



Detection and molecular characterization of chicken infectious anaemia virus in poultry flocks in Haryana

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ABSTRACT

Chicken infectious anaemia virus (CIAV) is an economically important pathogen affecting poultry industry worldwide. Commercial poultry flocks (26) suspected to be affected with chicken infectious anaemia (CIA) were processed for Polymerase Chain Reaction (PCR) using VP2 gene primers and characterized by partial gene sequencing. The PCR revealed 26.9% (7/26) poultry flocks positive for CIAV. Eleven PCR products of CIAV DNA were sequenced. The partial nucleotide sequence analysis of VP2 genes revealed that 11 field strains had 99.2 to 100% similarity among themselves and with the Indian strains. The VP2 gene sequences of 11 field strains showed 97.5 to 100% similarity to the field strains sequences reported from all over the world. On the basis of partial nucleotide sequencing analysis of VP2 gene, our findings suggest that the viral strains circulating in Haryana have similarity to other Indian strains.

Key words: Chicken infectious anaemia, PCR, Poultry, Sequencing, VP2 gene

The poultry in India is susceptible to various infectious diseases which include established viral agents and emerging viral agents. Chicken infectious anemia virus (CIAV) is an emerging viral pathogen affecting poultry population. The virus was first reported from contaminated vaccines in Japan (Yuasa *et al.* 1979). Since then virus has been isolated from various part of the world including India (Dhama *et al.* 2008; Dhama *et al.* 2011; Eltahir *et al.* 2011). The virus is responsible for severe immunosuppression which makes birds susceptible to many diseases and leads to heavy economic losses worldwide (Snoeck *et al.* 2012; Wani *et al.* 2013). The virus synthesizes a single major transcript of 2.0 kb with three overlapping reading frames (ORF1, ORF2, and ORF3), responsible for production of VP1, VP2 and VP3 proteins respectively. VP1 is a 54 kDa structural protein which forms the viral capsid. VP2, a non-structural protein (24 kDa), is a multifunctional protein with role in viral replication, antigen presentation, pathology and virus assembly (Peters *et al.* 2007). VP3 (13 kDa) is a non structural protein also known as “apoptin” having anti-neoplastic effect and responsible for the disease pathogenesis. Of these proteins, VP2 is conserved and less variable as compared to VP1 and VP3 (Farkas *et al.* 2008).

The Chicken infectious anemia (CIA) is vertically and horizontally transmitted disease among chickens. Although chicken of all age groups are affected but young chicks up to 3–4 weeks of age are the most susceptible (Todd, 2000; Dhama *et al.* 2008). Clinically the disease is characterized by anemia, atrophy or hypoplasia of lymphoid organs (thymus and bone marrow), subcutaneous or intramuscular hemorrhages, anorexia, lethargy and increased mortality (Schat 2003; Dhama *et al.* 2008). In India, CIA has been suspected on the basis of case history, clinical symptoms and post mortem lesions (Verma *et al.* 1981; Suresh *et al.* 1995). Venugopalan *et al.* (1994), first reported the existence of disease in Tamil Nadu by viral antigen detection. Later on, CIA was confirmed by virus isolation and detection of CIA DNA samples by PCR from different states of the country (Kataria *et al.* 1999; Dhama *et al.* 2002).

Haryana ranks 4th in broiler production (0.34 million tonnes per year) in India with an annual growth rate of 12% and per-capita availability of 188 eggs/person/year which is three times higher than the national average (Basic Animal Husbandry Statistics, 2015–2016). A high prevalence (73.3%) of CIA was reported in Haryana (Wani *et al.* 2013). The molecular characterization of CIAV isolates obtained from four different states of India including Haryana also reported high prevalence (93.7%) by PCR, warranting the urgent need for constant monitoring of this virus in the field for emergence of any new variants and consequent changes in pathogenicity (Gowthaman *et al.* 2014). However, no systematic study has been carried out in Haryana regarding

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viral DNA detection and molecular characterization of CIAV strains circulating in the Haryana. The present study was thus planned to detect CIAV in tissue samples collected from poultry flocks suspected for CIA by PCR using VP2 gene primers and partial nucleotide analysis of VP2 amplified PCR products.

MATERIALS AND METHODS

Collection of samples: Sixty six tissue samples (thymus, bone marrow, spleen, liver, and bursa) were collected separately from 26 commercial poultry flocks suspected of CIA from Ambala, Panchkula, and Hisar districts of Haryana, during the period from May 2016 to March 2017. The collected samples were stored at -20°C till further use.

Polymerase chain reaction (PCR): The DNA from tissue samples was extracted by PureLink Genomic DNA Mini Kit (Invitrogen, USA) as per manufacturer's recommendations. The primer pairs targeted VP2 gene was used in this study as described previously (Ottiger, 2010). The primers (Sigma Aldrich Pvt. Ltd., Bengaluru, India) were forward 5'-CTAAGATCTGCAACTGCGGA-3'; reverse 5'-CCTTGGGAAGCGGATAGTCAT-3'. The synthesized primer pairs were obtained in freeze dried and desalted form. All the primers were reconstituted with nuclease free water (NFW) as specified by the manufacturer. The PCR reaction was carried out in a 25 μl reaction containing 12.5 μl DreamTaq Green Master mix (2 $\times$), 0.5 μl of each primers, 5 μl template and 6.5 μl Nuclease free water. The PCR was performed in Biorad thermal cycler under the following conditions: initial denaturation for 10 min at 95°C ; 35 cycles of 1 min at 95°C (denaturation), 1 min at 60°C (annealing) and 90 sec at 72°C (extension); and a final extension step of 10 min at 72°C . The expected product size for PCR reaction was 419 bp. The PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose containing ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) in Tris Acetate- EDTA buffer (TAE) buffer and visualized using UV transilluminator. The size of the amplified product was ascertained by comparison with standard DNA marker (100 bp DNA ladder, Thermo scientific). The amplified products were observed and photographed by gel documentation system.

Sequencing: Purified PCR products (11) from all the CIAV positive samples were sequenced from Bioserve Biotechnologies (I) Pvt. Ltd. Hyderabad, using forward and reverse PCR primers to determine the nucleotide and amino acid sequences. Automated DNA Sequencer Applied Biosystem 3730 XL DNA analyzer was used for the purpose. The 11 sequences obtained were submitted to NCBI gene bank and following accession numbers were obtained MF770757, MF770758, MF770759, MF770760, MF770761, MF770762, MF770763, MF770764, MF770765, MF770766 and MF770767.

Sequence analysis: To confirm the identity of 11 CIAV sequences, all the sequences were analyzed using nucleotide BLAST tool on NCBI. The size of the sequenced product was 366 nucleotides and the position of these nucleotides

in VP2 gene is 674–1040. The percentage identity was computed by DNA Star software. The previously published sequences of VP2 gene of CIAV were retrieved from GenBank and these sequences were used for comparison. The CIAV nucleotide sequences were aligned using Clustal W method implied in MEGA 7 software. The Pair wise distance method using the Neighbour-Joining method (1000 replicates for bootstrap) was used for construction of phylogenetic tree of aligned sequences. The nucleotide sequences were translated to deduce amino acid sequences. The amino acid sequences of CIAV of this study corresponded to amino acid positions from 108- 216 of VP2 gene of GXC060821 strain (Accession no. JX964755).

RESULTS AND DISCUSSION

The DNA samples (66) from 26 poultry flocks were PCR amplified using primers specific for VP2 gene of CIAV. As expected with VP2 gene primers, the PCR product size of 419 bp was observed (Supplementary Fig.1). It was observed that 26.9% (7/26) flocks were positive for CIAV. Similarly, 26.6% prevalence of CIAV was reported in commercial broiler flocks in Egypt (Mohamed, 2010). In contrast to our study, lower prevalence (4%) of CIAV was observed in field samples of clinically infected broiler chicken in Malaysia (Chowdhury *et al.* 2002). Similarly, lower prevalence (10.22%) of CIAV was found in China (Eltahir *et al.* 2011). In India, Praveen *et al.* (2008) detected higher prevalence (56.25%) of CIAV by PCR in poultry flocks of Gujarat and Andhra Pradesh states of India. Likewise, Wani *et al.* (2013) also reported higher prevalence (73.3%) of CIAV from clinical samples from 12 different states of India. Higher prevalence of CIAV (51% - 93.7%) from the poultry affected with respiratory disease complex was reported from different states of India (Gowthaman *et al.* 2014; Krishan *et al.* 2015; Ganar *et al.* 2016; Hussein *et al.* 2016). This difference in prevalence of CIAV could be due to difference in types of sample collected, geographical distribution of the disease and detection method used.

In the present study, all the 11 purified PCR products of field CIAV positive samples were subjected for partial gene sequencing. Then comparison was performed with the published VP2 gene sequences of CIAV strains at NCBI nucleotide sequence data library. Nucleotide BLAST (online) revealed that all the 11 sequences belong to VP2 gene of CIAV (Supplementary Fig.1). The nucleotide sequence analysis of 11 field strains using VP2 gene revealed that all the strains of the present study had 99.2 to 100% similarity among themselves and with the Indian strains. Whereas, these CIAV sequences of 11 strains showed 97.5 to 100% nucleotide similarity to the field strains sequences reported from USA, Brazil, Taiwan, Bangladesh, Vietnam, Japan, Malaysia and China.

The nucleotide sequence showed the presence of three nucleotide substitutions (T to A at 676 position, A to T at 742, A to T at 804) in the field isolates MF770762 and MF770760 (742 A to T, 1037 G to C, 1038 C to T), two nucleotide substitutions in field isolates MF770758 (1037

G to C, 1038 C to T) and MF770757 (1037 G to C, 1038 C to T) and one nucleotide substitution in field isolate MF770766 (A to T at 742) (Supplementary Fig. 1). Based on observation of CIAV phylogenetic dendrogram, all 11 field strains were found closer to the sequences of strains reported from India, Brazil, Bangladesh, China, Taiwan, Vietnam, Japan, but far away from USA and Australia strains. In present study, with respect to amino acids, one unique amino acid substitution was observed, in which amino acid Lysine (K) was substituted by Methionine (M) at position 151, in the isolate MF770762 (Supplementary Fig.2). Similarly, in previous study phylogenetic analysis of VP2 gene of CIAV strains of Nagpur showed complete sequence identity with each other except one strain (NGP-10) showed 99.5% identity with each other (Ganar *et al.* 2016). In this study, Indian (Nagpur) isolates based on VP2 gene sequencing showed maximum identity with strain of China and minimum identity with strain of Australia. Similarly, Gowthaman *et al.* (2014) reported on the basis of VP2 gene analysis revealed that Indian CIAV isolates were having 98.5–99.7% similarity with isolates from China, USA and Australia where as with Brazil, Malaysia and Bangladesh shared 98.8–100% identity with Indian isolates.

The VP2 region contained 25 variable positions that were detected in the 13 isolates from southern China (Zhang *et al.* 2013). The amino acid alignment showed VP2 gene was relatively conserved among them. Natesan *et al.* (2006) analysis revealed that VP2 protein of Indian isolates revealed only 1 amino acid variation at the position 186 in CAV-E and the variation among Indian and foreign isolates ranged from 0 to 0.9%. Islam *et al.* (2002) sequenced full length genome of CIAV and found that VP2 with 98.6–99.5% sequence homology at the amino acid level appeared to be the most conserved and 6 out of 10 strains had only a single amino acid substitution.

We have not observed many changes in our CIAV strains at the molecular or nucleotide level. Our CIAV strains showed similarity with the previously published CIAV strains. This could be because of two reasons. First, VP2 gene is more conserved and less variable than VP1 and VP3 gene (Farkas *et al.* 1996). Second, fewer changes were observed at nucleotide and amino acid levels due to the small product size of VP2 gene in our research. In our study, VP2 gene has been used primarily for the diagnostics purpose (Gowthaman *et al.* 2014; Krishan *et al.* 2015) and less so for tracking of new CIAV strains. However, the importance of VP2 protein cannot be underestimated as VP2 alone or in combination with VP1 can be used as potential immunogen for vaccine production (Lee *et al.* 2009; Noteborn *et al.* 1998; Moeini *et al.* 2011).

On the basis of partial nucleotide sequencing analysis of VP2 gene of CIAV, our findings suggest that the CIA strains circulating in Haryana are closely related to previously characterized Indian strains. This information will be instrumental in implementation of biosecurity measures in Haryana to prevent the disease.

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REFERENCES

- Basic Animal Husbandry Statistics. 2015–16. Department of Animal Husbandry and Fisheries, Ministry of Agriculture, <http://dahd.nic.in/dahd/statistics/animal-husbandry-statistics.aspx>.
- Chowdhury S M, Omar A R, Aini I, Hair-Bejo M, Jamaluddin A A, Kono Y, Darus A and Yatim H M. 2002. Isolation, identification and characterization of chicken anaemia virus in Malaysia. *Journal of Biochemistry, Molecular Biology, and Biophysics* 6(4): 249–55.
- Dhama K, Kataria J M, Dash B B, Kumar N S and Tomar S. 2002. Chicken infectious anemia—A review. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* 23: 1–15.
- Dhama K, Mahendran M, Somvanshi R and Chawak M M. 2008. Chicken infectious anaemia virus: An immunosuppressive pathogen of poultry-A Review. *Indian Journal of Veterinary Pathology* 32(2): 158–67.
- Dhama K, Singh S D, Somvanshi R and Kataria J M. 2011. Chicken infectious anemia (an economically important immune-depressive viral disease of poultry), ICAR-NAE Project Publication, IVRI, Izatnagar, 1–36.
- Eltahir Y M, Qian K, Jin W, Wang P and Qin A. 2011. Molecular epidemiology of chicken anemia virus in commercial farms in China. *Virology journal* 8(1): 145.
- Farkas T, Tanaka A, Kai K and Kanoe M. 1996. Cloning and sequencing of the genome of chicken anaemia virus (CAV) TK-5803 strain and comparison with other CAV strains. *Journal of Veterinary Medical Science* 58(7): 681–84.
- Ganar K, Shah M, Kamdi B P, Kurkure N V and Kumar S. 2017. Molecular characterization of chicken anemia virus outbreaks in Nagpur province, India from 2012 to 2015. *Microbial Pathogenesis* 102: 113–19.
- Gowthaman V, Singh S D, Dhama K, Barathidasan R, Srinivasan P, Mahajan N K and Ramakrishnan M A. 2014. Molecular characterization of chicken infectious anemia virus isolated from commercial poultry with respiratory disease complex in India. *Advances in Animal and Veterinary Sciences* 2(3): 171–76.
- Hussein E, Arafa A E, Anwar N and Khafaga A. 2016. Molecular and pathological analysis of chicken anemia virus isolated from field infection in three Egyptian Provinces. *Advances in Animal and Veterinary Sciences* 4(5): 218–29.
- Islam M R, John R, Raue R, Todd D and Müller H. 2002. Sequence analysis of the full length cloned DNA of a Chicken Anaemia Virus (CAV) Strain from Bangladesh: Evidence for genetic grouping of CAV Strains based on the deduced VP1 amino acid sequences. *Journal of Veterinary Medicine, Series B* 49(7): 332–37.
- Kataria J M, Suresh R P, Verma K C, Toroghi R, Kumar N S, Kataria R S and Sah R L. 1999. Chicken infectious anaemia (CIA) in India: detection of the agent by polymerase chain reaction and transmission study. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* 20(2): 91–95.

- Krishan G, Shukla S K, Bhatt P, Wani M Y, Dhama K, Malik Y P S and Kumar R. 2015. Clinical association of chicken anaemia virus with other infectious poultry diseases in North India and Nepal: Its pathological studies, molecular epidemiology and RFLP pattern of PCR amplified full length viral genome. *Advances in Animal and Veterinary Sciences* **3**(7): 395–405.
- Lee M S, Lien Y Y, Feng S H, Huang R L, Tsai M C, Chang W T and Chen H. J. 2009. Production of chicken anemia virus (CAV) VP1 and VP2 protein expressed by recombinant *Escherichia coli*. *Process Biochemistry* **44**(4): 390–95.
- Moeini H, Omar A R, Rahim R A and Yusoff K. 2011. Development of a DNA vaccine against chicken anemia virus by using a bicistronic vector expressing VP1 and VP2 proteins of CAV. *Comparative Immunology, Microbiology and Infectious Diseases* **34**(3): 227–36.
- Mohamed M A. 2010. Chicken infectious anemia status in commercial broiler chickens flocks in assiut-upper Egypt: occurrence, molecular analysis using PCR-RFLP and apoptosis effect on affected tissues. *International Journal of Poultry Science* **9**(6): 591–98.
- Natesan S, Kataria J M, Dhama K, Rahul S and Baradhwaj N. 2006. Biological and molecular characterization of chicken anaemia virus isolates of Indian origin. *Virus Research* **118**(1–2): 78–86.
- Noteborn M H, Verschueren C A, Koch G and Van der Eb A J. 1998. Simultaneous expression of recombinant baculovirus-encoded chicken anaemia virus (CAV) proteins VP1 and VP2 is required for formation of the CAV-specific neutralizing epitope. *Journal of General Virology* **79**(12): 3073–77.
- Ottiger H P. 2010. Development, standardization and assessment of PCR systems for purity testing of avian viral vaccines. *Biologicals* **38**(3): 381–88.
- Peters M A, Crabb B S, Tivendale K A and Browning G F. 2007. Attenuation of chicken anemia virus by site-directed mutagenesis of VP2. *Journal of General Virology* **88**(8): 2168–75.
- Praveen B N, Dhama K, Kataria J M, Dash B B and Singh S D. 2008. Detection and isolation of chicken anemia virus from field samples of poultry flocks from Gujarat and Andhra Pradesh, India *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **29**: 23–26.
- Schat K A. 2003. Chicken infectious anemia, pp 182–202. *Diseases of Poultry*, 11th edn. (Eds) Saif Y M *et al.* Iowa State University Press, Ames, IA.
- Snoeck C J, Komoyo G F, Mbee B P, Nakouné E, Le Faou A, Okwen M P and Muller C P. 2012. Epidemiology of chicken anemia virus in Central African Republic and Cameroon. *Virology Journal* **9**(1): 189.
- Suresh R P, Kataria J M, Verma K C and Sah R L. 1995. Occurrence of chicken anemia agent (CAA) like virus infections in chicks, *Proceedings, XII Annual Conference of Indian Association of Veterinary Pathology, (IAVP)*, 9–11 Nov, 1995, Orissa, India, 89.
- Todd D. 2000. Circoviruses: immunosuppressive threats to avian species: a review. *Avian Pathology* **29**(5): 373–94.
- Venugopalan A T, Elankumaran S, Raj G D, Manohar B M, Thangavelu A, Ravikumar G, Koteeswaran A and Raj A S. 1994. Isolation of chicken anaemia virus in Tamil Nadu. *Indian Veterinary Journal* **71**(4): 411–12.
- Verma K C, Panisup A S, Mohanty G C and Reddy B D. 1981. Infectious bursal disease (Gumboro disease) and associated conditions in poultry flocks of Andhra Pradesh. *Indian Journal of Poultry Science* **16**: 385–92.
- Wani M Y, Dhama K, Barathidasan R, Gowthaman V, Tiwari R, Bhatt P, Mahajan N K, Chawak M M, Singh S D and Kataria J M. 2013. Molecular detection and epidemiology of chicken infectious anaemia virus in India. *South Asian Journal of Experimental Biology* **3**(4): 145–51.
- Yuasa N, Taniguchi T and Yoshida I. 1979. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Diseases*, **23**: 366–385.
- Zhang X, Liu Y, Wu B, Sun B, Chen F, Ji J, Ma J and Xie Q. 2013. Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. *Scientific Reports* **3**: 3519.