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Detection and Molecular Characterization of VP1 Gene of Chicken Infectious Anaemia Virus in Suspected Layer Birds

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ABSTRACT

Background: Chicken Infectious Anaemia Virus (CIAV) is one of the emerging pathogen of the poultry. The Genus of this virus is *Gyrovirus* and the family *Anelloviridae*. Viral DNA contains three open reading frames (ORFs) encoding for three proteins VP1, VP2 and VP3. VP1 protein is major structural protein and is a capsid protein having major role in growth and spread of the virus. Present study was aimed for molecular detection of VP1 gene of the CIA virus in the samples collected from the outbreaks occurred at four different district of Maharashtra.

Methods: Total 50 samples comprising of Spleen, bone marrow, thymus and liver, were collected. DNA extracted from those samples was used for PCR amplification targeting, VP1 gene. Selected positive purified PCR products of VP1 were subjected for Nucleotide sequencing and Phylogenetic analysis.

Result: Total out of 50 pooled samples, 10 samples were positive for VP1 gene *i.e.* 20%. Sequencing of coding region of four CAV positive samples was conducted. Genetic analysis showed three isolates had high similarity with Del-Ros vaccine strainfrom USA; while one isolate shared close similarity to attenuated cloned isolate 10 of Cux-1 strain. The data also indicated Q139 and Q144 and Q394 amino acids substitutions among the VP1. Phylogenetic analysis of the sequenced viruses based on either the coding nucleotide sequence or VP1 coding sequence, suggested the circulation of III genotypes in Maharashtra.

Key words: Amino acid analysis, Chicken infectious anaemia, PCR, Phylogenetic analysis, VP1 gene.

INTRODUCTION

Chicken anaemia virus (CAV) is a widespread pathogen that causes immunosuppression in chickens. The virus-induced immunosuppression often results in secondary infections and a suboptimal response to vaccinations, leading to high mortality rates and significant economic losses in the poultry industry (Schat and Santen, 2020). CAV is transmitted either vertically or horizontally and infects birds of all ages. CAV infected young chickens display depression, muscle hemorrhage, pale bone marrow and thymus atrophy. In older chickens, CAV infection results in subclinical disease followed by immunosuppression (Adair, 2000). In addition, reduced immune responses to Newcastle disease and Marek's disease virus vaccines in affected chickens (Zhang *et al*., 2017).

CAV belongs to the Gyrovirus genus of the *Anelloviridae* (Rosario *et al*., 2017). They are non-enveloped icosahedral particle with a single-stranded, circular, negative-sense DNA genome that is approximately 2.3 kb nucleotides in length. The viral genome consists of three overlapping open reading frames (ORFs), ORF1, ORF2 and ORF3, that encode the structural protein VP1 (51.6 kDa) and two non-structural proteins VP2 (24 kDa) and VP3 (13.6 kDa), respectively.

The major capsid protein VP1 plays a critical role in viral capsid assembly and inducing neutralization antibodies in the host. The capsid VP1 protein is highly variable and contains the neutralizing epitopes (Todd *et al*., 1990). These epitopes map mainly in the hypervariable region of the protein that extends from position 139 to position 151 (Renshaw *et al*., 1996). Further, the amino acid substitutions at positions 133 and 144 of VP1 can affect the ability of CAV spreading in cell culture (Renshaw *et al*., 1996). Later, it was documented that amino acid at position 394 in VP1 could be a major genetic determinant of virulence (Yamaguchi *et al*., 2001). Thus, the VP1 gene has been selected for genetic characterization and evaluation of CAV.

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CAV isolates were thought to belong to a single serotype, However, Phylogenetically, based on the nucleotide sequence of the VP1 gene, four distinct genogroups/ genotypes (I, II, III and IV) have been identified (Ou *et al*., 2018). In the current study, we investigated CAV infection in commercial layer chickens from different district of Maharashtra and furthermore, conducted molecular characterization of identified CAV strains based on the entire protein-coding regions of the viral genome.

MATERIALS AND METHODS

Details of samples

For this study, 50 pooled tissue samples were collected from the outbreaks, occurred at different poultry farms of different regions of Maharashtra *viz.* Satara, Solapur, Nashik and Pune. These tissue sample included thymus, spleen, liver, bone marrow which were collected from the post mortem of suspected birds showing clinical signs like ruffled feathers, pallor comb and wattles, anorexia, anaemia, depression, stunted growth *etc*. The collected tissue samples were pooled together in a way that 4-5 same organs collected from same place came under one pooled sample. Samples were collected from Department of Pathology, KNPCVS, Shirwal. Maharashtra. Samples were collected in sterile containers containing 50% GPBS, which were carried to the laboratory along with icepacks and were stored at -20°C refrigerator till further processing.

The method used for extraction of DNA was Phenol-Chloroform-Isoamyl alcohol method of DNA extraction from tissue, taken from Sambrook *et al*. (1989) with few modifications. The obtained DNA, were used as template for PCR detection of VP1 gene. 25 µl for PCR mix was prepare containing containing 1.5 units of Taq polymerase, 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 100 ng DNA and nuclease free water up to 25 µl for 35 cycles (Table 1). The primer sequence used was VP1F 5′-AGCCGACC CCGAAC CGCAAGAA-3′ and VP1R 5′-ATCAGGGCTGCGT CCCCC AGTACA-3′. The primers used here was published primer. The results of PCR reaction were recorded with the help of electrophoresis, which was further visualized under the trans-illuminator (Hiremath *et al*., 2013).

Among the Positive PCR products, three representative samples from three regions (Nashik, Pune and Solapur) were selected and were purified using HiPurA® PCR Product Purification Kit and purified PCR products were sent for Nucleotide sequencing at BioResource Biotech Pvt. Ltd.

DNA sequencing and genetic analysis

The amplified PCR fragments purified with HiPurA® PCR Product Purification Kit (Himedia, India) according to the manufacturer instructions. The purified PCR products of 04 CAV positive were bidirectionally sequenced by BioResource Biotech Pvt. Ltd.

The sequencing results were compiled using Lasergene v7.0 software (DNASTAR, Madison, WI, USA). Multiple sequence alignments were performed using the ClustalX algorithm, followed by phylogenetic analysis using the Maximum likelihood algorithm in MEGAX (Version 10.1.8) and based on the Tamura-Nei model and 1000 bootstrap replications. Reference CAV strains of genogroups I-IV were included for phylogenetic classification (Olszewska-Tomczyk *et al*., 2016; Ou *et al*., 2018).

RESULTS AND DISCUSSION

Present study was conducted with the aim of, detection of VP1 gene of CIA virus from different outbreaks and its molecular characterization.

PCR amplification of VP1 gene

All samples were subjected to PCR amplification of VP1 gene using specified primers. In most of the studies, researchers targeted VP1 gene, as it is the structural protein and plays an important role in the pathogenicity of the disease. Most of the researches showed the importance of VP1 region in the virulence of the virus; hence this region of the viral genome is mostly targeted for research purpose. PCR amplification of VP1 gene showed positive amplicons of 1390 bp (Fig 1). Out of 50 pooled samples 10 samples were positive for VP1 gene *i.e.* 20%. According to Sreekala *et al*. (2019) opined about involvement of VP1 region of viral genome in multiple mutations which are not observed with other two genes of the viral genome. They targeted VP1 gene for their PCR studies. Hiremath *et al.* (2013) stated that VP1 is the region most prone for mutations and is very important for pathogencity. Wani *et al*. (2013) mentioned the importance of VP1 gene saying that, it is a major structural protein and is prone for mutations at certain points. They further added that such mutations can determine the pathogenicity of the virus. CIAV grows mostly in the lymphoid tissue. Out of all the samples collected highest positivity rate of VP1 was observed in bone marrow 40%, followed by thymus 30%. Goryo *et al*. (1985) opined about pathogenesis of CIA virus. They stated that during CIA virus replication, haemopoietic and thymic precursors were mainly targeted which are present in bone marrow and thymus respectively.

Table 1: Cycling conditions of PCR reaction for VP1 gene.

Gene	Thermal cycling				
VP1	Initial denaturation	Denaturation	Annealing	Extension	Final extension
	94°C 4 min	94°C 1 min	60°C 1 min 35 Cycles	72°C 1 min	72°C 8 min

This could be the reason for the maximum detection of viral genome in these two organs. Smyth *et al.* (2006) opined about the pathogenesis of the virus and spread of the virus in different tissues. According to them the infection spreads through viraemia and the antigen is detected within 4-5 days in primary lymphoid organs like bone marrow and thymus.

Nucleotide and amino acid sequence analysis

For molecular characterization, four CAV-positive samples representing locations of Maharashtra were selected for nucleotide sequencing. The nucleotide sequence annotated were obtained of 1823 bp length containing entire CAV coding region. Further, nucleotide sequences, obtained in this study, were compared to complete representative CAV sequences from different countries.

Identities analyses were conducted at both the nucleotide and amino acid levels. At the nucleotide level, three viruses, (Solapur-12, Nashik-24, Pune-29) were found closely match to low pathogenic C369 infectious clone CAV from Japan and the Del-Ros vaccine strain with >99.1% maximum identity. One virus (Pune-7) revealed a higher identity with a attenuated cloned isolate 10 of Cux-1 strain with a high identity of 98.9%. At the amino acid level of the VP1 coding protein, a similarity level of >99% where find among Solapur-12, Nashik-24, Pune-29 Pune-7. Amino acid variation based on the VP1 sequence (450 AA) indicated an overall variation of 0.4-2.9% among all isolates compared with other representative CAV sequences. In this study, we found glutamine (Q) at both positions. Also had T and Q at position 89 and 394 of VP1, respectively (Table 2), (Fig 2).

Phylogenetic analysis

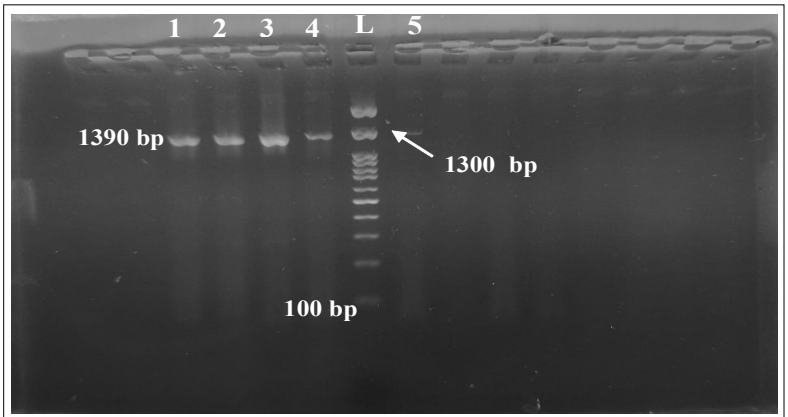
Phylogenetic analyses of the full-length VP1 gene sequences (1350 bp) and the protein coding region of the CAV genome indicated that CAV isolates obtained in this study belongs to genotype III. The three isolates (12, 24 and 29) were clustered with low pathogenic C369 infectious clone CAV from Japan and the Del-Ros vaccine strain while, isolate 7 with isolate 10 from UK.

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 (Zhang *et al.,* 2012). The present study reports the molecular characterization of CAV isolates obtained from Maharashtra of India. The investigated layer flocks showed with severe atrophy of bursa of the Fabricius and thymus and discoloration of the bone marrow, as well as subcutaneous haemorrhages, further, detection of CAV by PCR, were in coordination with caused by CAV.

Previous studies have demonstrated that amino acid at position 394 in VP1 is crucial for CIAV virulence. When the amino acid was glutamine (Q), the virus showed high pathogenicity and when it was histidine, the virus had low pathogenicity (Eltahir *et al*., 2011; Yamaguchi *et al*., 2001). In our isolates, we have a found Threonine at 394 instead

Table 2: The amino acid motif in the VP1 protein of the CAV.

Sequences	Genotypes	Amino acid positions in VP1														
Consensus		22	75	89	97	125	139	141	144	287	290	370	376	394	413
		H	V	T	M	I	K		E	S	A	G	L	Q	A
3711 Australia (EF683159)	I	.	.	.	.	.	.		.	.	.	.	.	.	S
98D06073 USA (AF311900)	II	.	I	.	L	.	Q		Q	T	P	S	.	.	.
Del-Ros_USA_AF313470	III	.	.	.	.	.	.		.	.	.	.	.	.	.
Netherland/ D10068/26P4	III	.	.	.	.	.	.		.	.	.	S	.	.	.
Cux-1_Germany_M55918	III	.	.	.	.	.	.		.	A	.	S	.	.	.
BD-3_Bangladesh_AF395114	III	.	I	.	L	.	Q		Q	A	.	T	.	.	.
CAV- Pune-7	III	.	.	.	.	L	.		.	A	.	.	.	.	.
CAV- Solapur-12	III	.	.	.	.	L	.		.	.	.	.	I	.	.
CAV- Pune-24	III	.	.	.	.	L	.		.	.		.	I	.	.
CAV- Nashik-29	III	.	.	.	.	L	.		.	.	.	.	I	.	.
1102PT01_Taiwan_KY888892	IV	.	.	.	.	L	.		.	A	.	S			



Lane 1,2,3,4 and 5: Positive samples. L-ladder (100 bp).

Fig 1: Agarose gel electrophoresis showing VP1 gene amplicon (1390 bp).

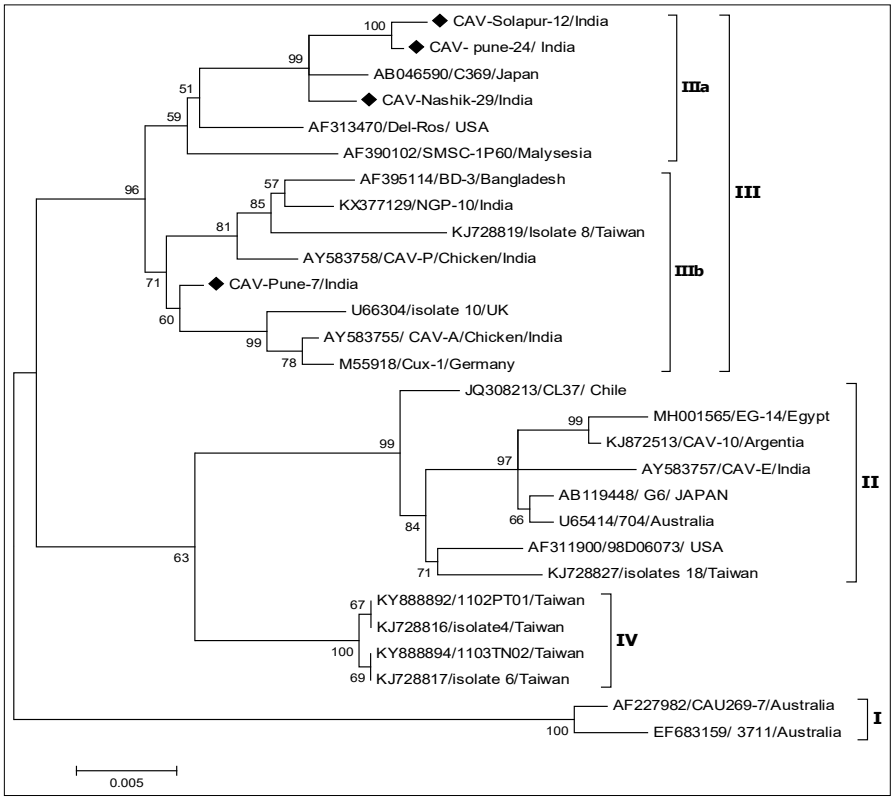


Fig 2: The phylogentic tree construction based on the complete coding nuclotide sequence of CAV.

of Alanine, which is indicative of attenuated strain of CIAV.

Phylogenetic and molecular analyses revealed, that the current isolates belongs to genotype III. Additional investigation should be conducted to understand CAV infection among chicken farms and the evaluation of the pathogenicity of CAV strains and also improve the control of CAV.

CONCLUSION

In this study, detection and molecular characterization of Chicken infectious anaemia virus circulating among commercial layer farms from different districts of Maharashtra state has been done on the basis of the VP1 gene. Phylogenetic analyses of the full-length VP1 gene sequences revealed, that the current isolates belong to genotype III.

Conflict of interest

All authors declare that they have no conflict of interest.

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