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Short Paper

Cloning and sequence analysis of VP1, VP2 and VP3 genes of Indian chicken anemia virus

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Summary

Chicken anemia virus was detected by PCR in tissue samples collected from poultry flocks in Gujarat, India. The VP1, VP2 and VP3 gene sequences of CAV from Anand, Gujarat were obtained after cloning the PCR products in pDrive cloning vector. Nucleotide sequence alignment with other CAV sequences demonstrated overall identity of 95-98.8%, 98.8-99.8% and 98.8-100% for VP1, VP2 and VP3 regions, respectively. Deduced amino acid sequences revealed 91.7-99.7%, 99-100% and 97.3-100% homologies for VP1, VP2 and VP3 proteins, respectively, indicating high level of genome conservation. Further, placement of critical nucleotides and amino acids at particular positions indicated that Anand CAV is possibly of more pathogenic potential. The CAV isolates were phylogenetically grouped together independent of their geographic origin.

Key words: Chicken anemia virus, Chicken infectious anemia, India

Introduction

Chicken infectious anemia (CIA) is an economically important, highly immuno-suppressive disease of young chickens caused by chicken anemia virus (CAV). Clinically, CIA is characterized by increased mortality, reduced weight gain, aplastic anemia, generalized lymphoid atrophy of all hematopoietic and lymphoid organs, and intramuscular and subcutaneous hemorr-hages (Schat, 2003).

Chicken anemia virus is a non-enveloped virus with a circular single stranded DNA of about 2.3 k bases, and is the sole member of the genus *Gyrovirus* within the family *Circoviridae*. A polycistronic polyadenylated mRNA of 2100 bases which comprises three partially or completely overlapping open reading frames (ORFs) is transcribed from the CAV genome, encoding three proteins, VP1, VP2 and VP3. ORF1 encodes the capsid VP1, the major viral structural protein (Todd *et al*., 1990), ORF2 encodes the VP2, a scaffolding protein and overlaps with ORF3, which encodes the VP3, a non-structural protein named apoptin. Recently, Eltahir *et al*. (2011) categorized CAV into four genotype groups (A-D) and five subtypes (A1, A2, A3, D1 and D2). In the recent past, certain poultry flocks, in the Anand area of Gujarat, India were found to be anaemic and increased mortality. The gross pathological lesions were suggestive of CIA. Confirmatory diagnosis was undertaken based on polymerase chain reaction (PCR) followed by cloning and sequencing of VP1, VP2 and VP3 genes.

Materials and Methods

Samples

Eighty field clinical samples consisting of bone marrow, spleen, liver, thymus and bursa of fabricius from commercial layer chickens collected from 16 different farms suspected for CIA.

DNA extraction and detection of CAV by PCR

Viral DNA was extracted from homogenized tissues by the method described by Todd *et al*. (1991) with minor modifications. The extracted DNA was tested for the presence of CAV DNA by PCR using three specific oligonucleotide primer sets. The primer sets VP1 (VP1F 5' AGC CGA CCC CGA ACC GCA AGA A 3', VP1R 5' ATC AGG GCT GCG TCC CCC AGT ACA 3'), VP2 (VP2F 5' AGC GCA CAT ACC GGT CGG CAG T 3', VP2R 5' AGG GGT TCG GCA GCC TCA CAC TAT 3') and VP3 (VP3F 5' ATG AAC GCT CTC CAA GAA G 3', VP3R 5' ACT TAC AGT CTT ATA CAC CTT 3') were used to amplify VP1, VP2 and VP3 genes, respectively. The PCR buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.625 U/µl *Taq*DNA polymerase and 10 pmoles of each primer was used for 25 µl PCR reaction. The reactions were started with denaturation (4 min at 94°C) followed by 35 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 57°C for VP1, 1 min at 63°C for VP2 and 1 min at 58°C for VP3 primers), extension (1 min at 72°C) and final extension (8 min at 72°C).

Cloning and sequencing of PCR products

Purified PCR products of VP1, VP2 and VP3 genes of a representative sample designated as Anand CAV were cloned into pDrive vector 3.85 kb (QIAGEN, Germany) with U overhangs, using QIAGEN PCR Cloning Kit and was propagated in *E. coli* host (DH5-α). The recombinants obtained were screened by colony PCR as per the standard procedure (Sambrook *et al*., 1989). The plasmid DNA was extracted using QIAprep® Spin Miniprep Kit (QIAGEN, Germany) after growing transformants in Luria-Bertani (LB) ampicillin broth. Recombinant plasmids isolated were confirmed by PCR using M13 primers. PCR products obtained were purified using Eppendorf Perfectprep® PCR Cleanup 96 kit. Bidirectional sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) using M13 primers on capillary sequencer (ABI PRISM® 310 Genetic Analyser, Applied Biosystems, USA).

Data analysis and sequence alignment

The VP1, VP2 and VP3 gene sequences of Anand CAV and their deduced amino acid sequences (using ExPASy proteomics tools) were aligned with the other 26 CAV sequences from GeneBank database using Clustal W Programme (Chenna *et al*., 2003). Sequences were compiled and analysed using Bioedit software (Hall, 1999).

Phylogenetic tree, based on amino acid sequences of the VP1 gene was obtained using the same Clustal W Programme. The tree was visualized and edited using Tree View Programme (Page, 1996). CAV isolates used in the study were CAV-A (India), CAV-B (India), CAV-E (India), CAV-P (India), Cux1(N) (Germany), Cux1(M) (Germany), BD3 (Bangladesh), SMSC-1 (Malaysia), TR20 (Japan), A2 (Japan), Harbin (China AF475908), AH4 (China DQ124936), DelRoss (USA), 014201 (USA), 3711 (Australia), Clone 27 (UK) for VP2 (16 sequences). In addition, Karnataka (India), Punjab (India), NIE/100 (Nigeria), L-028 (USA), Nobilis P4 (Vaccine), Pallister (Australia) for VP1 (22 sequences) and SN0161NSW (Australia), Namakkal (India), Haryana (India) and BR 367/03 (Brazil) for VP3 (20 sequences) were used for sequence analysis.

Results

Detection of CAV by PCR

All the 80 samples from 16 different farms, turned out to be positive for CAV by at least one primer set. VP1, VP2 and VP3 amplification yielded a specific product of 1390 bp, 713 bp and 367 bp, respectively.

Sequence analysis and comparison

Nucleotide sequences of VP1 (1350 bp), VP2 (721 bp) and VP3 (349 bp), of the Anand CAV obtained were submitted to the GeneBank, NCBI, USA, with the accession numbers EU424059, EU424060 and EU424061, respectively. Anand CAV demonstrated overall nucleotide sequence identity of 95-98.8%, 98.8-99.8%, and 98.8-100% for VP1, VP2 and VP3 regions, respectively.

Analysis of the deduced amino acids of VP1 (449aa) sequences of 22 CAV strains submitted to the GeneBank identified eleven positions at which more than one strain had amino acid substitutions. Most variations were observed at positions 75, 97, 139, 144, 251, 254, 287, 290, 370, 413 and 447. The amino acid identity of CAV isolate with other sequences ranged from 91.7-99.7%, 99-100%, and 97.3-100% for VP1, VP2 and VP3 proteins, respectively.

Phylogenetic analysis

An unrooted phylogenetic tree based on VP1 deduced amino acid sequences (Fig. 1) revealed three distinct clusters, except CAV-P isolate, which formed a separate branch away from other CAV isolates. Anand CAV and seven other strains (CAV-E, SMSC-1, T-20, AH4, NIE/100, CAV-B and BD-3) formed a separate well-defined cluster (I). Second cluster (II) was formed by L-028, Clone 27, Cux-1(M), CAV-A and Cux-1(N), while the remaining strains *i.e.* Harbin, Novilis P4, A2, 3711, Del-Ross, 01-4201, Pallister, Karnataka and Punjab isolates were grouped together into a third loose cluster (III).

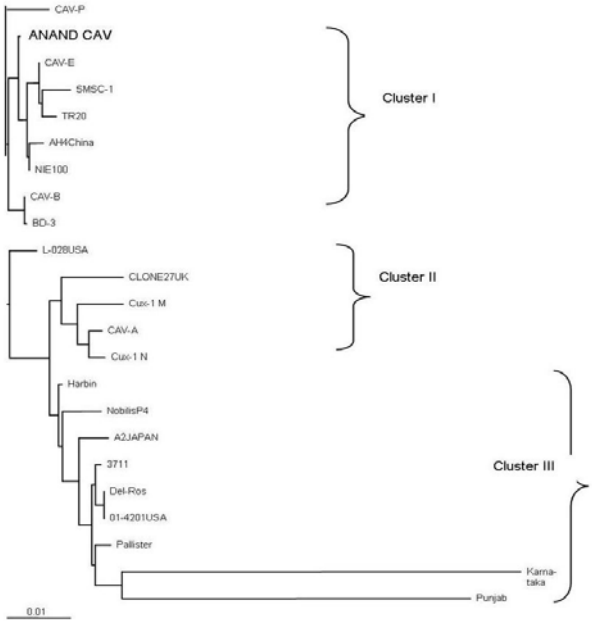

Fig. 1: Unrooted phylogenetic tree based on predicted aminoacid sequences of VP1 region of CAV. The bar represents 1% difference in the aminoacid homology between two sequences

Discussion

Gross lesions like gangrenous dermatitis, pathological changes of subcutaneous haemorrhages, atrophied thymus and bursa with pale liver were found that were suggestive of CIA disease and detection of CAV by PCR indicated that recent outbreaks in Anand poultry farms were caused by CAV.

The VP1 amino acid sequence alignment confirmed the previous findings from Renshaw *et al*. (1996) which described hyper-variable (HV) region located at the 5´ portion of VP1, spanning 13 amino acids from positions 139 to 151. They also suggested that amino acid Glutamine (Q) at positions 139 and 144 are associated with decreased rate of spread in cell cultures. Anand CAV has Q at positions 139 and 144 similar to other Indian isolates CAV-B and CAV-E. In addition, it was observed that the amino acids at position 139 and 144 do not appear to be independent of each other; ^{144}Q is always accompanied by ^{139}Q (except CAV-P). Glutamic acid (E) or aspartic acid (D) at position 144 is always accompanied by lysine (K) at position 139. The present study also supports the finding of Eltahir *et al*. (2011), of maximum variability at position 144 (Q, D, H and E). However, we also observed another variable region from positions 75 to 97, which was in agreement with earlier findings of Simionatto *et al*. (2006), who reported variable region from positions 75 to 98 among the Brazilian sequences studied.

Anand CAV sequence of the VP1 and all other strains analysed in the study were found to have Q at position 394, a major genetic determinant of virulence and if it is Q, the CAV isolates are highly pathogenic

and if it is histidine (H), they have less pathogenicity (Yamaguchi *et al*., 2001).

Most variability between compared isolates could be found in the VP1 coding region thus, phylogenetic analysis using VP1 amino acid sequences was performed. Anand CAV along with seven other strains, CAV-E, SMSC-1, TR-20, AH4, NIE/100, CAV-B and BD-3 formed a distinct lineage away from other sequences, despite the great geographical distances between the origins. Signatory amino acids ^{75}I, ^{97}L, ^{139}Q, ^{144}Q and ^{251}R (arginine) could be identified with this group. This observation is in agreement with the findings of Eltahir *et al*. (2011), on the 25 Chinese isolates. Signatory amino acids ^{75}V (valine), ^{97}M (Methionine), ^{139}K and ^{144}E were associated with the cluster II. Compared CAV strains were not grouped on the basis of their geographic origin (Islam *et al*., 2002; Chowdhury *et al*., 2003; Eltahir *et al*., 2011).

It can be concluded that placement of critical nucleotides and amino acids at particular positions as indicated earlier coupled with the necropsy findings in the birds included in the study reveals that Anand CAV is possibly of more pathogenic potential. All the Indian sequences of CAV including Anand CAV did not fall into a separate group, and highlight the possible relevance in the future for molecular epidemiology of CAV.

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