased mortality. The causative agent of the disease is Chicken Anaemia Virus (CAV), belonging to the genus Gyrovirus of the family Circoviridae. CAV has been included in the list of emerging and important viruses that are a severe threat to the Indian poultry industry. Although a few sequences of CAV from India are available in GenBank, no systematic analysis of Indian CAV strains has been performed to the best of our knowledge. Thirty–two commercial poultry flocks with a history of respiratory disease complex (RDC) from four different states of India were included in this study. Necropsy was carried out on freshly dead and ailing birds. Tissue samples were collected aseptically for direct tissue PCR detection of CAV. The PCR products were further subjected to sequencing to study the molecular epidemiology. PCR amplification of VP2 gene from the clinical tissues yielded expected product size of 419 bp in 30 out of 32 clinical cases screened. The Indian CAV viruses grouped with the major branch that consists of viruses from China, Brazil, USA, Malaysia, Bangladesh and Australia. The 16 Indian isolates shared 98.8 ± 100 % homology among them. The percent identity matrix calculated for Indian isolates with CAV isolates from various parts of the world indicated closest relationship with isolates from China, USA and Australia (98.5–99.7 %), whereas Brazil, Malaysia and Bangladesh shared (98.8–100%) homology with Indian isolates. Further epidemiological and molecular studies are suggested to know the magnitude of infection and design appropriate disease prevention and control strategies for this economically important pathogen of poultry.

ABSTRACT

Chicken infectious anemia (CIA) is a highly contagious disease of young chicken, characterized by severe anemia, generalized lymphoid atrophy and increased mortality. The causative agent of the disease is Chicken Anaemia Virus (CAV), belonging to the genus Gyrovirus of the family Circoviridae. CAV has been included in the list of emerging and important viruses that are a severe threat to the Indian poultry industry. Although a few sequences of CAV from India are available in GenBank, no systematic analysis of Indian CAV strains has been performed to the best of our knowledge. Thirty–two commercial poultry flocks with a history of respiratory disease complex (RDC) from four different states of India were included in this study. Necropsy was carried out on freshly dead and ailing birds. Tissue samples were collected aseptically for direct tissue PCR detection of CAV. The PCR products were further subjected to sequencing to study the molecular epidemiology. PCR amplification of VP2 gene from the clinical tissues yielded expected product size of 419 bp in 30 out of 32 clinical cases screened. The Indian CAV viruses grouped with the major branch that consists of viruses from China, Brazil, USA, Malaysia, Bangladesh and Australia. The 16 Indian isolates shared 98.8 ± 100 % homology among them. The percent identity matrix calculated for Indian isolates with CAV isolates from various parts of the world indicated closest relationship with isolates from China, USA and Australia (98.5–99.7 %), whereas Brazil, Malaysia and Bangladesh shared (98.8–100%) homology with Indian isolates. Further epidemiological and molecular studies are suggested to know the magnitude of infection and design appropriate disease prevention and control strategies for this economically important pathogen of poultry.
failures or aggravation of the residual pathogenicity of attenuated vaccine viruses (Todd, 2000; Schat, 2003; Toro et al., 2006; Dhama et al., 2008).

In India, the disease has long been suspected, on the basis of clinical manifestations and lesions (Verma et al., 1981; Khanna, 2010), virus detection by immunoperoxidase test, PCR and isolation. The disease has been reported from poultry flocks of some states of the country, and included in the list of emerging and important viruses that are a severe threat to the Indian poultry industry (Venugopalan et al., 1994; Kataria et al., 1999; Verma et al., 2005; Natesan et al., 2006; Praveen et al., 2008; Bhatt et al., 2011; Wani et al., 2013). Although a few sequences of CAV from India are available in GenBank (NCBI), no systematic analysis of Indian CAV strains has been performed to the best of our knowledge. Therefore, need was felt for further molecular characterization of these viruses to find out the genetic variation among them, if any, which would help devising suitable control strategy to prevent losses by this virus in the poultry industry. Sequencing was used as a tool to study the molecular epidemiology of Indian CAV isolates with the history of respiratory disease complex (RDC) in the present study.

MATERIALS AND METHODS

Field Samples

Thirty-two commercial poultry flocks with a history of respiratory disease complex (RDC) during May 2010 to September 2011 from four different states of India viz. Uttar Pradesh, Haryana, Rajasthan, and Tamil Nadu were included in this study. Selected flocks consisted of multi-aged layers and broilers and they were reared under cage system and deep litter system, respectively. The age of the RDC affected poultry birds of the 32 poultry flocks under investigation ranged between 4 and >72 weeks. All the birds were reared under standard managemental conditions recommended by the breeding companies. All the poultry flocks were vaccinated against respiratory pathogens viz, Newcastle disease, infectious bronchitis, infectious coryza and fowl cholera.

Collection and Transportations of Clinical Samples

Necropsy was carried out on freshly dead and ailing birds with symptoms of RDC. Tissue samples such as trachea, lungs, and spleen were collected aseptically for direct tissue PCR detection of CAV. The tissue samples were transported on ice from the field to Avian Diseases Section of the Division of Pathology, IVRI, Izatnagar. The samples were properly labelled and stored at −20 °C until used for PCR testing.

Polymerase Chain Reaction and Sequencing

Figure 2: The CAV affected grower display pale and icteric liver

Figure 3: The CAV affected grower display pale and icteric kidney

Figure 1: Severe multifocal intra-nuclear hemorrhages in the breast muscle
The clinical tissues were cut into small pieces and 5% homogenates were prepared in phosphate buffered saline (pH 7.2). Viral DNA from tissue homogenate was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) as per the manufacturer’s instructions. The primer pair targeting the VP2 gene of CAV viz., CAV_P1 3’CTA AGA TCT GCA ACT GCG GA5’ and CAV_P2 3’CCT TGG AAG CGG ATA GTC AT5’ were used in the PCR detection to amplify a CAV virus specific 419-bp fragment (Ottiger, 2010). The amplification was carried out using PCR Master Mix (2X) (Fermentas, USA), with an initial denaturation at 95º C for 3 minutes, followed by 35 cycles at 94 ºC for 1 min, 60 ºC for 1 min, 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. Ten micro liters of the PCR products were analyzed by electrophoresis in 1.5% agarose gel with 0.5 mg/mL ethidium bromide. PCR products were purified by using ExoSAP–IT® (Affymetrix, USA) and sequenced using BigDye Terminator v3.1 kit (Applied Biosystem, USA) as per the manufacturer’s instructions.

**Phylogenetic Analysis**

The nucleotide sequence data generated were edited and aligned using Sequencing Analysis Software v5.3 (Applied Biosystems, USA) and MEGA 5 Softwares. Reference sequences were downloaded from NCBI database. Phylogenetic analysis and evolutionary associations were inferred in MEGA 5.0 using the Maximum Likelihood algorithm with Kimura–2P correction and 1000 bootstrap replications.

### RESULTS

#### Clinical Signs

Affected birds showed dullness, depression, somnolascence, stunted growth, prostration, facial swelling, decreased feed intake and water consumption, hock sitting posture, staggering gait, watery diarrhoea, cyanotic combs and wattles, and drop in egg production. The respiratory signs including of sneezing, gasping, corza and rales were indicative of involvement of the respiratory system. One of the pre-layer flock aged 17 weeks old out of the 32 flocks investigated showed signs of pale bird syndrome along with respiratory distress.

#### Gross Lesions

No appreciable/ minimal lesion was found in majority of the birds that died in peracute disease. Many affected birds showed emaciation, haemorrhagic/catarrhal tracheitis, diffuse pulmonary congestion and oedema, airsacculitis, fibrinous adhesive pericarditis and fibrinous periephatapitits, petechiae on the epicardial surfaces and abdominal fat, haemorrhagic proventriculitis, egg peritonitis, oophoritis, splenic atrophy and or mottling and nephritis–nephrosis complex. The flock affected with pale bird syndrome displayed highly pale and icteric carcass with severe multifocal intramuscular haemorrhages in the pectoral region (Figure 1), and thigh muscles, widespread petechial haemorrhages on the junction of proventriculus and gizzard with increased mucous content. The liver, kidney and bone marrow were markedly pale and icteric (Figure 2 and 3).

**PCR Detection and Phylogenetic Analysis**

PCR amplification of VP2 gene from the pooled tissues with primer pair described in materials and methods yielded expected product size of 419 bp (Figure 4). CAV specific nucleic acid was detected in 30 out of 32 clinical cases screened. Phylogenetic tree that was constructed using MEGA 5 program with 29 viruses available in the Gen Bank indicated multiple groups. The Indian CAVs were grouped with the major branch that consists of viruses from China, Brazil, USA, Malaysia, Bangladesh and Australia (Figure 5). The 16 Indian isolates shared 98.8–100% homology among them. The percent identity matrix calculated for Indian isolates with CAV isolates from various parts of the world indicated closest relationship with isolates from China, USA and Australia (98.5–99.7%), where as Brazil, Malaysia and Bangladesh shared (98.8–100%) homology with Indian isolates.
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**Other Respiratory Agents**

The samples were also positive for several respiratory agents like, FAV, LPAI, NDV, IILT, MG and MS, the data for which is not shown.

**DISCUSSION**

CAV has been reported from major poultry producing countries of the world including India and is being documented as emerging and an economically important pathogen from several countries (Kataria et al., 2005; Dhamma et al., 2008; Schat, 2009; Oluwayelu, 2010; Bhatt et al., 2011; Snoeck et al., 2012; Gowthaman et al., 2013; Nayabian and Mardani, 2013; Wani et al., 2013). Recently, Bhatt et al. (2011) reported high prevalence (86.88%) of CAV antibodies in Northern India while Wani et al. (2013) reported a prevalence rate of 73.3% by PCR detection of CAV from clinical samples from different states of the country. The present study reports the molecular characterization of CAV isolates obtained from few states of India. Out of the 32 clinical samples screened by PCR, 30 were positive for CAV. Overt clinical signs of CAV could be recorded in a single farm only. All the cases were associated with RDC, this denotes that CAV is ubiquitous and mainly cause subclinical infection in India. Because of the widespread practice of vaccination for breeders and the presence of maternal antibodies, the clinical form of CAV is rare today (Sommer and Cardona, 2003). Although CAV is present as subclinical infections, in concurrence with other respiratory pathogens as identified in the present study, it measurably decreased the flock performance, increased the incidence of vaccine failures and other immunosuppressive and respiratory pathogens. Similar observations were also reported by several researchers in subclinical cases of CAV with other agents (De Boer et al., 1994; Toro et al., 2000).

Close phylogenetic relationship of Indian CAV isolates with USA, Brazil and China which are the leading poultry producers of the world denotes that the source of virus might have evolved from a common origin and circulating in East Asia and Australia; it could be either infecting grant parent stock or contaminated vaccines (Brown et al., 2000).

The probable mechanism of transmission in Indian conditions could be vertical transmission from breeders or vaccine contamination, and CAV has been reported in SPF flocks and breeders in many occasions (Engström, 1999). CAV is an important extraneous pathogen that potentially contaminates the avian virus vaccines, particularly those prepared by inoculation of chicken embryos; CAV was detected as contaminant of NDV and IBV vaccines which were produced from embryo-derived chick eggs (ECEs) (Aamir et al., 2007).

Further extensive epidemiological studies are suggested for the virus in the country to know the magnitude of this important poultry virus, role of subclinical CAV infection in RDC along with isolation of viral strains and pathological studies. These would clearly define the disease status and need for the inclusion of CAV virus vaccination programs in India, and strengthening of R&D activities for development of rapid, sensitive and confirmatory diagnostics and modern generation vaccines to counter this economically important disease of poultry.

**REFERENCES**


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