

Research Article



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

GOPAL KRISHAN¹, SANTOSH KUMAR SHUKLA², PRAKASH BHATT³, MAQBOOL YAQOOB WANI⁴, KULDEEP DHAMA⁵, YASH PAL SINGH MALIK⁶, RAJESH KUMAR^{7*}

¹Department of Animal Husbandry, Himachal Pradesh, India; ²Department of Veterinary Medicine; ³Veterinary Clinics, ⁷Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand - 263 145, India; ⁴Immunology Section, ⁵Avian Diseases Section, Division of Pathology, ⁶Division of Biological Standardization, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.) – 243 122, India.

Abstract | The aim of the study was to assess the role of chicken anaemia virus (CAV) in precipitating important poultry diseases in commercial poultry flocks and its molecular surveillance in poultry flocks of North India (Uttarakhand and Uttar Pradesh states) and Nepal (Mahendra Nagar). Study was conducted during September-2012 to April-2013 on 13 randomly selected poultry flocks. Necropsy was performed for making tentative diagnosis of other concomitant diseases and samples were collected and epidemiological data was recorded. Flocks were screened for CAV by VP2 gene based PCR followed by PCR-RFLP analysis of viral genome. Haemato-biochemical parameters of flocks were also studied. Out of 185 tissue samples, PCR screening confirmed the presence of CAV genome in 95 samples, while 50 showed other viruses like infectious bursal disease (IBD), Inclusion Body Hepatitis (IBH) and Marek's Disease (MD). PCR-RFLP analysis of the full genome of different CAV isolates with *Hind* III, *Pst* I, *Nhe* I and *Mbo* II restriction enzymes showed indistinguishable restriction patterns. A significant decline in erythrocytic and leukocytic cell counts, and increase in the activities of degenerating enzymes was observed in CAV positive flocks. In conclusion, CAV was detected at different poultry farms of India and Nepal and its association with precipitation of other poultry diseases through its marked immunosuppressive potential is evident from findings of present study. Thus, the role of CAV in precipitating other infectious diseases in birds along with molecular epidemiology and immunosuppressive effects needs to be studied in detail so that a comprehensive strategy may be formulated for timely adoption of effective prevention and control measures, subsequently alleviating economic losses in poultry rearing countries.

Keywords | Chicken anaemia virus, Poultry, Immunosuppression, Haemato-biochemical parameters, PCR- RFLP, Molecular epidemiology

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***Correspondence** | Rajesh Kumar, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand- 263 145, India; **Email:** rajeshvet@rediffmail.com

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INTRODUCTION

Chicken anaemia virus (CAV), the causative agent of chicken infectious anaemia (CIA), belongs to the *Gyrovirus* genus of the *Circoviridae* family (Pringle, 1999;

Todd et al., 2000), and is an economically important and emerging pathogen affecting poultry industry worldwide (McNulty, 1991; Jiang et al., 2005; Dhama et al., 2008; Schat, 2009; Kim et al., 2010; Oluwayelu, 2010; Bhatt et al., 2013; Snoeck et al., 2012; Nayabian et al., 2013; Wani

et al., 2013). CAV is a non-enveloped virus with a circular, covalently linked, negative-sense, ss DNA genome of about 2,298 to 2,319 nucleotides, and is one of the smallest avian viruses (23–25 nm size). The major transcript from the CAV genome is an unspliced polycistronic mRNA of about 2100 nucleotides encoding VP1 (51.6 kDa), the viral capsid protein; VP2 (24.0 kDa), a scaffold protein; and VP3 or apoptin (13.6 kDa) that is responsible for causing apoptosis in chicken thymocytes and chicken lymphoblastoid cells (Todd et al., 1992; Natesan et al., 2006).

Chicken anaemia virus infection is characterized by lymphoid atrophy, immunosuppression, increased mortality, aplasia of the bone marrow and anaemia, reduced body weight gain, and the development of subcutaneous and intramuscular haemorrhages in chickens (Dhama et al., 2008; Schat, 2009; Pope, 1991; Miller and Schat, 2004). No specific treatment exists for CIA, supplementation with haematinics and immunostimulants can ameliorate anaemic condition and depressed immunity (Bhatt et al., 2013; Latheef et al., 2013). Vaccines including of live-attenuated and killed vaccines are being used but have some limitations, and therefore DNA vaccine and other advanced vaccine options are being explored (McNulty, 1991; Schat, 2003; Dhama et al., 2008, 2014, 2015; Sawant et al., 2015; Zhang et al., 2015). CAV is considered to play an important role in precipitation of number of poultry diseases (Dhama et al., 2008). Experimental co-infection of CAV with Marek's disease virus (MDV) (Zanella et al., 1999; Miles et al., 2001), dual infection of CAV with infectious bursal disease virus (IBDV) (Yuasa et al., 1980; Rosenberger et al., 1989; Imai et al., 1991; McNeilly et al., 1995), or adenovirus (Toro et al., 2000 and 2001) have resulted in increased morbidity and mortality. CAV infection in poultry has also been associated with poor efficacy of vaccines against Marek's disease, Newcastle disease and infectious laryngotracheitis (Box et al., 1988; Otaki et al., 1988; Cloud et al., 1992; Liu et al., 2001) Precipitation and/or increase in mortality due to these diseases by concomitant CAV infection owes to immunosuppression caused by CAV (McNulty, 1991; Dhama et al., 2008; Pope, 1991).

CAV has been reported from poultry flocks of India based on clinical signs, virus isolation, serological and molecular detection studies (Natesan et al., 2003; Dhama et al., 2004; Bhatt et al., 2011; Wani et al., 2013), however, detailed molecular epidemiological studies, immunosuppression, clinical presentation and association of the virus with other infectious pathogens/diseases of poultry is lacking. Applications of molecular tools like PCR along with RE analysis and sequencing have emerged as effective confirmatory tools for diagnosis and studying the molecular epidemiology of poultry pathogens including CAV, which help in designing and adapting appropriate disease prevention and control programme. However, applications of clinical diag-

nostic methods based on haemato-biochemical parameters help in devising the effective treatment strategies. Present study describes the molecular epidemiology of CAV and its role in precipitating important infectious diseases of poultry in commercial flocks of northern Indian states (Uttarakhand and Uttar Pradesh) and Nepal.

MATERIALS AND METHODS

SAMPLE COLLECTION

Epidemiological data of different infectious diseases from the poultry farms of Uttarakhand state (Udham Singh Nagar, Nainital districts), Uttar Pradesh (Bareilly, Pilibhit and Rampur districts), India and Nepal (Mahendra Nagar district) were recorded during the period of September-2012 to April-2013 (Table 1). Blood samples were collected from affected birds of all the flocks under investigation at the beginning of different disease outbreaks. Five apparently unhealthy birds were selected from each flock, and 2 ml of blood was collected in heparinised vials (10–20 IU/ml) for haemato-biochemical studies and in sterilized test tubes for separation of serum. Post-mortem examination of at least three dead birds from each farm was carried out. Detailed gross post-mortem lesions were recorded and tentative diagnosis was made. Tissue samples from liver, thymus, spleen, bursa of fabricius and bone marrow were collected in sterilized vial using aseptic conditions and kept in air tight containers at -20°C for PCR detection of CAV genome.

PCR DETECTION OF CAV

For the detection of CAV by PCR in clinical samples, whole tissue DNA was extracted from thymus, bursa, spleen, liver and bone marrow using GeneiPure™ Mammalian Genomic DNA Purification Kit (Bangalore Genei) as per manufacturer's recommendations. The absorbance of the extracted DNA was measured at 260 nm and 280 nm in UV-VIS spectrophotometer. The ratio of OD₂₆₀ and OD₂₈₀ was calculated to check the purity of extracted DNA. The DNA samples having ratio of ≥ 1.8 were used for PCR. The amplification of CAV VP2 gene was carried out using sense VP2F: 5'ATG CAC GGG AAC GGC GGA C3' and antisense VP2R: 5'TCA CAC TAT ACG TAC CGG GG3' primers as described previously (Basaraddi et al., 2013). The PCR products were confirmed by running in 1 % (w/v) agarose gel in 0.5X TBE buffer with DNA bands stained with ethidium bromide (0.5µg/ml) as per the method of Sambrook et al. (1989), and visualized and documented using Gel documentation system (Alpha Imager).

RESTRICTION ENDONUCLEASE (RE) ANALYSIS

Full genome of the virus was amplified by using primers viz., FGF: 5'-GAA TTC CGA GTG GTT ACT ATT CCA TCA-3 and FGR: 5'-GAT AGT GCG ATA AAT CTA TTT TCT GCG T-3'. The amplified products were subjected to restriction endonuclease (RE) enzyme diges-

Table 1: Details of poultry flocks

Sr. No.	Farm Code/Area	Code No.	System of management	Age of Birds	Total Flock Strength
1.	N (Baazpur, UK*)	N	Cage	14 weeks	6000
2.	SP (Baazpur, UK)	SP	Cage	12 weeks	12000
3.	KP (Kashipur, UK)	KP	Cage	23 weeks	9000
4.	NP (Jaffarpur, UK)	NP	Cage	17 weeks	8000
5.	IP (Punjabnagar, UP*)	IP	Deep litter	2 weeks	1000
6.	DP (Ramnagar, UK)	DP	Deep litter	3 weeks	1200
7.	AP (Bilaspur, UP)	AP	Deep litter	3 weeks	2400
8.	ZP (Pilibhit, UP)	ZP	Deep litter	5 weeks	2000
9.	PN (Golapar, UK)	PN	Deep litter	4 weeks	1200
10.	AH (Baazpur, UK)	AH	Cage	16 weeks	9000
11.	PI (Pilibhit, UP)	PI	Deep litter	6 weeks	1300
12.	PF (Mahendra Nagar, Nepal)	PF	Deep litter	2 weeks	1800
13.	MP (Bareilly, UP)	MP	Deep litter	7 weeks	900

* UK = Uttarakhand (India), UP = Uttar Pradesh (India)

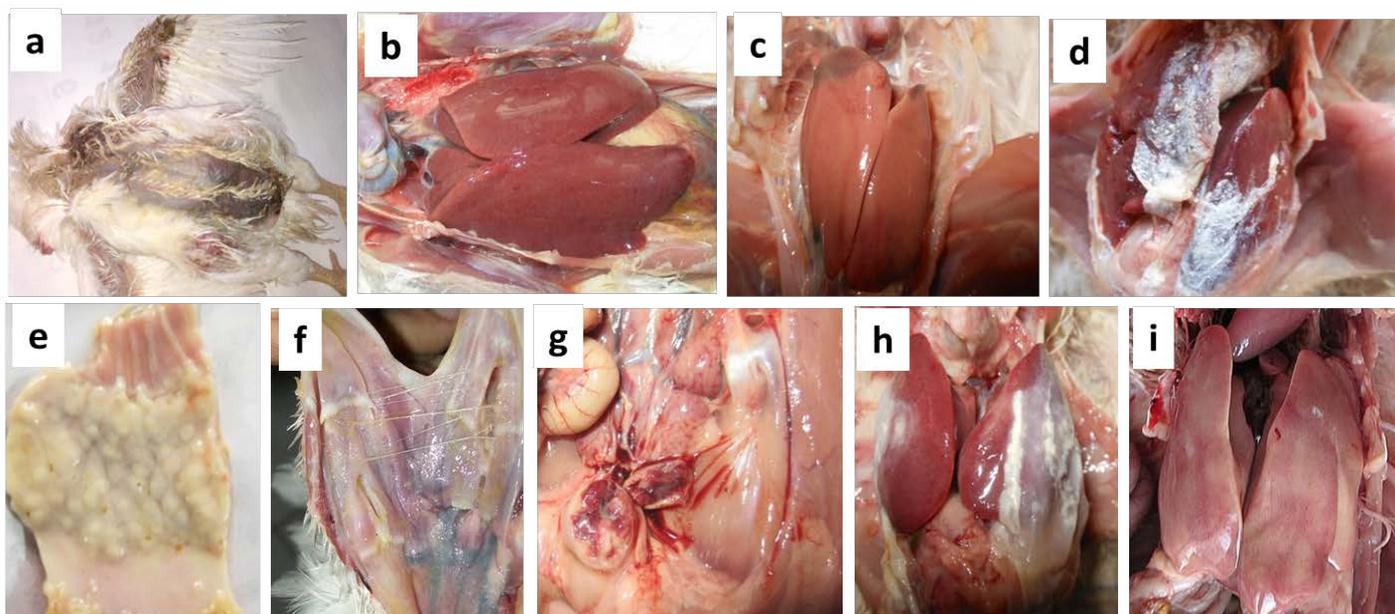


Figure 1: Necropsy findings of the suspected CAV infected poultry birds collected from different farms (a) Moist haemorrhages on the skin of CIA-GDS affected bird; (b) Nodular appearance of liver in Marek's disease affected bird; (c) Swollen liver with necrotic foci in CIA-GDS affected bird; (d) Urate deposits in Gout affected bird; (e) Thickened proventriculus in Marek's disease affected bird; (f) Caseous plug on opening of trachea in Fowl pox affected bird; (g) Haemorrhages on mucosal surface of bursa in IBD affected bird; (h) Pericarditis and perihepatitis in CCRD affected bird and (i) Punctate haemorrhages on the liver of IBH affected bird

tion using *Hind* III, *Pst* I, *Nhe* I and *Mbo* II restriction enzymes as per the manufacturer's protocol, using specific assay conditions and the buffers supplied (Merck Genei). The molecular weights of various fragments of the digested viral DNA were determined using Fortran Software and expressed in base pairs (bp).

HAEMATO-BIOCHEMICAL ANALYSIS

The various haematological parameters viz., haemoglobin (Hb) content, packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC) and differential

leukocyte count (DLC) were estimated by standard procedures. For biochemical parameters, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and uric acid (UA) were estimated using commercially available reagent kits.

RESULTS

PREVALENCE OF CAV AND CO-INFECTIONS

During the disease investigation in the affected poultry flocks, birds were found suffering from chicken infectious

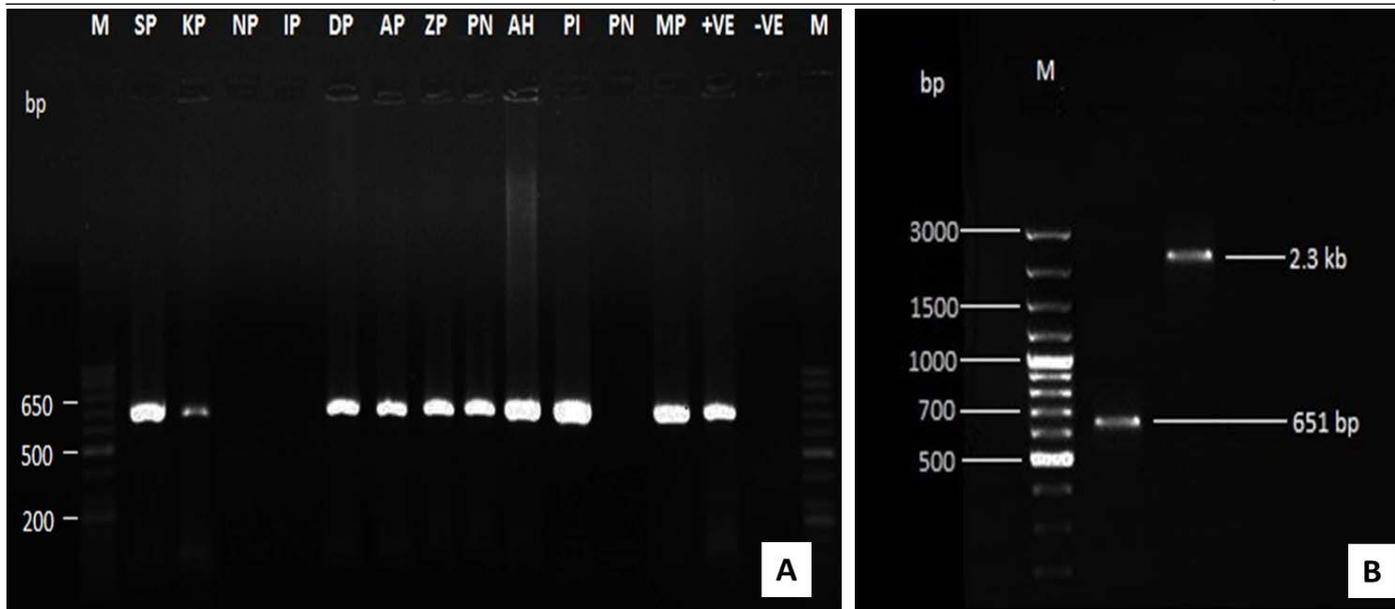


Figure 2: Amplification of CAV-DNA using PCR assay using VP2 specific primer (A) and by using both VP2 and full length genome primers (B)

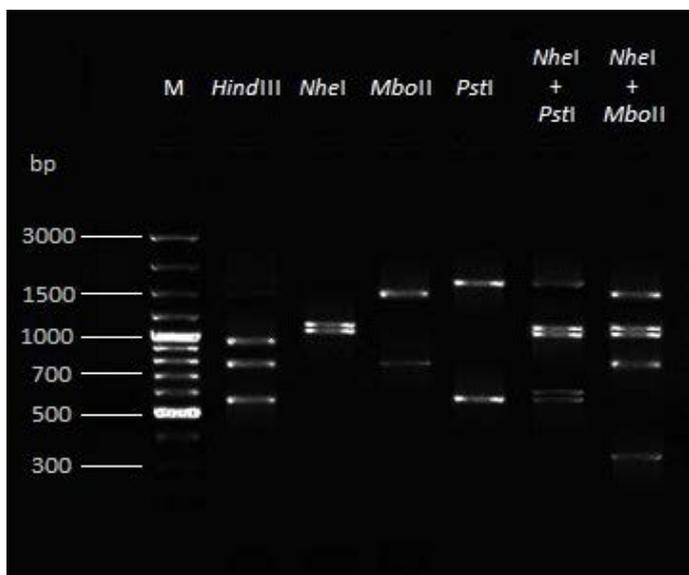


Figure 3: Restriction fragment length polymorphism (RFLP) pattern after RE digestion of amplified full length CAV genome with four different enzymes and their combinations

anaemia– gangrenous dermatitis syndrome (CIA-GDS), Marek’s disease, infectious bursal disease and inclusion body hepatitis (Figure 1 and Table 2). In the PCR based screening of a total of 185 tissue samples collected from twelve different flocks, 145 samples (78.38%) were found positive for CAV (95/95), Marek’s disease (15/15), infectious bursal disease (20/20) and inclusion body hepatitis (15/15), while 40 samples tested negative for fowl pox, CRD and gout. The CAV positive flocks showed virus positive PCR amplicons of 651 bp (VP2 gene) and ~2.3 kb (complete genome) (Figure 2). The detailed results of the molecular surveillance of CAV in the affected poultry flocks are presented in Table 3.

RESTRICTION ENZYME (RE) ANALYSIS OF FULL GENOME CAV ISOLATES

RE analysis of full genome amplicon (~2.3 kb) of all CAV isolates with *HindIII*, *PstI*, *NheI* and *MboII* restriction enzymes revealed identical RFLP patterns (Figure 3, Table 4). All amplicons contained three *HindIII* sites, one *PstI* site, one *NheI* site and one *MboII* site.

ANALYSIS OF HAEMATOLOGICAL PARAMETERS

Haematological parameters of CAV positive and negative groups revealed a significant ($P < 0.05$) decline in the Hb, PCV, TEC, mean Hb and mean PCV in the CAV positive groups as compared to the control group (Table 5). In comparison to healthy control, a significant ($P < 0.05$) reduction in the mean TEC values of CAV positive flocks was observed except the one farm (KP). In the myeloid lineages also, a significant ($P < 0.05$) decline in the TLC, percent lymphocyte count (PLC) and mean PLC was observed in all the CAV positive birds as compared to the negative group. The mean TLC was found significantly ($P < 0.05$) lower in the all the CAV positive flocks except in one positive flock (MP) which was suspected for IBH based on gross lesions. All the CAV positive flocks showed a significant ($P < 0.05$) increase in the mean percent heterophil count as compared to the negative control group.

ANALYSIS OF BIOCHEMICAL PARAMETERS

All serum samples showed significantly ($P < 0.05$) higher activities of AST, ALT and ALP enzymes in all the CAV positive flocks (Table 6). The mean uric acid value was found significantly ($P < 0.05$) higher in the CAV positive flocks except in one CAV positive farm (KP) when compared with the negative group.

Table 2: Necropsy findings and molecular surveillance of suspected twelve poultry flocks for CAV with their respective tentative diagnosis

Sr.No.	Farm-Code	Necropsy findings	Tentative diagnosis	Mortality (%)	Type of tissue sample	No. of samples screened for CAV		
						Total	Positive	Negative
1	SP	- Haemorrhagic and necrotic lesions on the wings and breast region - Severe atrophy of thymus and bursa - Bone marrow aplasia with yellowish discolouration - Pale discolouration of liver	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	5.72	Thymus, Bursa, Bone marrow, Spleen, Liver	20	20	-
2	KP	- Discolouration of liver with presence of nodules - Marked thickening of proventriculus	Marek's Disease	2.67	-do-	15	15	-
3	NP	- Cheesy plug on opening of trachea - Diphtheritic membrane with white nodules along mucosal lining of trachea, upper esophagus and nasal cavity	Fowl Pox	1.20	-do-	15	-	15
4	IP	- Chalky white deposition of urate crystals on heart, liver, kidneys and under the skin - Swollen kidneys with urate deposits in ureters giving white chord appearance	Gout	3.50	-do-	15	-	15
5	DP	- Haemorrhagic and necrotic lesions on the wings, breast and thighs - Thymus and bursal atrophy - Bone marrow hypoplasia with presence of pink colour	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	11.25	-do-	15	15	-
6	AP	- Haemorrhages on thigh muscles - Enlarged bursa with presence of haemorrhages on mucosal surface - Swollen kidneys	Infectious Bursal Disease	4.00	-do-	20	20	-
7	ZP	- Haemorrhagic and necrotic lesions on the wings, breast and thighs - Thymus and bursal atrophy - Pink coloured marrow cavity of femur - Slight enlargement of liver with pale discolouration	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	4.15	-do-	15	15	-
8	PN	- Haemorrhagic lesions on wings - Marked atrophy of thymus with mild bursal atrophy - Pinkish discolouration of bone marrow - Discolouration of liver	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	3.75	-do-	15	15	-
9	AH	- Thymus and bursal atrophy - Bone marrow hypoplasia with presence of pink - Enlargement and mottled appearance of liver - Haemorrhagic and necrotic lesions on wings and breast	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	6.39	-do-	15	15	-
10	PI	- Haemorrhagic and necrotic lesions on wings and breast region - Severe atrophy of thymus and moderate bursal atrophy - Bone marrow aplasia with yellowish discolouration - Pale discolouration of liver	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	45.69	-do-	15	15	-
11	PF	- Fibrinous pericarditis and perihepatitis - Presence of caseous exudates in the air sac - Catarrhal inflammation of nasal passage, trachea and bronchi - Cheesy exudate around the nostrils and eyes	Chronic Complex Respiratory Disease	3.33	-do-	10	-	10
12	MP	- Swelling of the liver with presence of punctiform haemorrhagic foci throughout the surface - Pale coloured marrow cavity of the femur - Enlarged and pale kidneys - Mild bursal atrophy	Inclusion Body Hepatitis	6.22	-do-	15	15	-

Table 3: Disease-wise analysis of samples tested by PCR for detection of CAV DNA

Sr. No.	Suspected Disease (Based on gross lesions)	No. of samples tested	No. of positive samples	No. of negative samples
1.	Chicken Infectious Anaemia – Gangrenous Dermatitis Syndrome	95	95	-
2.	Marek's Disease	15	15	-
3.	Fowl Pox	15	-	15
4.	Gout	15	-	15
5.	Infectious Bursal Disease	20	20	-
6.	Chronic Complex Respiratory Disease	10	-	10
7.	Inclusion Body Hepatitis	15	15	-
Total		185	145	40

Table 4: Digestion products of RE analysis with different enzymes

Sr. No.	Restriction Enzymes	Digestion products (bp)
1.	<i>Hind</i> III	966, 791, 562
2.	<i>Pst</i> I	1743, 576
3.	<i>Nhe</i> I	1191, 1128
4.	<i>Mbo</i> II	1522, 797
5.	<i>Nhe</i> I + <i>Pst</i> I	1743, 1191, 1128, 615, 576
6.	<i>Nhe</i> I + <i>Mbo</i> II	1522, 1191, 1128, 797, 331

DISCUSSION

CAV infection has been incriminated as the cause in several cases where infectious anaemia occurred in chicken flocks of up to 2-4 weeks of age (McNulty, 1991; Dhama et al., 2008; Schat, 2009; Pope, 1991; Rosenberger and Cloud, 1989). Age related resistance occurs in chicks as clinical disease does not occur in chicks above 4 weeks of age (Wani et al., 2014). Co-infections of CAV with other pathogens can overcome the effects of age and maternal antibody related resistance, thus increasing the susceptible age period for CAV and its persistency (Yuasa et al., 1980; Rosenberger and Cloud, 1989; Toro et al., 2000). In the field, CAV infection seems to cause few signs of disease, however, dual infections generally occur which are more serious in terms of economic losses to poultry producers (Dhama et al., 2008; Pope, 1991; Imai et al., 1999). Therefore, it becomes necessary to determine the existence of CAV in association with other pathogens in poultry population of any country. Although, postmortem findings act as first hand tentative diagnostic method for many infections in poultry, the association of CAV in mixed infections and its diagnosis is difficult especially during subclinical infection. Applications of molecular tools have paved a way for the rapid and convenient diagnosis in such mixed infections.

In India, chicken infectious anemia (CIA) has been suspected since 1990's after the emergence of virulent strains of IBDV and on the basis of clinical symptoms, lesions and

its detection by immunoperoxidase test (Venugopalan et al., 1994). Presently, the disease has been confirmed from many states using virus isolation, PCR detection and serological studies (Wani et al., 2013; Natesan et al., 2003; Dhama et al., 2004; Bhatt et al., 2011). The molecular techniques including PCR have been well employed by several workers (Wani et al., 2013; Todd et al., 1966; Dhama et al., 2004; Bhatt et al., 2011; Rozypal et al., 1997; Chowdhury et al., 2002; Miller et al., 2003) for detection of CAV-DNA in clinical samples. However, detailed molecular epidemiological status, immunosuppressive nature and clinical/field interactions of the virus with other infectious pathogens/diseases of poultry have not been performed earlier in the country.

In the present study, molecular surveillance of the CAV, IBD, IBH, fowl pox, gout, CRD and MD in suspected poultry flocks from north Indian states and Mahendra Nagar area of Nepal with the history of different disease outbreak, viz. bacterial, viral, protozoal, etc. was undertaken along with clinical, pathological and haemato-biochemical parameters studies. High prevalence of CAV recorded in present study is in accordance with the earlier reports from India (Wani et al., 2013; Bhatt et al., 2011). Amongst the nine CIA positive flocks, three flocks were suffering with MD, IBD and IBH based on necropsy examination results. Jin et al. (2001) found co-infection of MDV, CAV and REV in some tissue samples of bursa from the chicken flocks suspected to be infected by IBDV according to clinical symptoms and gross lesions by dot hybridization technique in some parts of China. Later, Jiang et al. (2005) also found co-infection of MDV, CAV and REV in tissue samples of 828 chickens from 42 different chicken flocks suspected to be infected by immunosuppressive diseases in 11 provinces of China.

In the present investigation, all the PCR amplified full genome DNA amplicons from CAV positive flocks showed identical RFLP pattern with restriction enzymes (*Hind* III, *Pst* I, *Nhe* I and *Mbo* II), signifying circulation of single serotype of the virus. Previous reports have shown pathogenic variations among the different isolates (Kim et al., 2010;

Table 5: Mean values of haemoglobin, PCV, TEC, TLC and DLC in different flocks

Sr. No.	Farm Code	Haemoglobin (Hb) (g/L)	Packed Cell Volume (PCV) (L/L)	Total Erythrocyte Count (TEC) ($\times 10^9/L$)	Total Leukocyte Count (TLC) ($\times 10^6/L$)	Differential Leukocyte Count (DLC)			
						Heterophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil + Basophil (%)
1.	N	118.33±0.24	0.32±0.00	3.20±0.05	21.47±0.81	33.67±0.58	60.00±0.33	3.67±0.33	2.67±0.33
2.	SP	66.00±0.12*	0.21±0.01*	1.92±0.04*	11.56±1.74*	53.00±1.53*	41.00±1.53*	3.33±0.33	2.67±0.33
3.	KP	116.00±0.12	0.30±0.01*	3.15±0.14	17.73±0.18*	42.00±1.15*	53.00±1.53*	2.66±0.67	2.33±0.33
4.	NP	109.00±0.25	0.32±0.01	2.74±0.09*	19.93±0.18	55.67±0.88*	39.33±0.88*	3.00±0.58	2.00±0.00
5.	IP	114.67±0.18	0.33±0.00	2.99±0.12	20.47±1.05	56.67±0.88*	37.67±1.45*	3.33±0.33	2.33±0.33
6.	DP	68.00±0.12*	0.24±0.01*	2.10±0.10*	9.53±0.42*	50.00±0.58*	44.67±0.88*	3.33±0.67	2.33±0.33
7.	AP	72.67±0.07*	0.27±0.02*	2.72±0.05*	9.60±0.38*	50.33±1.20*	44.00±1.15*	3.33±0.67	2.67±0.33
8.	ZP	68.00±0.12*	0.22±0.00*	1.95±0.08*	10.80±0.81*	49.00±1.00*	44.00±1.15*	3.67±0.33	2.67±0.33
9.	PN	68.00±0.12*	0.24±0.01*	2.09±0.09*	9.73±0.48*	50.67±0.88*	44.67±1.45*	3.00±0.58	1.67±0.33
10.	AH	68.67±0.18*	0.23±0.00*	2.05±0.04*	9.70±0.46*	49.67±0.67*	43.67±0.88*	4.00±0.58	3.00±0.58
11.	PI	65.33±0.37*	0.19±0.01*	1.69±0.22*	9.87±0.35*	49.00±1.73*	43.67±2.40*	4.00±0.58	2.67±0.33
12.	PF	114.00±0.23	0.32±0.00	2.89±0.15	25.67±0.70*	58.00±0.57*	34.33±1.20*	5.33±0.33*	2.33±0.33
13.	MP	78.00±0.23*	0.26±0.01*	2.81±0.10*	22.80±1.36	38.33±1.20*	53.00±1.00*	5.67±0.33*	2.67±0.33

* Significantly (P<0.05) different

Table 6: Mean value of AST, ALT, ALP, Uric Acid in different flocks

Sr. No.	Farm Code	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Uric acid (UA) (mg/dl)
1.	N	174.83±0.66	34.00±0.31	485.50±1.55	5.53±0.05
2.	SP	322.33±3.60*	70.97±0.84*	586.13±6.23*	7.00±0.11*
3.	KP	256.83±1.55*	59.91±0.78*	558.93±1.08*	5.90±0.17
4.	NP	178.30±2.16	34.54±0.83	488.13±1.30	5.70±0.04
5.	IP	279.67±2.03*	66.90±0.85*	572.80±1.64*	12.27±0.99*
6.	DP	263.43±2.75*	64.23±0.35*	567.87±3.46*	6.76±0.44*
7.	AP	241.03±4.36*	54.80±0.97*	537.10±3.40*	11.47±0.57*
8.	ZP	254.20±1.97*	57.23±1.28*	558.10±2.64*	6.83±0.08*
9.	PN	251.13±1.50*	56.13±1.86*	552.47±4.22*	6.73±0.04*
10.	AH	265.07±1.68*	64.23±0.32*	572.27±2.16*	6.96±0.09*
11.	PI	284.03±6.85*	54.73±1.36*	571.43±1.76*	6.96±0.09*
12.	PF	243.90±2.41*	70.20±0.54*	542.57±1.81*	5.82±0.04
13.	MP	324.33±3.60*	74.53±0.54*	603.37±2.59*	7.14±0.08*

* Mean values (±SE) are significantly different (P<0.05)

Natesan et al., 2003; Yuasa et al., 1986; McNulty et al., 1990; Adair, 2000; Connor et al., 1991; Davidson et al., 2004; Oluwayelu et al., 2005). A study from the USA reported an antigenically different CAV isolate (Spackman et al., 2002), which could be a prototype virus of serotype 2. It has been highlighted that due to recombination mechanisms the new genetic variants are arising (Eltahir et al., 2011). Recently, genetically similar variants of gyroviruses have been detected in the human populations, alarming their importance in human health (Biagini et al., 2013; Smuts, 2014). Todd et al. (1992) assigned 14 CAV isolates of seven countries in to seven groups by RE analysis of the PCR amplified DNA with *Hae* III, *Hinf* I and *Hpa* II

enzymes. Noteborn et al. (1992) used the enzymes, viz., *Eco*RI, *Acc*I, *Bgl*II, *Hind*III, *Sst*I, *Bam*HI, *Xba*I and noticed some interesting minor differences by *Acc*I, *Hind*III and *Eco*R I enzymes in spite of the overall high degree of similarity. Similarly, Imai et al. (1998) analyzed 14 CAV amplified DNA products to *Bgl*II, *Hind* III, *Pst* I and *Sac* I which showed the amplified region as highly conserved among all the isolates. However, in one of our study (Dharma et al., 2004) we found some variations among the six Indian CAV isolates on the basis of RE analysis of PCR amplified products. Similarly, Natesan et al. (2003) reported differences among Indian isolates of CAV by using three restriction enzymes (*Hha* I, *Dde* I and *Hae* III).

The previous reports are based on RE analysis of partial CAV genome segments and the novelty in RE digestion in this present study is that PCR products covering the whole viral genome were employed rather than any CAV gene specific smaller region PCR products, and such studies are lesser revealing RFLP pattern of the whole genome sequence, thus providing a useful molecular data regarding CAV RE analysis and comparison with other isolates of worldwide origin. Further studies including digestion with other specific restriction enzymes, sequence analysis and phylogenetic analysis of CAV genome are recommended for the differentiation and molecular characterization of all the CAV isolates.

During acute phases of the CIA especially in young chicks, CAV causes destructive effects on erythroid and myeloid tissues of bone marrow leading to suppression of differentiation and proliferation of haemopoietic precursor cells. This drastically affects erythropoiesis and myelopoiesis, leading to anaemia and (McNulty, 1991; Dhama et al., 2008). In the present study, a significant ($P < 0.05$) decline in the Hb, PCV and TEC was observed in the CAV positive groups. In the myeloid lineages also, a significant ($P < 0.05$) decline in the TLC and percent lymphocyte count was observed in all CAV positive birds as compared to the selected healthy group. Such haematological studies could be useful in determining the haematological effects of CAV in co-infections with other pathogens, for which purpose explorative studies are suggested.

It is well documented that AST, ALT and ALP are found in the liver and ALP is found in the kidney also (Kaneko et al., 1997). CAV has a wide spread distribution in the body resulting in damage and focal necrosis of liver, kidney and spleen. Haemorrhages in the proventricular mucosa and subcutaneous tissues and muscular haemorrhages within wing tips are sometimes associated with severe anaemia (Dhama et al., 2008; Yuasa et al., 1986; Engstrom et al., 1988). The present study demonstrated significant ($P < 0.05$) elevation in the activities of ALT, AST and ALP enzymes, and uric acid of CAV positive birds, which can be associated with the damage to the organs like liver, kidneys and muscles of CAV affected chicks. Elaborative studies in this direction could make understand the pathology and pathogenesis of the CIA more clearly.

CAV has been found to be associated with both clinical and sub-clinical infections and have substantial effects particularly on commercial broiler performance. Economic losses stem from increased mortality, condemnations, the cost of antibiotics used to control secondary bacterial infections due to immunosuppression, vaccine failures and poor production performance (McNulty, 1991; Dhama et al., 2008; McIlroy et al., 1992). In the present study, all the CAV positive flocks showed significantly lower value

of ND HI titres as compared to the normal healthy flocks. This outcome suggested that CAV is responsible for lower immune response against ND vaccination. The destructive effect of CAV due to suppression of the population of both helper ($CD4^+$) and cytotoxic ($CD8^+$) T-Lymphocytes in the thymus as suggested by Hu et al. (1993) and Adair (2000) could be the reason for poor but protective immune response in CAV inoculated chicks. The low antibody titer in CAV infected birds and marked depression of humoral immune response has also been reported by other (Bhatt et al., 2013; Cloud et al., 1992; Liu et al., 2001).

Overall, findings of the present study highlight the high prevalence of CAV in Indian poultry flocks with confirmation of its immunosuppressive role with other pathogens in field conditions. Furthermore, the active presence of CIA viral DNA in the tissue samples of diseased birds along with clinico-pathological and haemato-biochemical findings in the disease outbreaks investigated confirmed the involvement of CAV and its association with precipitation of other poultry diseases through its marked immunosuppressive potential. Though, this appears to be the first report from India indicating association of CAV in precipitating other secondary pathogens/diseases in commercial poultry flocks, additional studies are required to find out the subclinical infection of CAV in commercial poultry flocks by suitable diagnostic assays and its association with other immunosuppressive and opportunistic pathogens in causing significant economic losses to poultry population. The usage of full length viral genome based PCR-RFLP for studying molecular epidemiology of this emerging and economically important virus is the most significant finding of the present study. The recent reports regarding CAV related variants in human population and its potent role in precipitating other infectious diseases in birds further warrants in depth molecular epidemiological studies, antigenic and genomic characterization of viral isolates, so that an appropriate and effective strategy could be designed and adopted for the prevention and control of this important avian pathogen, and alleviate economic losses being suffered in poultry population.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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- Adair BM (2000). Immunopathogenesis of chicken anaemia virus infection. *Dev. Comp. Immunol.* 24: 247-255. [http://dx.doi.org/10.1016/S0145-305X\(99\)00076-2](http://dx.doi.org/10.1016/S0145-305X(99)00076-2)
- Basaraddi MS, Dhama K, Wani M, Sawant PM, Tiwari R, Tiwari R, Kumar D, Singh SD, Singh R (2013). Downregulation in cytokines profiles and immunopathological changes in chicks infected with chicken infectious anaemia virus. *Afr. J. Microbiol. Res.* 7(21): 2464-2474.
- Bhatt P, Shukla SK, Mahendran M, Dhama K, Chawak MM, Kataria JM (2011). Prevalence of chicken infectious anaemia virus (CAV) in commercial poultry flocks of Northern India: A serological survey. *Trans. Emerg. Dis.* 58: 458-460. <http://dx.doi.org/10.1111/j.1865-1682.2011.01215.x>
- Bhatt P, Shukla SK, Wani YM, Tiwari R, Dhama K (2013). Amelioration of chicken infectious anaemia virus induced immunosuppression by immunomodulator and haematinic supplementation in chicks. *Vet. Arhiv.* 83: 639-652.
- Biagini P, Bédarida S, Touinssi M, Galicher V, de Micco P (2013). Human gyrovirus in healthy blood donors, France. *Emerg. Infect. Dis.* 19(6): 1014-1015. <http://dx.doi.org/10.3201/eid1906.130228>
- Box PG, Holmes HC, Bushell AC, Finney PM (1988). Impaired response to killed Newcastle disease vaccine in chicken possessing circulating antibody to chicken anaemia agent. *Avian Pathol.* 17: 713-723. <http://dx.doi.org/10.1080/03079458808436488>
- Chowdhury SM, Omar AR, Aini I, Hair BM, Jamaluddin AA, Kono Y, Darus A, Yatim HM (2002). Isolation, identification and characterization of chicken anaemia virus in Malaysia. *J. Biochem. Mol. Biol. Biophys.* 6: 249-255.
- Cloud SS, Rosenberger JK, Lillehoj HS (1992). Immune dysfunction following infection with chicken anemia agent and infectious bursal disease virus. II. Alterations of *in vitro* lymphoproliferation and *in vivo* immune responses. *Vet. Immunol. Immunopathol.* 34: 353-366. [http://dx.doi.org/10.1016/0165-2427\(92\)90174-O](http://dx.doi.org/10.1016/0165-2427(92)90174-O)
- Connor TJ, McNeilly F, Firth GA, McNulty MS (1991). Biological characterisation of Australian isolates of chicken anaemia agent. *Aust. Vet. J.* 68: 199-201. <http://dx.doi.org/10.1111/j.1751-0813.1991.tb03192.x>
- Davidson I, Kedem M, Borochovit H, Kass N, Ayali G, Hamzani E, Perelman B, Smith B, Perk S (2004). Chicken Infectious anemia virus infection in Israeli commercial flocks: virus amplification, clinical signs, performance, and antibody status. *Avian Dis.* 48: 108-118. <http://dx.doi.org/10.1637/7072>
- Dhama K, Kataria JM, Senthilkumar N, Tomar S (2004). Differentiation of Indian isolates of chicken anaemia virus (CAV) by polymerase chain reaction (PCR)-restriction enzyme (RE) analysis. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 25: 75-79.
- Dhama K, Mahendran M, Somvanshi R, Chawak MM (2008). Chicken Infectious Anaemia Virus: an immunosuppressive pathogen of poultry - A review. *Indian J. Vet. Pathol.* 32(2): 158-167.
- Dhama K, Tiwari R, Khan RU, Chakraborty S, Gopi M, Karthik, K, Saminathan M, Desingu PA, Sunkara LT (2014). Growth promoters and novel feed additives improving poultry production and health, bioactive principles and beneficial applications: The trends and advances - A Review. *Int. J. Pharmacol.* 10(3): 129-159. <http://dx.doi.org/10.3923/ijp.2014.129.159>
- Dhama K, Saminathan M, Jacob SS, Singh M, Karthik K, Amarpal, Tiwari R, Sunkara LT, Malik YS, Singh RK (2015). Effect of immunomodulation and immunomodulatory agents on health with some bioactive principles, modes of action and potent biomedical applications. *Int. J. Pharmacol.* 11(4): 253-290. <http://dx.doi.org/10.3923/ijp.2015.253.290>
- Eltahir YM, Qian K, Jin W, Qin A (2011). Analysis of chicken anemia virus genome: evidence of intersubtype recombination. *Virology J.* 512-518. <http://dx.doi.org/10.1186/1743-422X-8-512>
- Engstrom BE, Fossum O, Luthman M (1998). Blue wing disease of chickens: experimental infection with a Swedish isolate of chicken anemia agent and an avian reovirus. *Avian Pathol.* 17: 33-50. <http://dx.doi.org/10.1080/03079458808436426>
- Hu LB, Lucio B, Schat KA (1993). Depletion of CD4+ and CD8+ T lymphocyte subpopulations by CIA-1, a chicken infectious anemia virus. *Avian Dis.* 37: 492-500. <http://dx.doi.org/10.2307/1591677>
- Imai K, Mase M, Tsukamoto K, Hihara H, Yuasa N (1999). Persistent infection with chicken anaemia virus and some effects of highly virulent infectious bursal disease virus infection on its persistency. *Res. Vet. Sci.* 67: 233-238. <http://dx.doi.org/10.1053/rvsc.1999.0313>
- Imai K, Mase M, Yamaguchi S, Yuasa N, Nakamura K (1998). Detection of chicken anaemia virus DNA from formalin-fixed tissues by polymerase chain reaction. *Res. Vet. Sci.* 64: 205-208. [http://dx.doi.org/10.1016/S0034-5288\(98\)90126-6](http://dx.doi.org/10.1016/S0034-5288(98)90126-6)
- Jiang SJ, Meng SS, Cui ZZ, Tian FL, Wang ZF (2005). Epidemic Investigation of co-infection of MDV, CAV and REV in spontaneous diseased chicken flocks in China. *Virology Sinica.* 20: 164-167.
- Jin WJ, Cui ZZ, Liu YL, Qin AJ (2001). Co-infection of MDV, CAV and REV in infectious bursal disease sample. *Chinese J. Vet. Sci.* 21: 6-9.
- Kaneko JJ, Harvey JW, Bruss ML (1997). Clinical biochemistry of domestic animals. Fifth edn. Academic Press.
- Kim HR, Kwon YK, Bae YC, Oem JK, Lee OS (2010). Molecular characterization of chicken infectious anemia viruses detected from breeder and broiler chickens in South Korea. *Poult. Sci.* 89: 2426-2431. <http://dx.doi.org/10.3382/ps.2010-00911>
- Latheef SK, Dhama K, Wani MY, Samad HA, Tiwari R, Singh SD (2013). Ameliorative effects of *Withania somnifera*, *Azadirachta indica*, *Tinospora cordifolia* and E care Se herbal preparations on chicken infectious anaemia virus induced haematological changes in chicks and their live body weights. *South Asian J. Exp. Biol.* 3(4): 172-182.
- Liu ZG, Zheng SM, Yang LP, Xu YB, Zhou ZY (2001). Study on immunoprotective efficacy and mechanism post Newcastle disease vaccination of chicken infected with chicken anemia virus. *Sci. Agri. Sinica.* 34: 81-83.
- McLlroy SG, McNulty MS, Bruce DW, Smyth JA, Goodall EA, Alcorn MJ (1992). Economic effects of clinical chicken anaemia agent infection on profitable broiler production. *Avian Dis.* 36: 566-574. <http://dx.doi.org/10.2307/1591750>
- McNeilly F, Smyth JA, Adair BM, McNulty MS (1995). Synergism between chicken anemia virus (CAV) and avian reovirus following dual infection of 1-day-old chicks by a natural route. *Avian Dis.* 39: 532-537. <http://dx.doi.org/10.2307/1591806>
- McNulty MS (1991). Chicken anemia agent: a

- review. *Avian Pathol.* 20: 187-203. <http://dx.doi.org/10.1080/03079459108418756>
- McNulty MS, Connor TJ, McNeilly F, McLoughlin MF, Kirkpatrick KS (1990). Preliminary characterisation of isolates of chicken anaemia agent from the United Kingdom. *Avian Pathol.* 19: 67-73. <http://dx.doi.org/10.1080/03079459008418657>
 - Miles M, Reddy M, Morgan W (2001). Coinfection of specific-pathogen-free chickens with Marek's disease virus (MDV) and chicken infectious anaemia virus: effects of MDV pathotype. *Avian Dis.* 45: 9-18. <http://dx.doi.org/10.2307/1593006>
 - Miller MM, Ealey KA, Oswald WB, Schat KA (2003). Detection of chicken anemia virus DNA in embryonal tissues and eggshell membranes. *Avian Dis.* 47: 662-671. <http://dx.doi.org/10.1637/7007>
 - Miller MM, Schat KA (2004). Chicken infectious anaemia virus: an example of the ultimate host-parasite relationship. *Avian Dis.* 48(4): 734-745. <http://dx.doi.org/10.1637/7271-090304R>
 - Natesan S, Kataria JM, Dhama K, Rahul S, Baradhvaj N (2006). Biological and molecular characterization of chicken anaemia virus isolates from Indian origin. *Virus Res.* 118(1-2): 78-86. <http://dx.doi.org/10.1016/j.virusres.2005.11.017>
 - Nayabian H, Mardani K (2013). Molecular characterisation of the chicken anemia viruses isolated from broiler farms of west Azerbaijan, Iran. *Avian Pathol.* 42(2): 108-113. <http://dx.doi.org/10.1080/03079457.2013.766668>
 - Noteborn MHM, Verschueren CAJ, Van Roozelaar DJ, Veldkamp S, Van Der Eb AJ, De Boer GF (1992). Detection of chicken anemia virus by DNA hybridization and polymerase chain reaction. *Avian Pathol.* 21: 107-118. <http://dx.doi.org/10.1080/03079459208418823>
 - Oluwayelu DO, Todd D, Ball NW, Scott AN, Oladele OA, Emikpe BO, Fagbohun OA, Owoade AA, Olaleye OD (2005). Isolation and preliminary characterization of chicken anemia virus from chickens in Nigeria. *Avian Dis.* 49: 446-450. <http://dx.doi.org/10.1637/7339-020705R.1>
 - Oluwayelu DO (2010). Diagnosis and epidemiology of chicken infectious anemia in Africa. *Afr. J. Biotechnol.* 9(14): 2043-2049.
 - Otaki Y, Nunoya T, Tajima M, Kato A, Nomura Y (1998). Depression of vaccinal immunity to Marek's disease by infection with chicken anaemia agent. *Avian Pathol.* 17: 333-47. <http://dx.doi.org/10.1080/03079458808436452>
 - Pope CR (1991). Chicken anaemia agent. *Vet. Immunol. Immunopathol.* 30: 51-65. [http://dx.doi.org/10.1016/0165-2427\(91\)90008-Z](http://dx.doi.org/10.1016/0165-2427(91)90008-Z)
 - Pringle CR (1999). Taxonomy at the XIth International Congress of Virology, Sydney, Australia. *Arch. Virol.* 144: 2065-2070. <http://dx.doi.org/10.1007/s007050050728>
 - Rosenberger JK, Cloud SS (1989). The effects of age, route of exposure, and co-infection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian Dis.* 33: 753-759. <http://dx.doi.org/10.2307/1591156>
 - Rozypl TL, Skeeles JK, Dash JK, Anderson EJ, Beasley JN (1997). Identification and partial characterization of Arkansas isolates of chicken anemia virus. *Avian Dis.* 41: 610-616. <http://dx.doi.org/10.2307/1592152>
 - Sambrook J, Fritsch EF, Maniatis TC (1989). *Molecular Cloning - A Laboratory Manual*, II Edn, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.
 - Sawant PM, Dhama K, Rawool DB, Wani MY, Tiwari R, Singh SD, Singh RK (2015). Development of a DNA vaccine for chicken infectious anemia and its immunogenicity studies using high mobility group box 1 protein as a novel immunoadjuvant indicated induction of promising protective immune responses. *Vaccine.* 33(2): 333-340. <http://dx.doi.org/10.1016/j.vaccine.2014.11.020>
 - Schat KA (2003). Chicken infectious anemia. In: *Diseases of Poultry*, 11th ed. (Editors) Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR and Swayne DE. Iowa State University Press, Ames, USA. Pp. 182-202.
 - Schat KA (2009). Chicken infectious anaemia. *Cur. Topics Microbiol. Immunol.* 331: 151-183.
 - Smuts HE (2014). Novel Gyroviruses, including chicken anaemia virus, in clinical and chicken samples from South Africa. *Adv. Virol.* 2014: 321284. <http://dx.doi.org/10.1155/2014/321284>
 - Snoeck CJ, Komoyo GF, Mbee BP, Nakouné E, Le Faou A, Okwen MP, Muller CP (2012). Epidemiology of chicken anemia virus in Central African Republic and Cameroon. *Virol. J.* 9: 189-204. <http://dx.doi.org/10.1186/1743-422X-9-189>
 - Spackman E, Cloud SS, Rosenberger JK (2002). Comparison of a putative second serotype of chicken infectious anemia virus with a prototypical isolate I. *Pathogenesis. Avian Dis.* 46: 945-955. [http://dx.doi.org/10.1637/0005-2086\(2002\)046\[0956:COAPSS\]2.0.CO;2](http://dx.doi.org/10.1637/0005-2086(2002)046[0956:COAPSS]2.0.CO;2)
 - Todd D, Mawhinney KA, McNulty MS (1992). Detection and differentiation of chicken anemia virus isolates by using the polymerase chain reaction. *J. Clin. Microbiol.* 30: 1661-1666.
 - Todd D, McNulty MS, Mankertz A, Lukert PD, Dale JL, Randles JW (2000). Family *Circoviridae* In: *Virus Taxonomy - Classification and Nomenclature of Viruses*. Seventh Report of the ICTV. F.A. Murphy et al. (Editors), Academic Press, New York.
 - Toro H, Gonzalez C, Creda L, Hess M, Reyes E, Geisse C (2000). Chicken anaemia virus and fowl adenoviruses: association to induce the inclusion body hepatitis/hydropericardium syndrome. *Avian Dis.* 44: 51-58. <http://dx.doi.org/10.2307/1592507>
 - Toro H, Gonzalez O, Escobar C, Cerda L, Morales MA, Gonzalez C (2001). Vertical induction of the inclusion body hepatitis/hydropericardium syndrome with fowl adenovirus and chicken anaemia virus. *Avian Dis.* 45: 215-222. <http://dx.doi.org/10.2307/1593031>
 - Venugopalan AT, Elankumaran S, Raj GD, Manohar BM, Thangavelu A (1994). Isolation of chicken anemia virus in Tamil Nadu. *Ind. Vet. J.* 71: 4-11.
 - Wani MY, Dhama K, Barathidasan R, Gowthaman V, Tiwari R, Bhatt P, Mahajan NK, Chawak MM, Singh SD, Kataria JM (2013). Molecular detection and epidemiology of chicken infectious anaemia virus in India. *South Asian J. Exp. Biol.* 3(4): 145-151.
 - Wani MY, Dhama K, Latheef SK, Singh, SD, Tiwari R (2014). Correlation between cytokine profile, antibody titre and viral load during subclinical chicken anaemia virus infection. *Veterinarni Medicina.* 59(1): 33-43.
 - Yuasa N, Imai K (1986). Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). *Avian Pathol.* 15: 639-645. <http://dx.doi.org/10.1080/03079458608436327>
 - Yuasa N, Taniguchi T, Noguchi T, Yoshida I (1980). Effect of infectious bursal disease virus infection on incidence of

- anemia by chicken anemia agent. *Avian Dis.* 24: 202-209. <http://dx.doi.org/10.2307/1589780>
- Zanella A, Caro R, Dall AP, Grilli G, Lavazza A, Lombardi G (1999). Interaction between Marek's disease and chicken infectious anaemia viruses. *Sel. Veterinaria.* 10: 727-742.
 - Zhang X, Wu B, Liu Y, Chen W, Dai Z, Bi Y, Xie Q (2015). Assessing the efficacy of an inactivated chicken anemia virus vaccine. *Vaccine.* 33(16): 1916-1922. <http://dx.doi.org/10.1016/j.vaccine.2015.02.066>