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Experimental Pathological Studies of an Indian Chicken Anaemia Virus Isolate and its Detection by PCR and FAT

[1]Mohd Yaqoob Wani, [2]Kuldeep Dhama, [1]Shyma K. Latheef, [2]Rajamani Barathidassan, [3]Ruchi Tiwari, [4]Sandip Chakraborty, [5]Milind Madhukar Chawak and [2]Shambhu Dayal Singh
[1]Immunology Section, Division of Veterinary Biotechnology,
[2]Avian Diseases Section, Division of Pathology, Faculty of Veterinary and Immunology,
Indian Veterinary Research Institute, Izatnagar, Bareilly Uttar Pradesh, 243122, India
[3]Department of Veterinary Microbiology and Immunology, College of Veterinary Sciences, Uttar Pradesh Pandit Deen Dayal Upadhayay Pashu Chikitsa Vigyan Vishwa Vidyalaya Evum Go-Anusandhan Sansthan (DUVASU), Mathura Uttar Pradesh, 281001, India
[4]Department of Animal Resources Development, Pt. Nehru Complex, 799006, Agartala, India
[5]Poultry Diagnostic Research Centre, Venkateshwara Hatcheries Private Limited,
Pune, 412201, Maharashtra, India

Abstract: Chicken Infectious Anaemia Virus (CIAV) is one of the potent immunosuppressive and economically important agents affecting poultry industry worldwide. Recent reports indicate the emergence of this virus in the poultry flocks of the country. The present study aimed to investigate the pathogenic potential of a recent isolate of CIAV obtained from poultry flock of Uttaranchal State, India. Twenty first day-old age Specific Pathogen Free (SPF) chicks were inoculated intramuscularly with $10^{4.5}$ median tissue culture infective dose ($TCID_{50}$) of CIAV passaged in the Marek's disease virus transformed chicken splenic T lymphocyte (MDCC-MSB1) cell line while 15 chicks were kept as control. The CIAV isolate produced consistent clinical signs, loss in body weight gain, anaemia, low haematocrit values, bone marrow aplasia and generalized lymphoid atrophy. Mean Packed Cell Volume (PCV) value of the infected chicks was significantly low (18.22±2.22) compared to control group (34.12±4.72) at 14 day post infection (dpi). The establishment of virus infection in chicks was confirmed both at molecular and antigenic levels by Polymerase Chain Reaction (PCR) and Indirect Immunofluorescent Test (IIFT), respectively. Characteristic apoptotic pattern was also detected in the affected organs and the virus was re-isolated successfully in MDCC-MSB1 cell cultures. The present results revealed that the virus circulating in poultry flocks of Uttaranchal state is both pathogenic and immunosuppressive in nature. Extensive epidemiological studies are suggested in the poultry flocks of the country along with adaptation of appropriate diagnostic, prevention and control strategies so as to prevent economic losses caused by this important poultry pathogen.

Key words: Chicken infectious anaemia virus, pathogenicity, immunosuppression, apoptosis, isolation, detection, PCR, FAT

INTRODUCTION

Chicken Infectious Anemia Virus (CIAV) is one of the most extensively studied viral pathogen of poultry and has gained much importance due to its an emerging and remerging nature (Schat, 2003; Dhama *et al*., 2002, 2008a; Bhatt *et al*., 2011a; Snoeck *et al*., 2012). It leads to major economic losses to the poultry industry due to its severe immunosuppressive potential, vaccine failures, production losses and increased mortality associated with multiple secondary infections (Todd, 2000; Schat, 2003; Natesan *et al*., 2006a). *Chicken Infectious Anemia Virus* (CIAV) is a single stranded naked DNA virus with circular genome, the smallest avian pathogen (23-25 nm size, 2.3 kb genome) and belongs to the family Circoviridae and the genus *Gyro virus* (Pringle, 1999). Chicken is the only recognized host of this virus. The disease, Chicken Infectious Anaemia (CIA), generally occurs in young chicks of upto 3-4 weeks of age and is characterized by increased mortality, poor weight gain, reduction in

Corresponding Author: Kuldeep Dhama, Avian Diseases Section, Division of Pathology, Faculty Veterinary Immunology, Indian Veterinary Research Institute, Izatnagar, Bareilly, 243122, Uttar Pradesh, India

haematocrit values, anaemia, aplasia of the bone marrow and generalized lymphoid atrophy (Pope, 1991; Schat, 2003; Oluwayelu, 2010). The CIAV infection shows notable characteristics such as vertical transmission, contamination of Specific Pathogen Free (SPF) eggs, highly contagious and hardy nature, ubiquitous distribution and the potential to produce marked immunosuppression (Yuasa, 1992; Schat, 2003; Miller and Schat, 2004; Dhama *et al*., 2008a).

The virus is worldwide in distribution and has been discovered from almost all poultry producing countries (Ducatez *et al*., 2006, 2008; Mohamed, 2010; Nayabian and Mardani, 2013). Recently, a very high serological incidence (86%) of CIAV infection in commercial poultry farms in the Northern region of India has been reported, indicating ubiquitous nature of the virus (Bhatt *et al*., 2011a). Though, CIAV isolates are serologically monotypic worldwide, variation in pathogenicity/severity of lesions has been reported among different stains (Natesan *et al*., 2006a). Apathogenic CIAV isolates have not been reported so far and also limited studies exist regarding the pathogenic potential of Indian field isolates (Dhama, 2002; Verma *et al*., 2005; Natesan *et al*., 2006a).

The existing literature indicates that genetically different CIAV strains are circulating in commercial poultry farms worldwide, including India (He *et al*., 2007; Hailemariam *et al*., 2008; Dos Santos *et al*., 2012). Hence, the current situation demands constant monitoring of this virus in the field to determine the emergence of new variants and consequent changes in their pathogenicity. So, the present study was carried out to find out the pathogenic potential of a recently isolated field isolate of CIAV from poultry flocks of Uttaranchal state, India. The techniques of Polymerase Chain Reaction (PCR) and Fluorescent Antibody Test (FAT) were employed to detect the establishment of virus infection in experimentally infected chicks. The virus was also re-isolated from the experimentally infected chicks employing Marek's disease virus transformed chicken splenic T lymphocyte (MDCC-MSB1) cell cultures.

MATERIALS AND METHODS

Virus isolate: An Indian field isolate of CIAV, isolated from Uttaranchal state and maintained in Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, was used for experimental infection purposes.

Experimental chicks: Embryonated Specific Pathogen Free (SPF) eggs (N = 40) were obtained from M/S Venkateshwara Hatcheries Private Limited (VHL), Pune, Maharashtra and were hatched in Hatchery Unit of Central Avian Research Institute, Izatnagar. The chicks were reared under strict isolation in the Experimental Animal Shed of Division of Pathology, Indian Veterinary Research Institute.

Experimental design: Day-old SPF chicks (n = 35) were randomly divided into two groups: Group A and Group B. Group A of 20 chicks was kept as CIAV infected and Group B of 15 chicks was kept as uninfected control. All the chicks of the Infected group were inoculated intramuscularly with $10^{4.5}$ median tissue culture infective dose ($TCID_{50}$) of the MDCC-MSB1 recovered Uttaranchal CIAV isolate, while the control chicks received phosphate buffered PBS as described previously (Dhama, 2002; Natesan *et al*., 2006a). All experimental procedures on animals were carried out according to the recommendations and approval of the Institute Animal Ethics Committee (IAEC) under the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Clinico-pathological studies: The CIAV infected Group A of chicks was observed regularly for the development of clinical signs of chicken infectious anaemia and the mortality, if any, was recorded. Simultaneously, Packed Cell Volume (PCV) (Campbell, 1995) and mean body weight gain for 10 chicks in each group were recorded weekly at 7, 14, 21 and 28 Days Post Infection (dpi) intervals. Five chicks from both the groups were sacrificed at 14 and 28 dpi for observing the gross lesions and histopathological changes in thymus, bone marrow, spleen, liver and bursa. The organs were collected in 10% formal saline for microscopic examination. The formalin fixed tissues, 4-5 μm thick, were routinely processed for H and E staining for observing histopathological lesions.

Virus detection by PCR and FAT: For confirmation of the establishment of CIAV infection, techniques of PCR and FAT were employed for detection of viral nucleic acid and antigen, respectively, in tissues collected from the experimentally infected chicks. Tissue samples of thymus, liver, bone marrow and spleen collected from the experimentally infected chicks at 14 dpi, were used for the CIAV detection at molecular level by PCR using VP3 gene specific primers (FP: atgaacgctctccaagaagata and RP: Cagtcttatacaccttcttgcg) of CIAV. Briefly, 25 mg of tissue sample was used for the isolation of DNA by DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufactures recommended protocol. Detection of CIAV at antigenic level was done by employing

fluorescent antibody test (FAT) as described previously (Dhama, 2002). Indirect immunofluorescent technique (IIFT) was employed for detecting viral specific antigen. Briefly, tissue sections (thymus, liver, spleen and impression smears of bone marrow) were fixed on clean microscopic slides by keeping in chilled acetone for 10 min, washed thrice in PBS and treated with 1:50 diluted anti-CIAV chicken serum. The slides were then incubated for 1 h at 37°C in a moist chamber, washed thrice with PBS, treated with 1:30 diluted anti-chicken FITC conjugate and incubated for 1 h at 37°C in a humidified atmosphere. Finally, the slides were again washed thrice with PBS, mounted with 50% glycerol saline and examined under the UV microscope (Nikon, Japan).

DNA fragmentation assay for apoptotic changes: The CIAV induced apoptosis in chicks of the infected group was determined by DNA fragmentation assay (laddering assay) as described previously (Jeurissen *et al*., 1992). In brief, 10 μL of each DNA sample extracted from the thymic tissues at 14 dpi from the infected chicks, were run in 2% agarose gel containing ethidium bromide (0.5 μg mL^{-1}) as tracking dye.

Virus re-isolation: The re-isolation of virus from the CIAV infected chicks was carried out in Marek's disease virus transformed chicken splenic T lymphocyte (MDCC-MSB1) cell line as described by Dhama (2002). Thymic tissues and bone marrow of the infected chicks were used for isolation of the virus. The presence of virus in the cell culture was confirmed both at antigenic and genomic level using PCR and IIFT as used earlier and described by Dhama (2002).

RESULTS

Clinico-pathological effects: Chicks of the group A infected with Uttaranchal isolate of CIAV showed acute course of disease with 100% morbidity and exhibited clinical signs of anorexia, anaemia and decrease in body weight gain at 14 dpi. A total mortality of 10% (2 out of 20 chicks died) was recorded in CIAV infected chicks during the experimentation. Uninfected chicks in group B remained healthy and no lesions and/or mortality were recorded during the course of study. The mean live body weights recorded in the experimental chicks showed significant reduction ($p<0.01$) in the infected group at 14, 21 and 28 dpi intervals as compared to respective control (group B) (Fig. 1a). The haematocrit values of the CIAV infected chicks (group A) decreased significantly ($p<0.05$) and mean PCV value was reduced to 18.22±2.22 at 14 dpi as compared to 34.12±4.72 in control group (Fig. 1b).

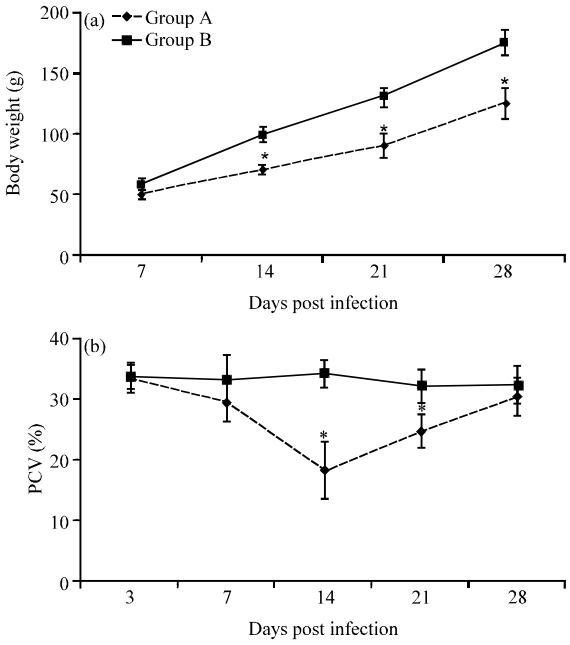

Fig. 1(a-b): Effect of CIAV (Chicken infectious Anaemia Virus) on body weight and PCV of experimentally infected chicks determined at various days post infection intervals (a) Body weights recorded of CIAV infected chicks (Group A) and uninfected control (Group B) chicks and (b) PCV (%) valued recorded of CIAV infected chicks (Group A) and uninfected control (Group B) chicks. Values are represented as Mean±SD; *$p< 0.05$

Gross lesions and histopathological changes: All the body organs viz., thymus, bone marrow, spleen, bursa and liver of the CIAV infected chicks (group A), sacrificed at 14 dpi, revealed typical gross lesions suggestive of CIA viz., pale and enlarged liver, atrophy of thymus, spleen and bursa along with pale to whitish bone marrow of the femur bone (Fig. 2a, b). The severity of gross lesion at 28 dpi was less compared to 14 dpi observations. All the chicks of the control group (B) appeared normal and healthy at all the intervals and no gross lesion was observed.

Histopathological studies reflected similar changes, both in intensity and severity in CIAV infected chicks compared to healthy control group as observed during gross pathology at 14 and 28 dpi. Microscopically, in the bone marrow marked depletion of haematopoietic cells including of both erythrocytic and granulocytic series was seen at 14 dpi in CIAV infected chicks with noticeable replacement by lipocytes. Histopathology of liver indicated moderate degeneration of hepatocytes, dilated

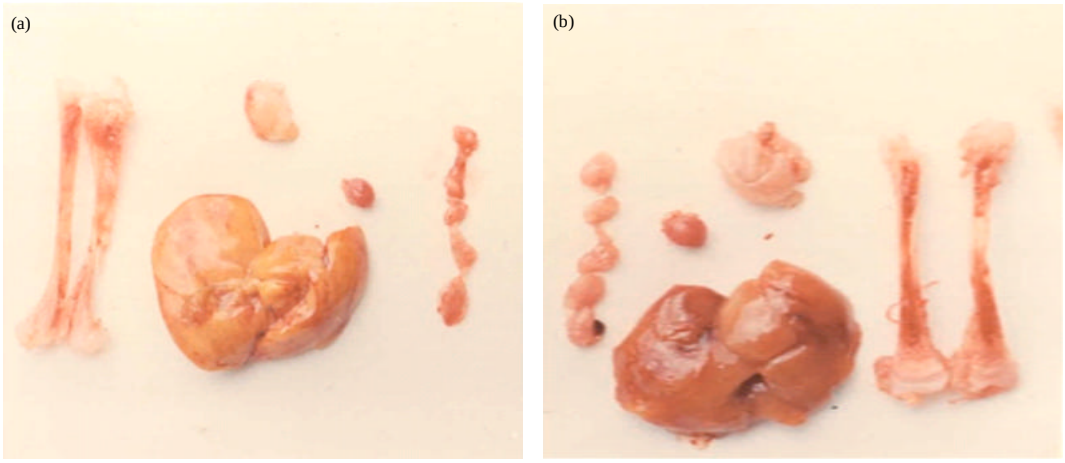

Fig. 2(a-b): Gross lesions in liver, bone marrow, spleen, bursa and thymus in chicks infected with CIAV (a) CIAV infected group and (b) Control group

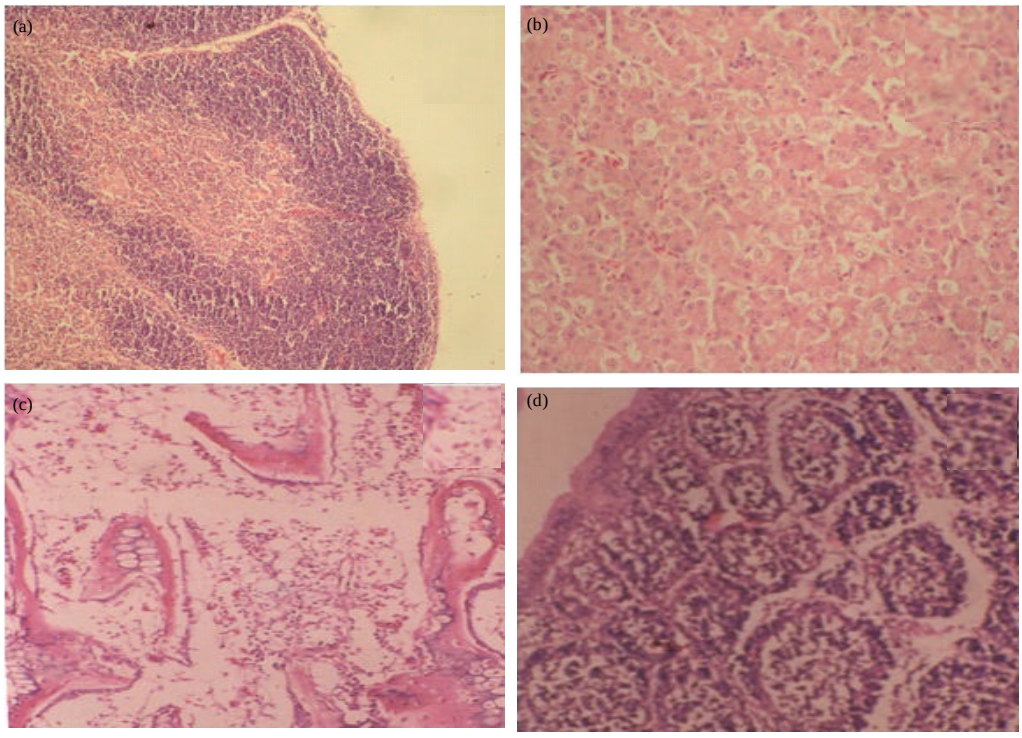

Fig. 3(a-d): Histopathological changes in the (a) Thymus, (b) Liver, (c) Bone marrow and (d) Bursa in the CIAV infected group at 14 dpi in experimentally infected chicks

blood capillaries and focal lymphoid aggregation. In the thymus and bursa, moderate atrophy and depletion of lymphocytes in cortex and medulla was observed at 14 dpi (Fig. 3a-d) and comparably less severe effects were observed at 28 dpi.

DNA fragmentation assay for apoptotic changes: The VP3 induced apoptosis in CIAV infection was determined in thymic tissues of the experimentally infected chicks by using DNA fragmentation assay both at 14 and 28 dpi. The thymic DNA showed pronounced nucleosomal

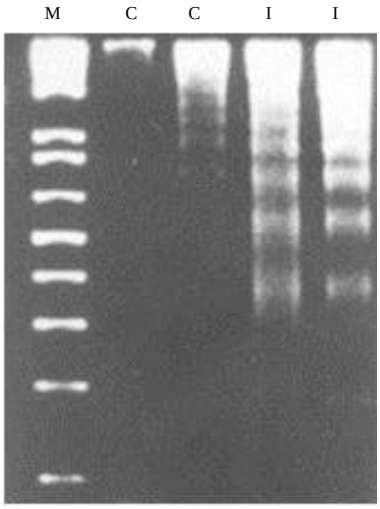

Fig. 4: Ladder pattern of thymic DNA indicating apotosis of lymphocytes. Genomic DNA from tissues of infected chicks (I), DNA from control chicks (C) and 1kb DNA Ladder (M)

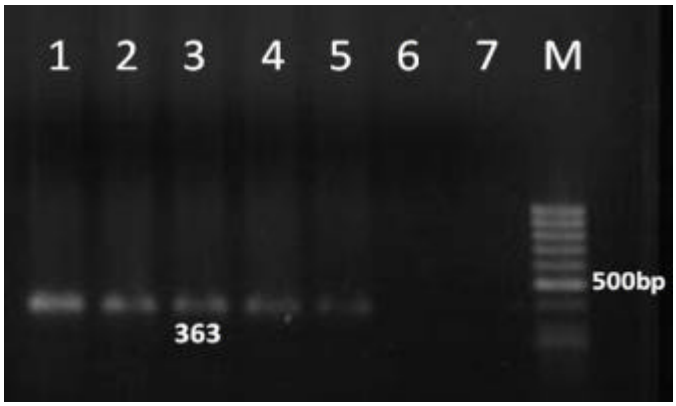

Fig. 5: Confirmation of CIAV infection in the tissues of experimentally infected chicks by PCR using VP3 gene specific primers at 14 dpi. Thymus, (1) Liver, (2) Bone marrow, (3) and Spleen, (4) Virus positive control, (5) Negative control, (6) Pooled tissues of control chick and (7) 100 bp DNA ladder (M)

laddering at 14 dpi compared to 28 dpi interval, while the tissue samples obtained from the chicks of control group did not show any DNA fragmentation (Fig. 4).

Virus detection by PCR and FAT: The expected amplicon of 368 bp size was obtained in PCR testing (using VP3 gene specific primers) of all the tissues (thymus, liver, bone marrow and spleen) obtained from the chicks of the infected groups as observed in the agarose gel electrophoresis (Fig. 5). All the tissue samples collected from control chicks were negative by PCR. Confirmation of the virus at antigenic level was done by IIFT staining using cryosections of thymus, liver, spleen and impression smears of bone marrow collected at 14 dpi, which gave the specific fluorescence. Characteristic intranuclear immunofluorescent (IF) antigen with fine fluorescent dotting (doughnut-shaped apoptotic bodies) pattern specific for CIAV was observed in all the collected tissues samples from infected birds. Tissues collected from control chicks (group-B) were negative for specific immunofluorescence (Fig. 6a-d).

Re-isolation of the virus in cell culture: The virus was re-isolated in MDCC-MSB1 cell cultures from the tissues of the experimentally infected chicks and was confirmed both at genomic and antigenic levels by PCR and IIFT tesing, respectively. Expected amplicon size of 363 bp was obtained during PCR amplification and the characteristic

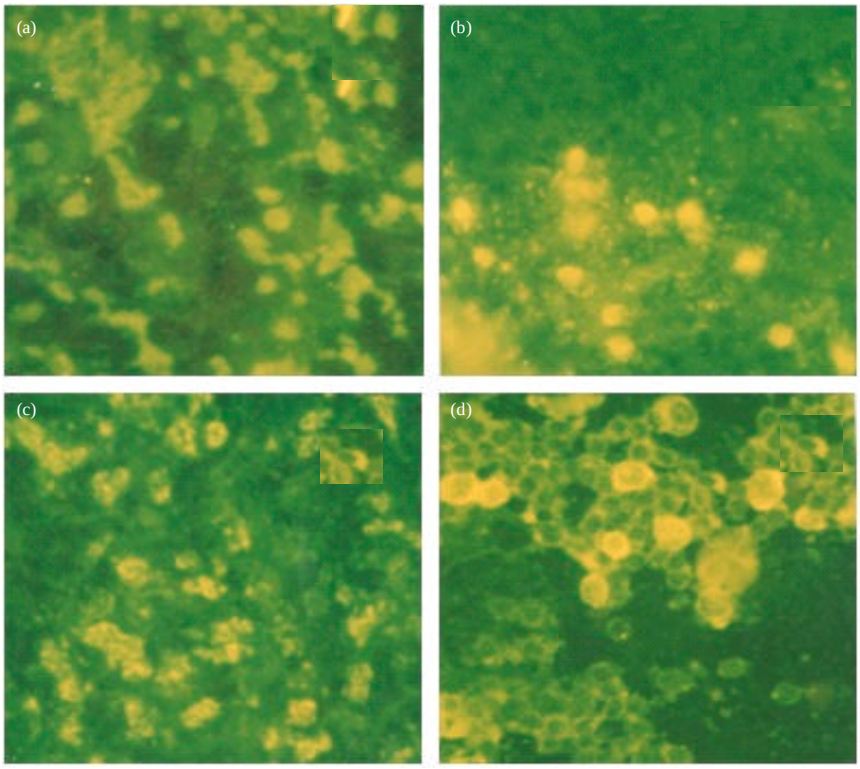

Fig. 6(a-d): Confirmation of CIAV at antigenic level in the, (a) Spleen, (b) Thymus, (c) Liver and (d) Bone marrow of the infected group as detected by IIFT at 14 dpi

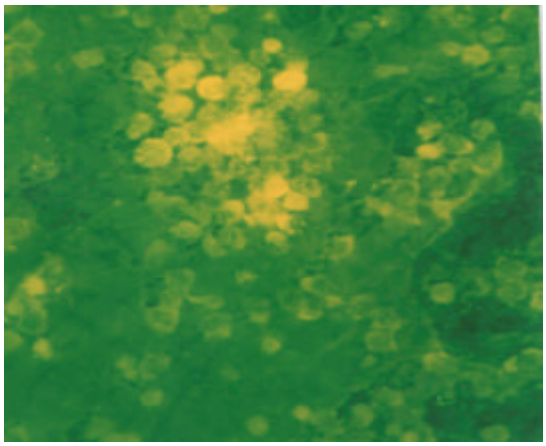

Fig. 7: Confirmation of CIAV at antigenic level by IIFT during re-isolation in the MDCC-MSB1 cell line from thymic tissue of the infected group

intranuclear immunofluorescent antigen with fine fluorescent dotting pattern specific for CIAV was obtained (Fig.7).

DISCUSSION

Infection with CIAV is of global level significance as it adversely affects the poultry health and production systems especially by increasing mortality pattern, immunosuppression leading to secondary bacterial complications and associated medication and vaccination failures to other viral diseases and also the production of SPF eggs (McNulty, 1991; Adair, 2000; Todd, 2000; Schat, 2003). In India, based on the virus detection, isolation and antibody detection in poultry flocks, the occurrence of CIAV has been reported from many states of the country, indicating that the virus is emerging and can cause economic losses to the poultry farmers and the industry (Kataria *et al*., 2005; Dhama *et al*., 2008a; Bhatt *et al*., 2011a). Only one serotype of the virus is said to exist worldwide, though, pathogenic variations among the different isolates have been observed (Connor *et al*., 1991; Oluwayelu *et al*., 2005; Natesan *et al*., 2006b; Kim *et al*., 2010). In this study, the pathogenic potential of the Indian field isolate of recent outbreak in Utarrakhand state was studied.

Specific clinical signs of CIAV infection are generally observed during first 3-4 weeks of age when susceptible chicks (lacking Maternally Derived Antibodies (MDA) or MDA has depleted from serum) are infected (McNulty, 1991; Hagood *et al*., 2000; Schat, 2003). In this study, similar age group of SPF chicks was used to simulate the natural conditions. It was found that the locally isolated strain produced the characteristic signs of CIA. The CIAV infected chicks showed signs of weakness, anorexia, ruffled feathers, anaemia (pallor) and stunted growth (Schat, 2003). There was decrease in haematocrit value (PCV < 20%) and body weight gain and 10% mortality rate was observed. All the lymphoid organs were atrophied, especially the thymus. Microscopic changes observed on histopathology included generalized lymphoid aplasia (lymphocyte depletion) in thymus, spleen and bursa. In bone marrow, hypoplasia or aplasia involving the haematopoietic cell lineages was observed and was in agreement with previous reports (Smyth *et al*., 1993; Liu *et al*., 1997; Dhama *et al*., 2003). Focal necrosis and vacuolar degeneration of the liver, as well as apoptosis in different organs were also noticed.

CIAV is believed to be a potent immunosuppressive agent as it causes marked damage both to stem cells in bone marrow and precursor T-lymphocytes in the thymus (Goryo *et al*., 1989a, b; Smyth *et al*., 1993). The immunosuppression caused by apoptosis induced by VP3 protein (apoptin) both in primary as well as in secondary lymphoid organs, thereby, damaging both the helper (CD4+) and cytotoxic (CD8+) T-lymphocytes due to its specific tropism for T-lymphocytes (Pope, 1991; Hu *et al*., 1993; Adair, 2000; Schat, 2003). In the present study, DNA fragmentation assay of the thymic tissue was carried out to confirm the apoptotic properties of Uttaranchal CIAV strain. It was found that the strain induced marked apoptosis of the lymphocytes in the thymus as observed by other workers previously. DNA fragmentation assay done in the present study revealed marked apoptosis of the lymphocytes in thymic tissue due to Uttaranchal viral strain as reported by other workers (Jeurissen *et al*., 1992; Natesan *et al*., 2006b; Basaraddi *et al*., 2013).

In the present study, confirmation of the establishment of the virus infection in the tissues of experimental chicks was carried out both at genetic level as well as at antigenic level using PCR and IIFT, respectively. CIAV was also re-isolated from the tissues of the experimental infected chicks employing cell culture. It was found that an expected amplicon of CIAV VP3 gene was amplified during PCR using gene specific primers. Demonstration of immunofluorescent antigen in CIAV infected tissues with appearance of characteristic fluorescent with doughnut bodies was in accordance with the reports of earlier workers (Brown *et al*., 2000; Dhama, 2002). Further, re-isolation of the virus using MDCC-MSB1 cell line gave final confirmation of the establishment of virus infection indicating that the pathology of the disease was indeed caused by CIAV used isolate.

In India, CIA has been suspected particularly after the emergence of virulent strains of infectious bursal disease virus and on the basis of clinical symptoms, lesions and its detection by immunoperoxidase test (Verma *et al*., 1981; Venugopalan *et al*., 1994). The disease has been confirmed from many states using virus isolation, PCR detection and serological studies (Kataria *et al*., 1999; Dhama *et al*., 2004; Natesan *et al*., 2006b). Recent serological report of Bhatt *et al*. (2011a) indicated that the virus is highly prevalent in the field conditions. Although, in adult birds CIAV does not cause clinical disease, subclinical infections usually occur and are responsible for economic losses (McNulty *et al*., 1991; Hoop, 1992; Hagood *et al*., 2000) and also acts as a source of infection for young birds. The immunosuppression induced by CIAV increases susceptibility to other pathogens, causes vaccination failures, aggravates the residual pathogenicity of avirulent strains and may have heterodynamic interactions with other viruses especially of oncogenic nature like Marek's disease virus (Todd, 2000; Haridy *et al*., 2012; Bhatt *et al*., 2013; Gowthaman *et al*., 2012). Advances in diagnosis and vaccinations along with appropriate disease prevention and control strategies need to be followed for this economically important and highly immunosusppressive pathogen (Dhama *et al*., 2008b, 2011, 2013a-c). Recently, herbs like ashwagandha, neem and guduchi have been found to be useful for prophylaxis and treatment of chicken infectious anaemia in chicks (Bhatt *et al*., 2011b; Mahima *et al*., 2012; Latheef *et al*., 2013). Other emerging and novel therapies including of virophages, cytokines and panchgavya elements have also been reported to be very effective against viral infections; these need to be explored for the treatment of CIAV in poultry (Dhama *et al*., 2013d, e, f, g; Tiwari *et al*., 2013).

Taken together, the results of the present study confirmed that the CIAV isolate from Uttaranchal state is highly pathogenic and produced anaemia, decrease in weight gain and characteristic lesions typical of CIA in almost all the inoculated chicks. Tissues from virus infected chicks were found positive for CIAV as confirmed by PCR and IIFT both at genetic and antigenic levels. The results of the present study supported the evidence of other workers about naturally occurring

isolates being pathogenic to day old susceptible chicks. Being an emerging pathogen circulating in the poultry flocks of the country, suitable prevention and control strategies including the development of cost effective pan side diagnostics and vaccines need to be explored.

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