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Virus Research 118 (2006) 78–86
Virus Research
www.elsevier.com/locate/virusres

Biological and molecular characterization of *chicken anaemia virus* isolates of Indian origin

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Received 18 July 2005; received in revised form 18 November 2005; accepted 18 November 2005
Available online 27 December 2005

Abstract

In the present study, four *chicken anaemia virus* (CAV) isolates (CAV-A, -B, -E and -P) recovered from different geographical regions of India were characterized. CAV genome of 1766 bp nucleotide region containing the complete coding region of VP2 and VP3 proteins, and partial coding region of VP1 protein were sequenced. The nucleotide and deduced amino acid sequence of the Indian CAV isolates were aligned and compared with CAV isolates of European, Asian, American and Australian origin. Phylogenetic analysis of the Indian CAV isolates were also carried out based on the nucleotide and deduced amino acid sequences. The results indicated that Indian isolates were genetically evolved from different parts of the world. Indian isolate, CAV-A was found closely related to European Cux-1 strain, CAV-B and -P were closely related to Bangladesh BD-3 strain and CAV-E was closely related to Australian 704 strain. The pathogenicity of the four CAV isolates was studied in day-old specific pathogen free (SPF) chicks. Day-old SPF chicks ($n = 50$) were divided into five groups comprised of 10 chicks in each group. Group 1 was kept as control and groups 2–5 were infected with each CAV isolate separately. The chicks were infected at a dose rate of 1 ml cell culture fluid ($10^{4.5}$ $TCID_{50}$/0.1 ml) per bird intramuscularly. The clinical signs, mortality and packed cell volume (PCV) and body weight gain were recorded on 5, 10 and 15 days post-infection. At 15th day, all the birds were sacrificed and various organs, viz., thymus, bone marrow, spleen, liver and bursa were examined for gross and microscopic changes. The pathogenicity study indicated that all the CAVs except CAV-B were able to produce clinical disease and immunosuppression in young chicks whereas the isolate CAV-B produced no clinical disease but only induced immunosuppression, which was revealed by microscopic examination of the lymphoid organs. The study showed valuable information on molecular epidemiological status of CAV isolates prevalent in India for the first time.

Keywords: *Chicken anaemia virus*; Characterization; India; Sequencing; Phylogenetic analysis

1. Introduction

Chicken anaemia virus (CAV), belonging to *Gyrovirus* genus of Circoviridae family, is not only the causative agent of chicken infectious anaemia (CIA), an immunosuppressive disease of young chickens, but also infects the chickens of all age groups. Chicken is the only recognized natural host, but serological survey has revealed the prevalence of this disease in domestic and wild birds (Farkas et al., 1998). The clinical disease is mainly noticed in young chicks of 10–14 days of age, which usually acquire the infection vertically. The disease is characterized by increased mortality, reduced weight gain, anaemia, aplasia of bone marrow and atrophy of thymus (McNulty, 1991; Pope, 1991; Coombes and Crawford, 1998; Von Bulow and Schat, 1997; Rosenberger and Cloud, 1998; Todd, 2000; Senthilkumar et al., 2002; Dhama et al., 2002). The major economic loss caused by this virus is associated with severe immunosuppression and increased mortality due to secondary infections.

This virus, first reported by Yuasa et al. (1979) from contaminated vaccines in Japan, has worldwide distribution. It is 23–25 nm in size, icosahedral, non-enveloped virus having a 2.3 kbp circular single stranded DNA genome. The genome codes for three viral proteins (VP1, VP2 and VP3) transcribed from single major transcript of 2.0 kbp size from three overlap-

Abbreviations: bp, base pairs; CAV, *chicken anaemia virus*; CIA, chicken infectious anaemia; DNA, deoxyribo nucleic acid; EDTA, ethylene dinitrilo tetra acetic acid; HVR, hyper variable region; PCR, polymerase chain reaction; PCV, packed cell volume; RE, restriction endonuclease; SPF, specific pathogen free; $TCID_{50}$, tissue culture infective dose-50

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doi:10.1016/j.virusres.2005.11.017

ping reading frames (ORF1, 2 and 3). It is believed that CAV genome replicates through rolling circle model (Meehan et al., 1992). Normally, the virus does not grow in any of the commonly used primary cells and cell lines. Marek's disease virus or avian leucosis virus transformed lymphoblastoid cell lines are susceptible to this virus and the virus usually multiplies with low titre. Among the viral proteins, VP1 is major capsid protein (54 kDa) and VP2 is non-structural protein found in the cells in early hours of virus replication cycle. VP1 and VP2 are the protective proteins inducing neutralizing antibodies (Koch et al., 1995). VP3 is the smallest protein of 13 kDa, called "apoptin", has unique apoptosis inducing property.

In India, Venugopalan et al. first reported the existence of CAV in 1994 and was later confirmed by Kataria et al. (1999) by polymerase chain reaction (PCR) detection and reproduction of disease with infected material. Dhama (2002) reported variation among six isolates of CAV recovered from different geographical regions of the country by restriction endonuclease (RE) analysis of PCR amplified 453 bp products. Not much information is available about the *chicken anaemia viruses* circulating in this subcontinent. In general, no significant antigenic or pathogenic difference was reported among the CAV isolates in the past. Thus, only one serotype was recognized previously. However, an antigenically different isolate (CAV-7) has been reported recently from USA (Spackman et al., 2002a, 2002b), which could be a prototype virus of serotype 2. An outbreak in 9 weeks old layer pullets with high morbidity (80%) and mortality (55%) in Taiwan has also been reported recently (JungPin et al., 2002). These reports insisted the need to study and monitor the CAVs circulating in the field. Renshaw et al. (1996) reported a hypervariable region (HVR) in VP1 protein spanning 13 amino acids (139–151) and showed that amino acids at position 139 and 144 play vital role in virus growth and spread. Later, it was documented that amino acid at position 394 in VP1 could be a major genetic determinant of virulence (Yamaguchi et al., 2001). If it is glutamine at this position, the isolates are highly pathogenic and if it is histidine, less pathogenic. Hence, molecular and biological characterization of the Indian CAV isolates was attempted in the present study in order to find out the epidemiological status of these viruses prevalent in India.

2. Materials and methods

2.1. Viruses

Four Indian field isolates of CAV (CAV-A, -B, -E and -P), isolated from different geographical regions of the country were used in the present study. All these isolates were adapted in Marek's disease virus transformed MSB-1 cell line. Details of these CAV isolates are given in Table 1.

Table 1
Description of the Indian and foreign CAV isolates/strains with EMBL accession numbers used in the present study

S. no.	CAV isolate/strain	Origin	Accession no.
1	CAV-A	India (Pune, Maharashtra)	AY583755
2	CAV-B	India (Noida, Uttar Pradesh)	AY583756
3	CAV-E	India (Palampur, Himachal Pradesh)	AY583757
4	CAV-P	India (Namakkal, Tamil Nadu)	AY583758
5	Cux-1	Germany	M55918
6	26P4	Netherlands	D10068
7	704	Australia	U65414
8	BD-3	Bangladesh	AF395114
9	SMSC-1	Malaysia	AF285882
10	SMSC-1P60 (attenuated)	Malaysia	AF390102
11	Harbin	China	AF475908
12	Del Ross	USA	AF313470
13	98D02152	USA	AF311892
14	A2	Japan	AB031296
15	TR-20	Japan	AB027470

2.2. Cultivation and titration of the virus

CAVs were propagated in MDCC-MSB-1 cells following the method described by Goryo et al. (1987). Virus inoculum (0.5 ml) was mixed to the MSB-1 cell pellet suspended in 0.5 ml of the medium and incubated at 39 °C for 1 h in a 15 ml centrifuge tube. Then, 5 ml of the RPMI medium was added into the tube, cells were suspended in the media, seeded into culture flask and incubated at 39 °C with 5% CO_2 concentration in a humidified atmosphere for 72 h. After 72 h of incubation, 1 ml of the cell suspension was transferred into 4 ml of fresh medium and incubated. The process was repeated 5–7 times to appreciate the cytopathic effect and virus was harvested at every passage level. Quantification of the CAV isolate was done as per the method of Imai and Yuasa (1990). Briefly, serial 10-fold dilutions (10^{-1} to 10^{-7}) of the virus were prepared in RPMI-1640 without serum (50 μl) followed by adding 4×10^4 MSB-1 cells in 150 μl culture medium to each well. Each dilution was tested in five concurrent cultures with eight passages at 3-day intervals. Cultures without virus were included as controls. Infectivity endpoints were based on the cytopathic effect as described by Yuasa (1983). Mean tissue culture infective dose ($TCID_{50}$) was calculated according to the method of Reed and Muench (1938).

2.3. Extraction of viral DNA

Viral DNAs of CAV isolates were extracted from the infected MSB-1 cells at each passage following the method as described by Tham and Stanislawek (1992) with some modifications. Briefly, the pelleted MSB-1 (infected) cells were lysed by treating with 900 μl of lysis buffer (10 mM Tris, 10 mM EDTA, 0.25% Triton X-100) and mixed by vortexing gently. The suspension was treated with 5 M stock NaCl (0.2 M final concentration), and centrifuged at $2000 \times g$ for 10 min at 4 °C. The supernatant (585 μl) was collected and treated with proteinase-K (0.5 mg/ml final concentration) at 56 °C for 1 h. Subsequently, the lysate was extracted with standard phenol:chloroform (1:1) and chloroform treatment, and precipitated by standard ethanol precipitation method as described by Sambrook et al. (1989).

2.4. PCR amplification of the CAV genome

Primers VP1F: AGC CGA CCC CGA ACC GCA AGA A and VP1R: TCA GGG CTG CGT CCC CCA GTA CA were used to amplify the VP1 region and primers VP2F: GCG CAC ATA CCG GTC GGC AGT and VP2R: GGG GTT CGG CAG CCT CAC ACT AT were used to amplify the VP2 region. These two primer sets covered the entire coding regions of CAV. The PCR amplification was carried out in PCR buffer containing 1.5 mM $MgCl_2$, 200 μM of each dNTPs, 10 pmoles of each primer and 1.0 unit of Taq polymerase in 25 μl of total reaction volume. The reaction was carried out in an automated thermal cycler (PTC 200, MJ Research, USA).

Amplification of the VP1 region was carried out with initial denaturation of 94 °C for 4 min followed by 34 cycles of denaturation, annealing and extension at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min, respectively, and the final extension was carried out at 72 °C for 8 min. The standardized PCR amplification yielded a distinct band of 1390 bp in size as expected. Amplification of the VP2 region was carried out using the primers VP2F and VP2R with initial denaturation of 94 °C for 4 min followed by 34 cycles of denaturation, annealing, extension at 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min, respectively, and final extension was carried out at 72 °C for 5 min. The standardized PCR amplification yielded a distinct band of 713 bp in size as expected.

2.5. Sequencing of the PCR products

The PCR amplified products of different CAV isolates were sequenced in the automated sequencer (Applied Biosystems, USA) by employing the big dye termination kit. Single reaction comprised of ready reaction mix (2.5×), 4 μl; big dye sequencing buffer (5×), 2 μl; primer, 3.2 pmoles; template DNA, 5 μl; and nuclease free water upto 20 μl. The reaction was carried out in a thermal cycler (Eppendorf, USA) for a total number of 50 cycles. The cycle steps followed were 94 °C for 4 min (initial denaturation), 94 °C for 30 s (cyclical denaturation), 55 °C for 10 s (cyclical annealing) and 60 °C for 4 min (cyclical elongation). After completion of the reaction, the amplicon was purified. EDTA (5 μl) and ethanol (100 μl) were added to the reaction mix (20 μl) directly and mixed well. The mixture was kept at room temperature for 15–30 min. Later, it was centrifuged at 12,000 rpm for 20 min and the supernatant was discarded carefully. The pellet was washed once with 70 μl of the 70% ethanol and air-dried. Hi-Di formamide was added to the pellet and mixed well by gentle vortexing. After a short spin, the mixture was denatured at 95 °C for 2 min and chilled on ice immediately for 5 min. The sample was loaded in the automated sequencer and the sequence was obtained.

2.6. Sequence alignment

All the nucleotide and amino acid sequences of Indian isolates and reference sequence from various countries were aligned by Clustal method using MegAlign programme of Lasergene Software (DNASTAR Inc., USA) and phylogenetic tree (cladogram) was drawn with unbalanced branches showing branch length proportionate to sequence divergence. Accession numbers and other details of the viruses used for comparison are given in Table 1.

2.7. Restriction endonuclease analysis

Restriction endonuclease enzymes were chosen on the basis of the sequence data that are useful for the differentiation of the isolates. RE analysis of the PCR amplified CAV regions were carried out as per the supplier recommendations.

2.8. Pathogenicity studies of Indian isolates of CAV

Day-old specific pathogen free (SPF) chicks ($n = 50$) were divided into five groups. Each group comprised of 10 chicks. Group 1 was kept as control and groups 2–5 were infected each with CAV isolate separately. The groups were maintained under strict isolated conditions and high biosecurity measures were taken to avoid the cross-infection. The CAV isolates were infected at a dose rate of 1 ml infected MSB-1 cell culture fluid ($10^{4.5}$ $TCID_{50}$/0.1 ml) per bird intramuscularly in thigh region. The chicks of all the groups were observed daily for clinical signs and mortality, and packed cell volume (PCV) and body weight gain were recorded on 5, 10 and 15 days post-infection. At 15th day, all the birds were sacrificed and various organs, viz., thymus, bone marrow, spleen, liver and bursa were examined for gross and microscopic changes. The data obtained were analyzed statistically, wherever needed.

3. Results

3.1. PCR amplification of different regions of CAV genome

PCR amplification of VP1 region using primers VP1F and VP1R yielded a specific product of 1390 bp (Fig. 1a) and similarly PCR amplification VP2 region using primers VP2F and VP2R yielded specific product of 713 bp (Fig. 1b). The authenticity of PCR amplification was confirmed by the expected size in agarose gel, RE analysis and nucleotide sequencing.

3.2. Sequence analysis

Nucleotide sequence obtained for the four Indian isolates (CAV-A, -B, -E and -P) were annotated and a length of 1766 bp containing almost entire CAV coding region except few amino acids at the C-terminal end of VP1. Analysis of the nucleotide sequence of 1766 bp from position 386 to 2151 (as per Meehan et al., 1992) containing the complete coding region of VP2 (which also includes the VP3) and partial coding region of VP1 protein, revealed 12–52 nucleotides variation among Indian isolates. A maximum variation of 52 nucleotides was observed in CAV-E isolate followed by 15 nucleotides in CAV-P, 13 in CAV-A and 12 nucleotides in CAV-B. The 52 nucleotide changes in CAV-E resulted in 5 amino acid changes.

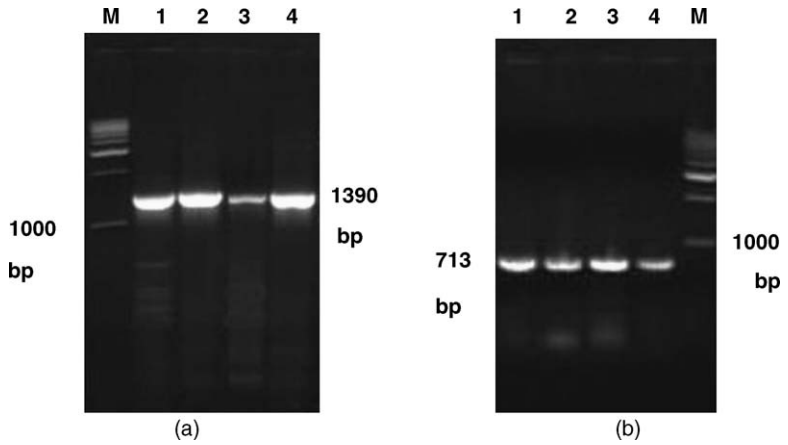

Fig. 1. (a) PCR amplification of *chicken anaemia virus* VP1 region; (M) 1 kbp DNA ladder marker (MBI Fermentas); (1) CAV isolate, CAV-A; (2) CAV isolate, CAV-B; (3) CAV isolate, CAV-E; (4) CAV isolate, CAV-P. (b) PCR amplification of *chicken anaemia virus* VP2 region; (M) 1 kbp DNA ladder marker (MBI Fermentas); (1) CAV isolate, CAV-A; (2) CAV isolate, CAV-B; (3) CAV isolate, CAV-E; (4) CAV isolate, CAV-P.

CAV-E showed a maximum variation of 4% among the Indian isolates where the total variation ranged from 0.5 to 4.0%. CAV-E isolate had a maximum identity with the Australian 704 strain (99.3%) followed by Japanese strain TR-20 (99.2%) and Malaysian SMSC-1 (99%). All the Indian and foreign CAV isolates used for analysis in the present study showed an overall nucleotide variation of 0.2–4.1%. The Indian isolates CAV-B and -P had maximum identity of ≥99% with Bangladesh isolate BD-3, whereas the isolate CAV-A was very closely related to the European Cux-1 strain.

The alignment of deduced 431 amino acid (AA) partial sequence of VP1 of 4 Indian CAV isolates (A, B, E and P) showed a maximum variation of 7 amino acids in CAV-P, of which at amino acids positions 144 (H), 262 (D) and 275 (F) were found specific to the isolate. The other isolates CAV-A, -B and -E showed only 5, 6 and 5 amino acids variation, respectively. The amino acid variation at position 144 (D) was specific for CAV-A isolate. Amino acid variation based on the partial VP1 sequence (431 AA) indicated an overall variation of 0–2.8% among all isolates compared. Among the Indian isolates, the amino acid variation ranged from 0.7 to 2.1%. CAV-E showed 100% identity with the Australian 704 strain. Similarly, CAV-B showed 100% identity to Bangladesh BD-3 strain and CAV-A had 99.5% identity to the European Cux-1 strain. Maximum variation among the CAV isolates was found in amino acid positions 139, 144, 287 and 290 (Fig. 2).

Analysis of deduced 216 amino acids sequence of VP2 protein of the Indian isolates revealed only 1 amino acid variation at position 149 in CAV-A and one at position 186 in CAV-E. Amino acid alignment of the VP2 (216 AA) region showed variation ranging from 0 to 0.9% among the Indian and foreign isolates. Among the Indian isolates CAV-E showed maximum variation

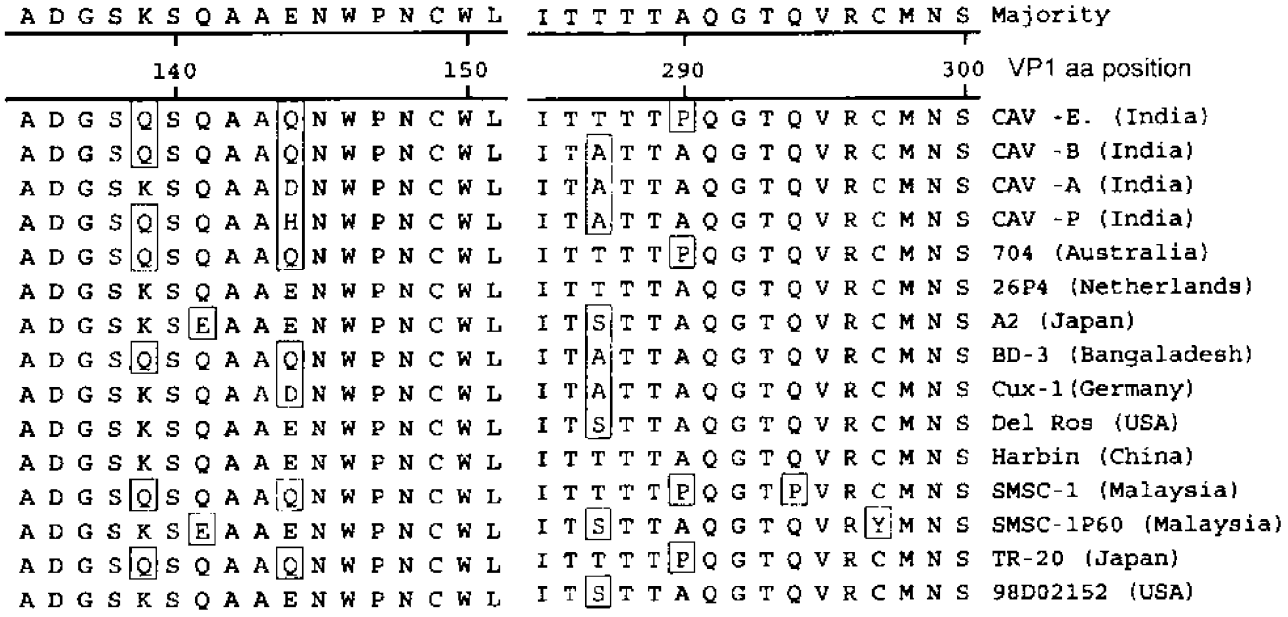

Fig. 2. Alignment of amino acid sequences of highly variable regions in VP1 coding sequence of different *chicken anaemia viruses*. Amino acid residues differing from majority are boxed.

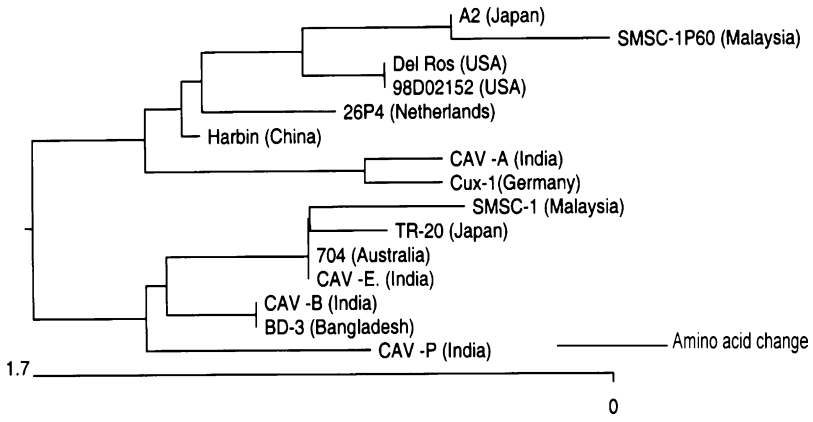

Fig. 3. Phylogenetic tree drawn based on the partial amino acid sequence of VP1 region from different *chicken anaemia viruses*. Branch distances corresponded to sequence divergence.

of 0.9%. CAV-B, -C and -P were 100% identical among themselves and also with foreign strains, viz., BD-3 (Bangladesh), TR-20 (Japan) and SMSC-1 (Malaysia).

3.3. Phylogenetic analysis

Phylogenetic analysis based on the nucleotide sequence of 1766 bp region of Indian CAV isolates along with foreign isolates revealed that CAV-A and Cux-1 had similar lineage and were very closely related since they formed one cluster whereas CAV-B and -P formed a separate cluster along with the Bangladesh strain BD-3. CAV-E formed a different cluster along with 704 (Australian), TR-20 (Japanese) and SMSC-1 (Malaysian) isolates which had a separate unique lineage. The USA isolates (Del Ross, DS2152), Japanese (A-2) and Malaysian attenuated SMSC-1 P60 strain formed another separate group. Similarly, phylogenetic analysis based on the 431 amino acids sequence of VP1 (Fig. 3) indicated that CAV-A was closely related to European whereas CAV-B, -P and -E were closely related to Australian, Malaysian and Bangladesh strains. Phylogenetic analysis based on the amino acid sequences of VP2 region did not reveal any major difference.

3.4. Restriction endonuclease analysis

The PCR amplified and purified VP2 region of CAV isolates A, B, E and P were digested with *Hae* III and *Mbo* I enzymes. Analysis of the amplified VP2 region of different isolates using the restriction enzyme *Hae* III revealed that CAV-E was different from other isolates (Fig. 4a) whereas *Mbo* I enzyme did not reveal any difference among the CAV isolates (Fig. 4b).

Restriction profile study of PCR amplified VP1 region of different isolates revealed differences among the Indian isolates. *Hha* I enzyme differentiated CAV-A, -B or -P and -E isolates by yielding different patterns (Fig. 5a). Using *Dde* I enzyme, the isolates could be assigned into two groups in which CAV-A and -E were in one and CAV-B and -P were in another (Fig. 5b). *Sac* I digestion grouped CAV-A and -P isolates together

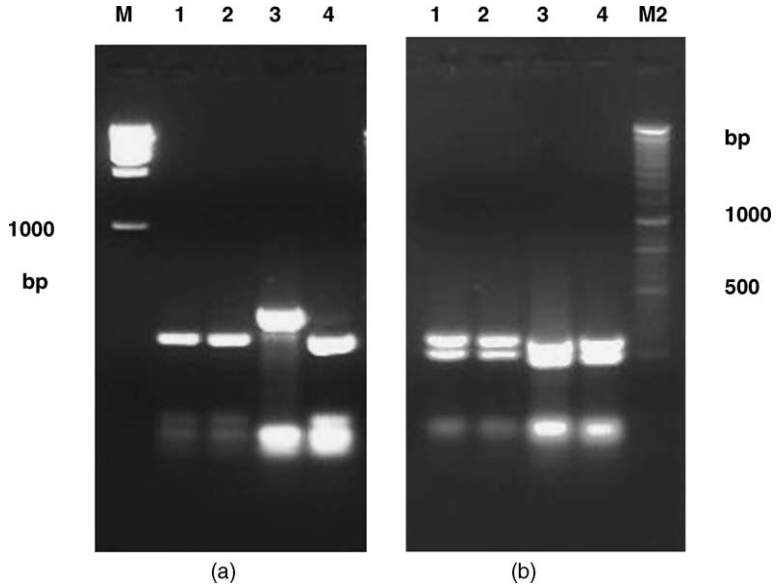

Fig. 4. Restriction endonuclease analysis of PCR amplified VP2 region using *Hae* III (a) and *Mbo* I (b) enzyme; (M) 250 bp DNA ladder marker (Invitrogen); (M2) 1 kbp DNA ladder marker (MBI Fermentas); (1) CAV isolate, CAV-A; (2) CAV isolate, CAV-B; (3) CAV isolate, CAV-E; (4) CAV isolate, CAV-P.

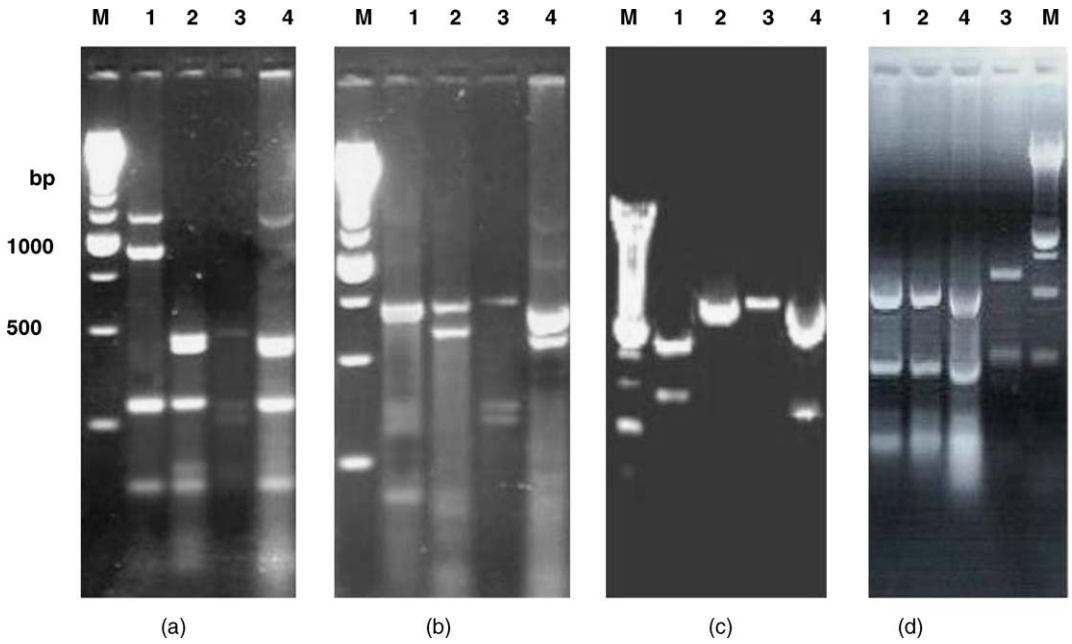

Fig. 5. Restriction endonuclease analysis of PCR amplified VP1 region using *Hha* I (a), *Dde* I (b), *Sac* I (c) and *Hae* III (d) enzymes; (M) 250 bp DNA ladder marker (Invitrogen); (1) CAV isolate, CAV-A; (2) CAV isolate, CAV-B; (3) CAV isolate, CAV-E; (4) CAV isolate, CAV-P.

in one group, CAV-B and -E isolates together in another group (Fig. 5c). *Hae* III digestion of the PCR amplified VP1 differentiated CAV-E from CAV-A, -B and -P isolates (Fig. 5d). All these Indian isolates could be differentiated using these three restriction enzymes *Hha* I, *Dde* I and *Hae* III.

3.5. Pathogenicity studies

Pathogenicity studies in day-old SPF chicks indicated that all the four Indian isolates of CAV are pathogenic and no significant variation was found among them except CAV-B in

Table 2
Clinical signs, mean body weight, mean PCV values, mortality, gross and histopathological lesion of day-old SPF chicks experimentally infected with different CIAV isolates

Observations	Days post-infection	CAV isolates				Control
		CAV-A	CAV-B	CAV-E	CAV-P	
Clinical signs	5	Healthy	Healthy	Healthy	Healthy	Healthy
	10	Healthy	Healthy	2 chicks, dull depressed	3 chicks were dull	Healthy
	15	2 dull and depressed	Healthy	3 dull and depressed	2 depressed	Healthy
PCV (%) ($N=6$, mean ± S.D.)	5	28.83 ± 2.32	30.67 ± 2.16	31.17 ± 3.06	31.66 ± 2.50	31.00 ± 2.00
	10	18.50 ± 1.52^{b}	18.33 ± 2.88^{b}	18.17 ± 3.97^{b}	18.00 ± 3.27^{b}	30.83 ± 2.79^{a}
	15	15.33 ± 2.07^{b}	14.33 ± 3.14^{b}	14.83 ± 2.93^{b}	12.83 ± 3.32^{b}	32.33 ± 3.39^{a}
Mean body weight (g) ($N=10$, mean ± S.D.)	5	37.33 ± 11.00^{a}	42.83 ± 2.13	44.33 ± 3.93	42.00 ± 2.28	43.33 ± 2.16^{b}
	10	54.50 ± 4.68^{b}	58.00 ± 3.03^{b}	52.16 ± 5.03^{b}	54.33 ± 3.93^{b}	65.50 ± 2.51^{a}
	15	85.0 ± 5.62^{b}	88.50 ± 4.46^{b}	89.00 ± 4.90^{b}	88.33 ± 5.57^{b}	103.50 ± 5.43^{a}
Mortality		1	0	2	1	0
Gross lesion						
Thymus		6/10	8/10	8/10	9/10	0/10
Bone marrow		4/10	5/10	4/10	7/10	0/10
Liver		9/10	9/10	8/10	6/10	2/10
Spleen		8/10	6/10	5/10	7/10	0/10
Bursa		6/10	4/10	4/10	5/10	0/10
Histopathology						
Thymus		10/10	10/10	10/10	10/10	0/10
Bone marrow		10/10	10/10	10/10	10/10	0/10
Liver		8/10	4/10	4/10	7/10	0/10
Spleen		7/10	8/10	10/10	10/10	0/10
Bursa		10/10	10/10	10/10	10/10	0/10

N, number of chicks. Values with different superscript (a and b) differs significantly at $P < 0.05$.

which clinical disease was not observed and only microscopic lesions were observed in lymphoid organs. The results of the comparative pathogenicity of all the four isolates are given in Table 2. All the isolates produced severe anaemia, reduced weight gain, gross and microscopic lesions in the experimentally infected chicks. The mean PCV value of CAV infected groups ranged from 12.83 ± 3.32 to 15.33 ± 2.07 as compared to the PCV of control chicks, 32.33 ± 3.39, on 15th day post-infection. Similarly, the mean body weight of the CAV infected groups was significantly lower (85.0 ± 05.62 to 89.00 ± 4.90) than the control chicks (103.50 ± 5.43) on 15 DPI. All the Indian isolates invariably produced atrophy of thymus, aplasia of bone marrow, spleen and bursal atrophy and paleness of liver. All the isolates also produced severe lymphocyte depletion in the lymphoid organs, viz., thymus, bone marrow, spleen and bursa of all the infected chicks. Statistical analysis of the PCV and body weight of chicks infected with different CAV isolates did not reveal significant difference among the groups. All the isolates produced lymphocyte depletion in the lymphoid organs, which indicated their immunosuppressive potential.

4. Discussion

Chicken anaemia virus is an economically important avian pathogen worldwide due to its highly immunosuppressive nature. It belongs to *Gyrovirus* genus of the Circoviridae family having circular single stranded DNA genome of 2.3 kbp in size. In India, the disease was first reported by Venugopalan et al. (1994) and thereafter not much information was available on CAVs circulating in the poultry population of the country. Later, Kataria et al. (1999) confirmed the wide prevalence of this virus from various states of this country. Dhama (2002) found some variation among the Indian isolates based on RE analysis of the PCR products. Therefore, need was felt for further molecular and pathogenic characterization of these viruses to find out the variation among them, if any, which would help devising suitable control strategy to prevent losses in the poultry industry. Sequencing was used as a tool to study the molecular epidemiology of Indian CAV isolates in the present study and also in vivo pathogenicity of these isolates was studied in day-old SPF chicks.

A nucleotide sequence portion of 1766 bp from position 386 to 2151 as per Meehan et al. (1992) in the genome of CAV was sequenced in the present study. The sequences obtained for the four Indian CAV isolates (CAV-A, -B, -E and -P) were aligned with several CAV isolates of different countries. Variation among the Indian isolates ranged from 12 to 52 nucleotides (0.5–4.0%) which revealed that Indian isolates were unique as the maximum variation among the CAV isolates worldwide is 5% (Dhama et al., 2002). Indian isolate CAV-E showed maximum variation of 4% among the Indian strains and it showed maximum identity of 99.3% with Australian 704 strain. It also showed more than 99% identity with Japanese TR-20 and Malaysian SMSC-1 strains. It is quite possible that these CAV strains might have evolved from a common origin and circulating in east Asia and Australia. Brown et al. (2000) sequenced a pathogenic Australian CAV isolate and reported an overall nucleotide sequence identity of approximately 95%. Kato et al. (1995) sequenced a Japanese isolate CAA82-2 and found 98% identity with European Cux-1 strain.

CAV-B and -P showed 12 and 15 nucleotides variation, respectively, among the Indian isolate but had more than 99% identity with Bangladesh strain (BD-3). This could be a common strain circulating in south-east Asia. CAV-A had 13 nucleotides variation and showed 99.8% identity to European Cux-1 strain.

The analysis of 431 amino acid sequences of the VP1 region of the Indian strains indicated only 5–7 amino acids variation (0.7–2.1%). A maximum variation of seven amino acids was observed in CAV-P and amino acids at positions 144 (H), 262 (D) and 275 (F) were found specific to the strain. CAV-A, -B and -E showed only five, six and five amino acids variation, respectively. The 52 nucleotide changes observed in CAV-E resulted only in 5 amino acid changes since most of the nucleotide mutations were synonymous mutations, which did not result in amino acid change, and only five mutations were non-synonymous. However, 15 nucleotide changes observed in CAV-P resulted in 7 amino acid changes due to seven non-synonymous mutations out of total 15 nucleotide mutations. Pallister et al. (1994) sequenced the PCR product of an Australian CAV isolate and compared the sequence of VP1 region with the German and American isolate and reported 12 amino acid differences in a total 449 amino acid, 8 of which were consecutive. It has been found that CAV-E had 100% identity to Australian 704 strain, CAV-B had 100% identity to Bangladesh BD-3 strain and CAV-A showed 99.5% identity to the European strain Cux-1.

Farkas et al. (1996) determined the complete nucleotide sequence of a CAV strain and concluded that N-terminal half of VP3 and N-terminal three-quarter of VP2 are well conserved and might sustain an essential function of these proteins. They also suggested that amino acid changes in VP1 might influence the antigenic variations among the CAV isolates. The present sequencing study also supports this fact. Recently, Spackman et al. (2002a, 2000b) have reported an antigenically different strain CAV-7 from the American reference CAV strain, Del Ross, and suggested that it could be a prototype virus of serotype-2. This report emphasized the need of reviewing the epidemiological status of CAV isolates worldwide.

Renshaw et al. (1996) reported a hypervariable region spanning 13 amino acids in VP1 region from position 139 to 151. Naturally occurring amino acid changes at position 139 and 144 has been shown to markedly affect the growth and spread of CAV isolates in cultured cells. In culture, it has been proven that VP1 Q-139 and/or Q-144 are associated with decreased rate of spread. Also, it is unclear which of the five amino acids upstream of the hypervariable region (VP1, N-23, I-75, Y-93 and L-07; VP2, E-175) are involved in the block of certain CAV permissible cell lines. The Indian isolates CAV-E and -B had Q-139 and Q-144 indicating that they might spread slowly in MSB-1 cells. CAV-A had K-139 and D-144, and CAV-P had Q-139 and H-144. The isolate CAV-P was adapted well in cell culture easily than all other isolates used in the present study, which suggested that Q-139 and H-144 might play a role for the increased permis-

sibility of MSB-1 cells to CAV isolates. Remarkable variation was found at position 144 in the present study as reported earlier (Brown et al., 2000).

Yamaguchi et al. (2001) reported that amino acid at position 394 in VP1 could be a major genetic determinant of virulence. If it is glutamine (Q) at this position, the isolates are highly pathogenic and if it is histidine (H) than less pathogenic. All the Indian isolates had glutamine (Q) at this position indicating that all are highly pathogenic. This finding also confirmed the results of pathogenicity study of these isolates in day-old SPF chicks as has been discussed in the later part.

RE analysis has been proven useful to differentiate CAV isolates at genome level (Todd et al., 1992; Noteborn et al., 1992; Van Santeen et al., 2001). On the basis of the sequence data, few useful restriction enzymes were selected for analysing the PCR products. RE digestion of the PCR amplified VP1 region using *Hha* I differentiated CAV-A, -B or -P and -E isolates by yielding different pattern. *Dde* I enzyme was found to group CAV-A and -E, and -B and -P separately in two groups, whereas *Sac* I digestion grouped CAV-A and -P together, CAV-B and -E together. Among these enzymes, *Hha* I was found very useful to differentiate more number of isolates. *Hae* III was also found useful to differentiate CAVs using both VP1 and VP2 regions. Noteborn et al. (1992) used the enzymes, viz., *Eco* RI, *Acc* I, *Bgl* II, *Hin*d III, *Sst* I, *Bam* HI, *Xba* I and detected variation among the isolates by *Acc* I, *Hin*d III and *Eco* RI enzymes. Similarly, Todd et al. (1992) analyzed 14 CAV isolates obtained from different parts of the world and assigned them to 7 groups based on the restriction pattern of the PCR amplified 675 bp region of N-terminal half of VP1 using the enzymes, viz., *Hae* III, *Hin*f I and *Hpa* I. The present study also indicated the usefulness of restriction enzymes, viz., *Hha* I, *Dde* I, *Sac* I and *Hae* III to differentiate the Indian field isolates, which would be helpful for the less equipped diagnostic laboratories.

The comparative pathogenicity study of all the five CAV isolates was carried out in day-old SPF chicks inoculated with a constant dose of 1 ml of the cell culture fluid having titre of $10^{4.5}$ $TCID_{50}$/0.1 ml. All the isolates produced consistent low PCV values, reduction in body weight, aplasia of bone marrow and generalized lymphoid atrophy and histopathological lesions of lymphocyte degeneration and depletion from the lymphoid organs. The lesions closely resembled those as has been described by other workers in SPF chicks inoculated with CAV at day-old age (Yuasa et al., 1979; Taniguchi et al., 1982, 1983; Dhama, 2002).

All the four Indian CAV isolates had amino acid Q at position 394 in VP1. In vivo pathogenicity study in SPF chick also revealed that they are highly pathogenic in nature. Therefore, the present study revealed good correlation between the molecular study as reported by Yamaguchi et al. (2001) with respect to the amino acid Q (glutamine) at position 394 in VP1 as major determinant of pathogenicity.

5. Conclusion

The present study indicated that genetically different strains of chicken anemia viruses are circulating in Indian subcontinent and they might have originated from different parts of the world, viz., Europe, Australia and south-east Asia. CAV-E was closely related to Australian, Malaysian and Japanese CAV strains. CAV-B and -P were closely related to Bangladesh strain, which could be a predominantly circulating strain in south-east Asia, and CAV-A was closely related to European strain (Cux-1). All the four Indian CAV isolates were found highly immunosuppressive to young chicks. All Indian CAVs had amino acid Q (glutamine) at position 394 in VP1, supporting the fact that amino acid at position 394 in VP1 as a major determinant of pathogenecity. Identification of amino acid at position (394) followed by in vivo pathogenicity study in SPF chicks for any newly emerging CAV isolate would help to identify low pathogenic or apathogenic CAV isolate in the field, which could be used for developing a live vaccine. The study suggests the need for constant monitoring of these genetically different viruses circulating in the field where there is considerably more chance to evolve new strain.

Acknowledgements

The authors are thankful to the Director, Indian Veterinary Research Institute for kindly providing the facility to carry out this research work. The authors also thank Dr. Siba K. Samal and Dr. D. Govindarajan, VA-MD Regional College of Veterinary Medicine, College Park, MD, for all the help and support during this project.

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