Full Length Research Paper

Downregulation in cytokines profiles and immunopathological changes in chicks infected with chicken infectious anaemia virus

Manjunath Sangappa Basaraddi[1], Kuldeep Dhama[2]*, Mohd Yaqoob Wani[1], Pradeep Mahadev Sawant[1], Ruchi Tiwari[3], Deepak Kumar[4], Shambhu Dayal Singh[2] and Rajendra Singh[5]

[1]Immunology Section, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.)–243 122, India.
[2]Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.)-243 122, India.
[3]Department of Microbiology, College of Veterinary Sciences, UP Pandit Deen Dayal Upadhyaya Veterinary University (DUVASU), Mathura (U.P.)-281001, India.
[4]Division of Veterinary Biotechnology, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.)-243 122, India.
[5]Division of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.)-243 122, India.

The present study was designed to study cytokine profile and lymphocytic proliferation activity in chicken infectious anaemia virus (CIAV) infected chicks at various intervals of clinically susceptible age. Briefly, ninety specific pathogen free (SPF) chicks were divided into five experimental groups (A to E). Groups A to D were inoculated with 0.5 ml of $10^{4.5}$ TCID$_{50}$ of CIAV intramuscularly at 1, 14, 21 and 28-day-old, respectively, while group E served as uninfected control. Comparative cytokine gene expression levels of interleukin (IL)-1β, IL-10, IL-12β and granulocyte macrophage-colony stimulating factor (GM-CSF) were measured at 4 and 7 days post infection (dpi) by qRT-PCR. Also, lymphocytic proliferation activity was measured at 1, 2 and 3 week post infection (wpi) by lymphocyte transformation test (LTT). The down regulatory changes in cytokines and depressed cell mediated immune (CMI) responses were observed in chicks of all infected groups (A to D), which were significantly (p<0.05) low as compared to control group. Higher intensities and more pronounced effects on cytokine downregulation and blastogenic responses of lymphocytes were seen at younger ages. Apoptosis in thymic DNA as detected by nucleosomal fragmentation was highest on 7 and 10 dpi and lowest on 14 dpi, which correlated with down regulated cytokine profile in all infected groups. The development of clinical signs and lesions along with PCR testing confirmed the establishment of CIAV infection in experimental chicks. Increased apoptosis, decreased PCV, depletion of lymphocytes, down regulated cytokine profile and reduced lymphocyte activity explain involvement of thymic and hematopoietic precursor cells leading to CMI suppression caused by CIAV. Altogether, immunopathological changes were more severe in chicks infected at younger ages as compared to later ages of the clinically susceptible period. However, further studies are suggested regarding the impact of viral load on the various cytokine profiles and revealing more about immunopathogenesis of CIAV both in young and adult birds.

Key words: Chicken infectious anemia virus, cell mediated immune response, polymerase chain reaction (PCR), qRT-PCR, cytokine, apoptosis.

INTRODUCTION

Chicken Infectious Anemia (CIA) is an economically important, immunosuppressive disease especially of young chicks, characterized by severe anemia, aplasia of bone marrow and generalized lymphoid atrophy (Schat, 2003; Dhama et al., 2008). The disease is transmitted both horizontally and vertically and has worldwide distribution affecting both layer and broiler industries (Singh et al., 1996; Dhama et al., 2008; Schat, 2009; Hegazy et al., 2010; Oluwayelu, 2010; Bhatt et al., 2011; Yassir et al., 2011 Gholami-Ahangaran and Zia-Jahromi, 2012; Snoeck et al., 2012). The disease affects poultry industry through increased mortality, production losses, complication associated with immunosuppresssion like secondary bacterial infections and vaccine failures, addition medical costs and adversely affecting production of specific pathogen free (SPF) birds and eggs (Hagood et al., 2000; Todd, 2000). Various studies have shown that CIAV is an important co-factor for a number of poultry diseases like infectious bursal disease (IBD), Marek's disease (MD) and leads to their vaccine failures (Bülow et al., 1986; Otaki et al., 1988a, 1988b).

The disease is caused by chicken infectious anemia virus (CIAV) that belongs to genus *Gyrovirus* of family *Circoviridae* (Pringle, 1999). CIAV is a non-enveloped, icosahedral DNA virus and is one of the smallest known avian pathogen with 23 to 25 nm diameter (Pringle, 1999; Schat, 2009). The virus has a 2.3 kb single stranded circular genome which encodes VP1 (the major capsid protein), VP2 (scaffold protein) and VP3 (apoptin) which causes p53 independent apoptosis in hematopoietic and thymic precursor cells (Schat, 2003, 2009). The virus has gained much importance due to unique features like highly contagious and hardy nature, vertical transmission and immunosuppressive potential. Due to these features, the virus has been placed in the list of emerging viruses that can cause severe threat to the Indian poultry industry (Bhatt et al., 2011).

CIAV being a potent immunosuppressive agent affects both cellular and humoral immune functions of the central as well as peripheral immune organs leading to decreased immunoprotective efficacies (Adair, 2000; Dhama, 2002; Hoerr, 2010). The virus has tropism for haematopoietic and thymic precursors (Noteborn and Koch, 1995; Scott et al., 1999). Earlier reports indicated that CIAV infection specifically causes severe defects in splenic T-lymphocyte functions leading to decreased responsiveness to non-specific mitogens like phytohema-gglutinin, concanavalin A, and fall in interleukin pro-duction (Adair et al., 1991). Macrophage concentration and functions are also severely reduced after *in vivo* or *in vitro* exposure to the virus (Cloud et al., 1992; McConnell et al., 1993a, 1993b). The virus has been found to be associated with the impaired generation of concurrent pathogen-specific cytotoxic T lymphocytes (Markowski and Schat, 2003). However, the cytokine responses and their role in the pathogenesis of CIAV have not been extensively studied yet. Therefore, the present study was designed with the aim to determine the effect of CIAV infection on cell mediated immune responses like blastogenic responses and cytokines viz., interlukin (IL)-1β, IL-10, IL-12β and granulocyte macrophage-colony stimulating factor (GM-CSF), and apoptosis in chicks infected with the virus at various intervals of their clinically susceptible period.

MATERIALS AND METHODS

Virus and experimental chicks

Indian field isolate of CIAV (strain A; accession No. AY583755) maintained in Avian Diseases Section, Indian Veterinary Research Institute, Izatnagar, was used for experimental infection purposes. The SPF chickens obtained from M/S Venkateshwara Hatcheries Group Limited (VHL), Pune, Maharashta, were reared in the infection and control sheds of Avian Disease Section, Division of Pathology, under strict isolated conditions. Feed and water were provided *ad libitum*. 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 by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental design

The SPF chickens (n = 90) were randomly divided into five groups (A to E) with 15 chicks each in Groups A to D and 30 in control Group E. All the chicks of Groups A to D were inoculated intramuscularly (i/m) with 0.5 ml of $10^{4.5}$ $TCID_{50}$ Marek's disease virus transformed -MSB1 cell culture passaged CIAV isolate as described previously (Natesan et al., 2006) at 1, 14, 21 and 28-day-old, respectively (Table 1). Chicks of the group E were kept as uninfected control, administered with 0.5 ml PBS i/m. Chicks of all the groups were regularly monitored for clinical signs of the disease and mortality, and cell mediated immune (CMI) responses at different intervals post infection by studying cytokine expression profile, lymphocyte transformation test (LTT), along with confirmation of virus infection by observation of haematological, gross, microscopic and apoptotic changes and polymerase chain reaction (PCR) testing.

Cell mediated immune response studies in CIAV infected chicks

PBMC separation and RNA extraction

For cytokine studies in CIAV infected chicks, peripheral blood mononuclear cells (PBMCs) were collected from three chicks of each group on 4 and 7 days post infection (dpi) as described previously (Kaiser et al., 2006) with slight modification. PBMCs

*Corresponding author. E-mail: kdhama@rediffmail.com. Tel: +91-581-2310074 or +919837654996. Fax: 0091-581-2303284 or 2302179.

Table 1. Experimental design.

Groups	Number of SPF chicks	Age of CIAV infection (days) (I/M)	Day of examination
A	15	1st day	4th day PI 7th day PI
B	15	14th day	4th day PI 7th day PI
C	15	21st day	4th day PI 7th day PI
D	15	28th day	4th day PI 7th day PI
E	30	Uninfected control	Same day with corresponding groups

I/M: Intramuscularly; PI: post inoculation.

Table 2. List of primers used for amplification of different genes by real time PCR.

Gene	Primer sequence	Product (bp)	Annealing Temp. (°C)	Accession no. of gene	Reference (if any)
β-actin	F 5' GCA CCA CAC TTT CTA CAA TGA G 3' R 5' ACG ACC AGA GGC ATA CAG G 3'	184	59	NM-205518	Self designed
IL-1β	F 5' AAC ATC GCC ACC TAC AAG 3' R 5' GAC GGT AAT GAA ACA TAA ACG 3'	185	55	NM-204524	Self designed
IL-10	F 5' CAT GCT GCT GGG CCT GAA 3' R 5' CGT CTC CTT GAT CTG CTT GAT G 3'	90	52	AJ-621735	Rothwell et al. (2004)
IL-12β (p40)	F 5' TGC CCA GTG CCA GAA GGA AAA C 3' R 5' CTT CGG TGT GCT CCA GGT CTT G 3'	214	58	NM-213571	Self designed
GM-CSF	F 5' CGC CCA CCA CAA CAT ACT C 3' R 5' ACG ATT CCG CTT TCT TCC T 3'	202	55	AJ-621740	Self designed

were also collected for CMI studies at 1st, 2nd and 3rd week post infection (wpi) from five chicks of each group. Briefly, about 3 ml blood samples were collected, diluted with equal volume of sterile PBS (pH 7.2), over-layered on 3 ml of histopaque (1.077 g/ml, Sigma Co., USA) and centrifuged at 400 ×g for 30 min. The gradient interface of PBMCs were collected and washed twice with PBS by centrifugation at 100 ×g for 10 min and finally resuspended in 1 ml of Trizol (Invitrogen, Carlsbad, CA). The resulting Trizol-PBMC mixture was incubated at room temperature for complete cell lysis. RNA was extracted using chloroform for aqueous phase separation, precipitated with isopropanol, washed with 75% ethanol, dried at 37°C and finally resuspended in RNase-free water.

Complementary DNA (cDNA) synthesis

The total RNA of the PBMCs obtained from the experimental chicks was reverse transcribed for cDNA synthesis using M-MLV RT enzyme (M1705, Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, 2 µg of total RNA and 0.5 µg of random hexamer primer were taken in 15 µl nuclease free water, incubated for 5 min at 70°C then chilled on ice. Resulting reaction mixture was combined with 5 µl of M-MLV 5X Reaction Buffer, 10 mM dNTP mixture, 40 U/µl of RNasin®, 200 IU of M-MLV

RT and RNase-free water to final volume of 25 µl. The reaction mixture was incubated at 37°C for 1 h, and the reaction was finally stopped by heating at 72°C for 10 min in the thermal cycler (MJ Research, San Gabriel, USA). The prepared cDNAs were checked by PCR using housekeeping gene, chicken β-actin specific primers (Table 2) and stored at -20°C until used.

Quantification of cytokines by real time PCR

For quantifying of the expression levels of cytokines (IL-1β, IL-10, IL-12β and GM-CSF), the cDNA samples of the respective PBMCs were subjected to real time PCR analysis using gene specific primers. The cDNAs prepared were run in duplicate sets on STRATAGENE Q-CYCLER with MX 3000P software using SYBR green master mix (QuantiFast SYBR Green PCR Master Mix, Qiagen, USA). The reaction mixture consisted of 12.5 µl of SYBR green master mix, 3 pmole each of gene specific forward and reverse primers, 100 ng of c-DNA and nuclease free water to 25 µl along with at least one non-template control. The quantitative SYBR green master mix contained hot start Taq DNA polymerase, hence the real-time PCR reaction started with initial incubation at 95°C for 5 min followed by 35 cycles of amplification with denaturation at 95°C for 10 s, combined annealing and extension at 60°C for 30 s

for all genes. The specificity of the target gene specific product was confirmed by dissociation curve generated by the software at annealing temperature through 95°C. Primers sets used in this study were either previously published primer sequences or were self designed (Table 2). Data analysis was performed by $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001), and presented as the mean relative fold change in gene expression normalized to an endogenous reference gene and relative to uninfected control.

Lymphocytic transformation test (LTT)

Lymphocytic transformation test (LTT) was conducted as described previously (Bounous et al., 1992) with slight modification. Briefly, PBMCs were separated from five chicks of each group at 1st, 2nd and 3rd wpi as mentioned above, and collected in RPMI-1640 growth media (without phenol red, with $NaHCO_3$, Sigma, USA) to which 0.25 g/100 ml HEPES, 100 IU/ml of benzyl penicillin and 100 µg/ml of streptomycin sulphate were added. Viability of the cells was ascertained by trypan blue dye exclusion method and the cells were adjusted to give 5×10^6 viable cells/ml and platted in 96-well flat-bottomed tissue culture plate (Nunclon, Denmark). Cells were stimulated (10 µg ml) either with Con-A (Sigma-Aldrich, St. Louis, Missouri, USA) or CIAV antigen (whole purified CIAV-A inactivated virus) dissolved in RPMI in triplicate wells along with unstimulated control wells. The plates were incubated at 37°C in a humidified chamber at 5% CO_2 tension. After 72 h, 20 µl (5 mg/ml) of MTT (3-[4, 5-Dimethyl thiazolyl]-2, 5-diphenyl tetrazolium bromide) solution was added to all the wells and incubated further for 4 h, following which the plate was centrifuged at 1200 rpm for 15 min. The supernatant was removed carefully from all the wells. The MTT formazan was extracted from the cells, using DMSO (150 µl/well). The optical densities (OD) of wells were measured at a test wavelength of 510 nm and a reference wavelength of 650 nm. Stimulation index (SI) was calculated by dividing the OD value of stimulated with the OD of unstimulated.

Confirmation of CIAV infection in chicks

Clinical, haematological, gross and histopathological changes

Chicks of all the groups (A to D) were observed for clinical signs and mortality, if any along with similar examination in the control (Group E) chicks. At 12 dpi, 1 ml of blood was collected from three chicks of each group for estimation of packed cell volume (PCV) using Wintrobe's macrohaematocrit method (Wintrobe, 1967). Wintrobe tubes were filled with blood with the help of long needled syringe to avoid air bubbles and centrifuged at 900 xg for 45 min to determine the PCV (%) values. For observation of gross and histopathological lesions, three chicks from each groups were sacrificed at 12th dpi and lesions recorded in various organs. Tissue samples (about 10 × 5 mm thick pieces) of thymus, liver, spleen and bursa were collected in 10% neutral buffered formalin and processed for histopathological examination following standard protocol and Haematoxylin and Eosin (H&E) staining.

Detection of CIAV using PCR

PCR was employed for confirmation of the establishment of CIAV infection in experimentally inoculated chicks. Total DNA was isolated from the pooled tissues of liver, thymus, spleen and bone marrow of at least three birds from each group sacrificed at 12 dpi, using DNeasy® Blood and Tissue Kit (QIAGEN, Germany) following manufacturer's instructions. The eluted DNA samples were stored at -20°C until used. PCR amplification of VP2 was

performed using gene specific primers (F:ATGCACGGGAACGGCGGAC and R: TCACACTATACGTACCGGGG). Briefly to 12.5 µl of 2×PCR master mix, 10 pmol/µl of each primer and 1µg of DNA was added using nuclease free water to the final volume of 25 µl in an automatic thermal cycler (PTC 200 MJ Research, USA). A known CIAV DNA and DNA isolated from control birds were used as positive control and experimental negative control, respectively.

DNA fragmentation assay for apoptotic changes

For determining CIAV induced apoptosis in infected chicks, DNA fragmentation assay (laddering assay) was performed using the DNA extracted from the thymus of chicks of all five groups (A-E) at an interval of 7, 10 and 14 days post infection (DPI). 10 µl of each DNA sample was analyzed in 2% agarose gel containing ethidium bromide (0.5 µg/ml) as described previously (Jeurissen et al., 1992).

Statistical analysis

All the data are given as mean ± SD. Different experimental groups were compared by ANOVA followed by a post hoc Tukey's test using SPSS v.16.0 statistical software, and values with $p \leq 0.05$ were considered statistically significant.

RESULTS

Quantitation of cytokine gene expression by real time PCR

The cytokine gene expression levels in CIAV infected chicks of all the groups were determined at 4 and 7 dpi. CIAV was found to down regulate the mRNA expression levels of IL-1β, IL-10, IL-12β and GM-CSF, significantly ($p<0.05$) in all infective groups at both intervals as compared to control group. The highest fold change was observed in IL-1β m-RNA expression level in infected chicks of group A (4924.8 fold) as compared to Group B (1060), Group C (2369) and Group D (596) at 4 dpi.

At 7 dpi, all CIAV infected groups showed similar decrease in IL-1β levels (~5000). Downregulatory change in IL-10 expression levels was greater at 4 dpi which was decreased by 33.11, 17.63, 13.90 and 6.75 folds in groups A to D, respectively, as compared to 7 dpi levels decreasing only by 16.31, 3.53, 2.98 and 2.73 folds, respectively. IL-12β expression profile was also downregulated like IL-10 and was more pronounced at 4 dpi, while GM-CSF followed the pattern of IL-1β. However, the effect of CIAV infection on IL-12β and GM-CSF as compared to IL-1β was very low (Figure 1).

Lymphocytic proliferation assay

The blastogenic responses of lymphocytes stimulated with non-specific (Con A) and specific (CIAV antigen) antigen were significantly ($p<0.05$) low in chicks of CIAV infected groups as compared to control group at all the three intervals of 1st, 2nd and 3rd wpi (Figure 2). The SI
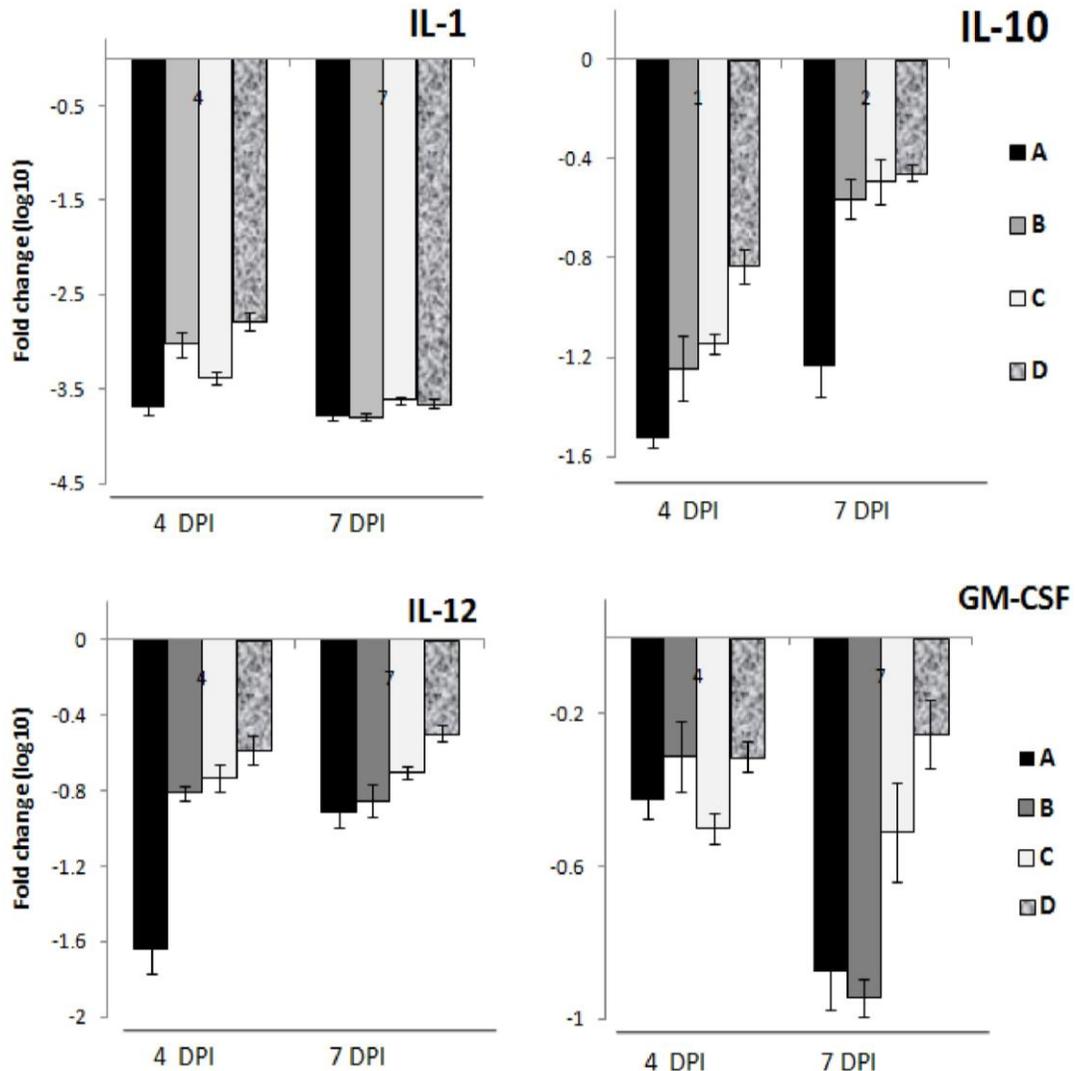
Figure 1. Relative fold change in PBMCs mRNA expression of cytokine levels (IL-1β, IL-10, IL-12β and GM-CSF) in CIAV infected chicks (Groups A to D) at 4 and 7 dpi as determined by quantitative real time PCR.
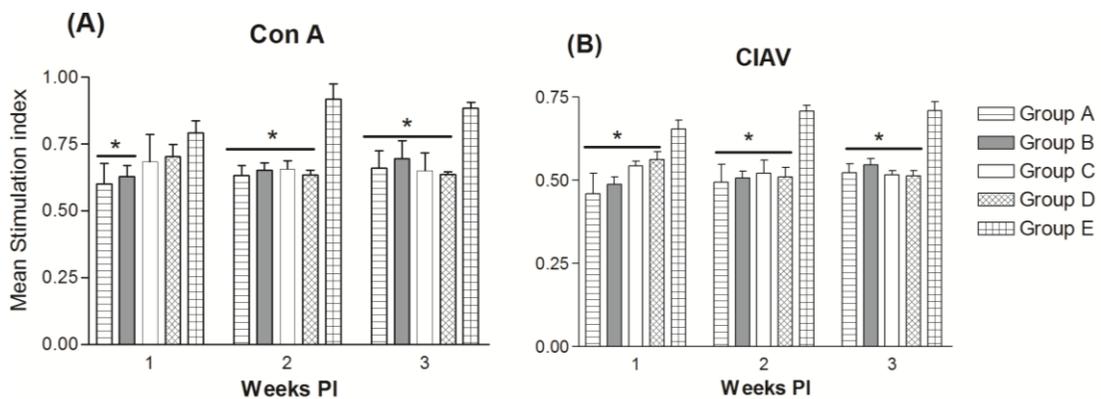
Figure 2. Blastogenic responses of peripheral blood mononuclear cells (PBMCs) of the chicks (Groups A to E) at different intervals of post infection to the non specific antigen ConA (A) and specific antigen (B) stimulation as determined by LTT using MTT dye. Values are represented as mean ± 1SD; * p ≤ 0.05.
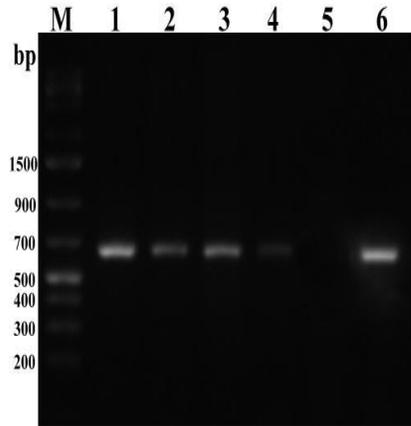
Figure 3. Amplified PCR products of DNA extracted from pooled tissues (liver, thymus, spleen and bone marrow) of CIAV infected (Groups A to D) and control (Group E) chicks on 12th dpi using VP2 gene specific primers. Lane M: 1 kb DNA ladder; Lanes 1 to 4: VP2 (651 bp) amplified product from Groups A to D chicks, respectively; Lane 5: uninfected control (group E); Lane 6: positive control (known CIAV DNA).

values ranged from 0.459 ± 0.062 to 0.563 ± 0.023 and 0.601 ± 0.076 to 0.702 ± 0.046 in infected groups as compared to 0.653 ± 0.027 to 0.710 ± 0.025 and 0.792 ± 0.045 to 0.917 ± 0.056 in control (Group E) on stimulation with CIAV antigen and Con A antigens, respectively. Higher SI values were observed on Con A stimulation as compared to stimulation CIAV antigen both in infected and control groups at all post infection intervals.

Clinical, hematological, gross and histopathological changes

Chicks of the CIAV infected groups (A to D) showed clinical signs of CIA viz., weakness, anorexia with ruffled feathers, anemic (pallor), and stunted growth, which were more pronounced in chicks infected at 1 and 14-day-old as compared to chicks infected at 21–day-old, and were lower in chicks infected at 28-day-old. The PCV (%) values in all the CIAV infected chicks were significantly low as compared to that of uninfected healthy control (26.67 ± 0.88) at 12 dpi. Among the infected groups, the mean PCV value was lowest (8 ± 0.58) in Group A chicks inoculated on 1st day of age and it showed increasing trends in Group B (11 ± 0.57), Group C (16.33 ± 0.88) and Group D (20 ± 0.58) inoculated at 14th, 21st and 28th day, respectively (Figure S1).

The gross lesions observed in various organs like thymus, liver, spleen, bone marrow and bursa of CIAV infected chicks (Group A to D) at 12 dpi included thymus

atrophy, whitish pale bone marrow, mild to moderately atrophied spleen and bursa, and pale liver (Figure S2). The lesions were more intense and characteristic in chicks inoculated at 1st and 14th days of age (Group A and B) as compared to those inoculated at 21st and 28th day (Group C and D). Histopathological changes were also similarly reflecting the gross lesions in both intensity and severity in chicks infected at various age intervals. Histopathology of liver indicated mild degeneration of hepatocytes, dilated blood capillaries and focal lymphoid aggregation. In thymus and bursa, mild to moderate atrophy and depletion of lymphocytes in cortex and medulla was observed (Figure 6). The microscopic lesions were less severe in chicks of Group C than that of Groups A and B, and were least in Group D. All the chicks of uninfected control Group E remained healthy throughout the experimental period and did not show any signs and lesions of CIA.

CIAV detection by PCR

PCR testing of the pooled tissues (liver, thymus, spleen and bone marrow) from chicks of CIAV inoculated groups (A to D) were found positive for viral DNA at 12 dpi. The CIAV VP2 specific PCR showed a distinct amplicon of 651 bp in 1% agarose gel electrophoresis. No such amplification from samples of uninfected control chicks (Group E) was observed (Figure 3).

Apoptotic changes

The cell death due to VP3 induced apoptosis caused by CIAV infection was investigated by testing thymic tissues for apoptotic changes in chicks of Groups A to D. The thymic DNAs showed nucleosomal laddering at all the three time intervals of 7, 10, 14 dpi only in chicks of Groups A to D. This laddering pattern was more marked and highest at 7 and 10 dpi and was lower at 14 dpi (Figures 4 and 5). This laddering pattern was lower in the chicks inoculated at 28 days of age (Group D).

DISCUSSION

CIAV is an important pathogen that poses threat to the poultry industry worldwide. It causes immuno-suppression, directly by severe depletion of lymphocytes from primary and secondary lymphoid organs (Taniguchi et al., 1982), or indirectly by participating with other immunosuppressive viruses (Bülow et al., 1986; Cloud et al., 1992). For the control of viral infections, cell mediated response is regarded as an integral and important component of immune system and it mainly involves various cytokines in comparison with the humoral immune response which acts through
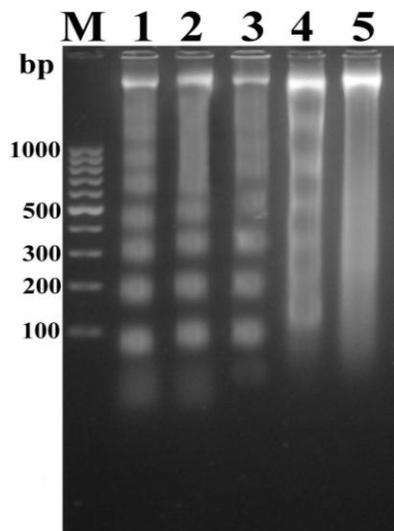
Figure 4. DNA laddering/fragmentation assay for the DNA extracted from thymus of CIAV infected and control chicks at 7 dpi. Lane M: 100 bp DNA ladder; Lanes 1 to 4: DNA extracted from chicks of Groups A to D, respectively; Lane 5: uninfected control (Group E).
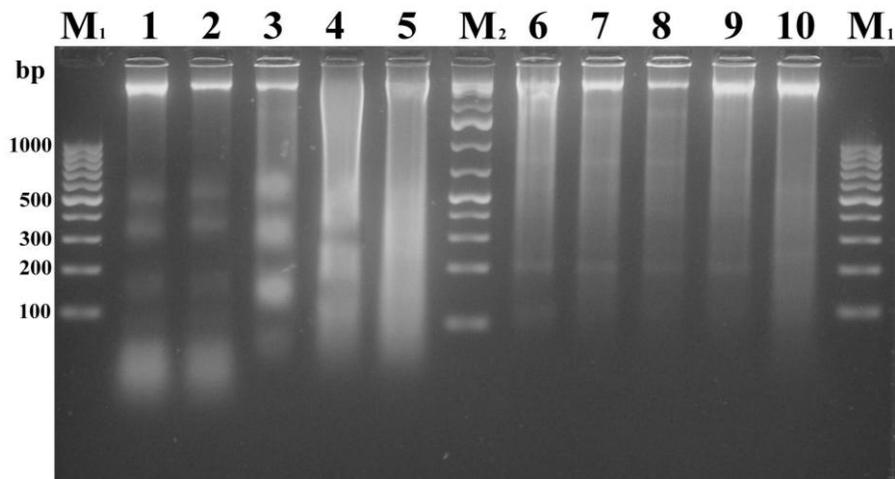
Figure 5. DNA laddering/fragmentation assay for the DNA extracted from thymus of CIAV infected and control chicks. Lane M_1: 100 bp DNA ladder; Lane M_2: 1 kb ladder; Lanes 1 to 4: DNA extracted from chicks of Groups A to D at 10 dpi; Lanes 6 to 9: DNA extracted from chicks of Group A to D at 14 dpi; Lanes 5 and 10: uninfected control (Group E).

antibodies. However, the effect of CIAV infection on various cytokines has not been well studied in detail. The data presented in this work showed that CIAV has marked effect in down regulation of IL-1β, IL-10, IL-12 and GM-CSF in virus infected chicks. The down regulation of cytokines was more pronounced in chicks infected at 1st

and 14th days of age as compared to chicks inoculated at 21st and 28th days of age, supporting the well established fact that chicks are more susceptible to disease during their first two weeks of age. Previously, it was reported that natural exposure of chickens to CIAV resulted in impaired functional activity and reduction of
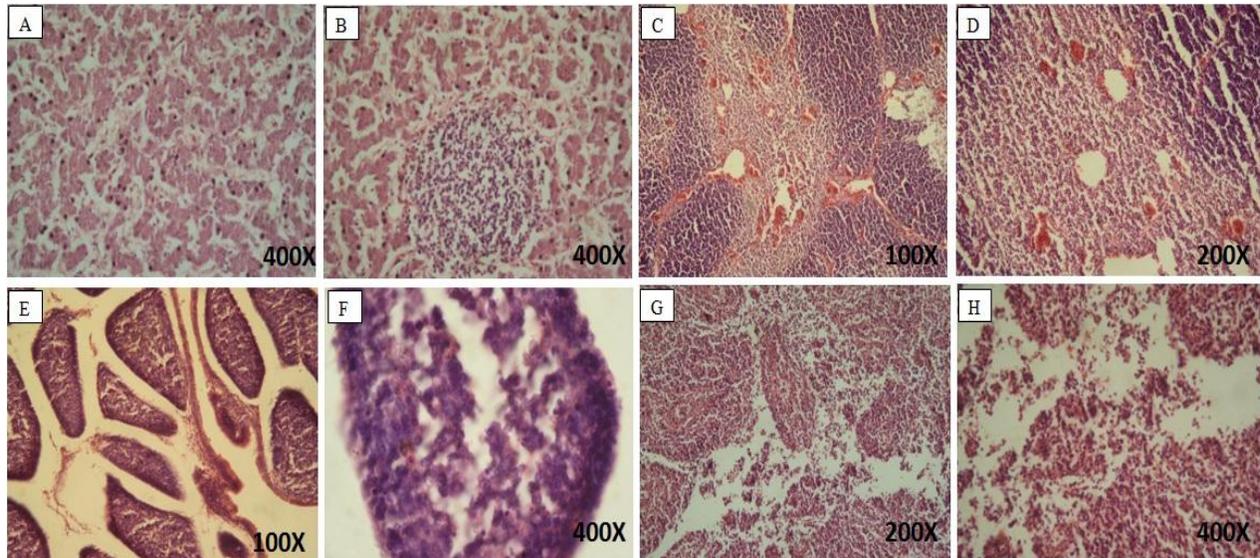
Figure 6. Histopathological changes in various tissues from the CIAV infected chicks at 12 dpi. Mild degeneration and focal lymphoid aggregation in liver (A and B), mild depletion of lymphocytes in the thymic cortex and medulla (C and D), mild depletion of lymphocytes in the bursal cortex and medulla (E and F); mild to moderate depletion of lymphocytes in the spleen (G and H).

IFN-γ, IL-1β and IL-2 expression (McConnell et al., 1993a; Ragland et al., 2002). The present experimental data indicated not only IL-1β decreases but also IL-10, IL-12 and GM-CSF at various post infection intervals of the clinically susceptible age, and the effects were more pronounced in young chicks. The immuno-regulatory cytokine, IL-10, decrease was more pronounced during earlier stages of infection (4 dpi) than at the latter stages (7 dpi), indicating higher immunosuppressive stage at 7 dpi. A change in cytokine expression levels may be closely related to replication of CIAV, but the mechanism is still not fully understood. However, it could be due to depletion of lymphocytes in lymphoid organs and there by decreased count in the peripheral blood.

Pathogenesis of CIAV indicates that primary target cells are haematopoietic precursor (haematocytoblasts) and thymic precursor (lymphoblasts) cells in the bone marrow and thymic cortex (Jeurissen et al., 1992; Noteborn and Koch, 1995). In this study, it was found that thymus is indeed primary target organ for the viral replication as confirmed by nucleosomal fragmentation, an indicator of apoptosis. Nucleosomal fragmentation was higher at 7, 10 dpi than at 14 dpi in the CIAV infected groups; also, the apoptotic pattern was least in chicks of Group D, infected at 28 days of age. This may be due to refractoriness of thymocytes to CIAV infection or depletion of the thymocytes in the thymus at latter stages and as age progresses (Goryo et al., 1985; Jeurissen et al., 1992; Noteborn and Koch, 1995). This type of apoptosis is an important phenomenon during the pathogenesis of CIAV and is associated with VP3 protein, apoptin (Noteborn and Koch, 1995). Due to this apoptosis

in thymic and haematopoietic precursor cells, possibly lower circulating lymphocyte were present in peripheral blood of infected groups as certain correlation was found between apoptotic pattern in the thymus and cytokine down regulation in the peripheral blood.

The blastogenic responses, as an indicator of cell mediated immunity (CMI) indicated lower stimulation indices in chicks of CIAV infected groups than the control chicks. The depression in CMI response in groups infected at younger age was more pronounced than those groups infected at later ages. Also, it was found that blastogenic responses were lower at 1 and higher at 3 wpi in young chicks infected at 1st and 14th day of age, while in older chicks infected with the virus at 21 and 28 days of age, the stimulation indices were comparatively higher which infers that older birds are comparatively less vulnerable to virus infection. This may be due to lower T lymphocytes in the peripheral blood associated with higher apoptosis of thymic and haematopoietic precursor cells in younger chicks as compared to older birds in which the virus may have replicated slowly because of development of age resistance to CIAV and due to maturation of immune cells.

After adsorption and penetration, CIAV enters the haematopoietic and thymic precursors and multiplies in their nucleii by a rolling circle model (Noteborn and Koch, 1995; Scott et al., 1999). The virus then affects both cellular and humoral immune function directly by affecting helper (CD4^{+}) and cytotoxic (CD8^{+}) T-lymphocytes in the thymus and indirectly through cytokine responses, respectively (Hu et al., 1993; Todd, 2000). By inhibiting
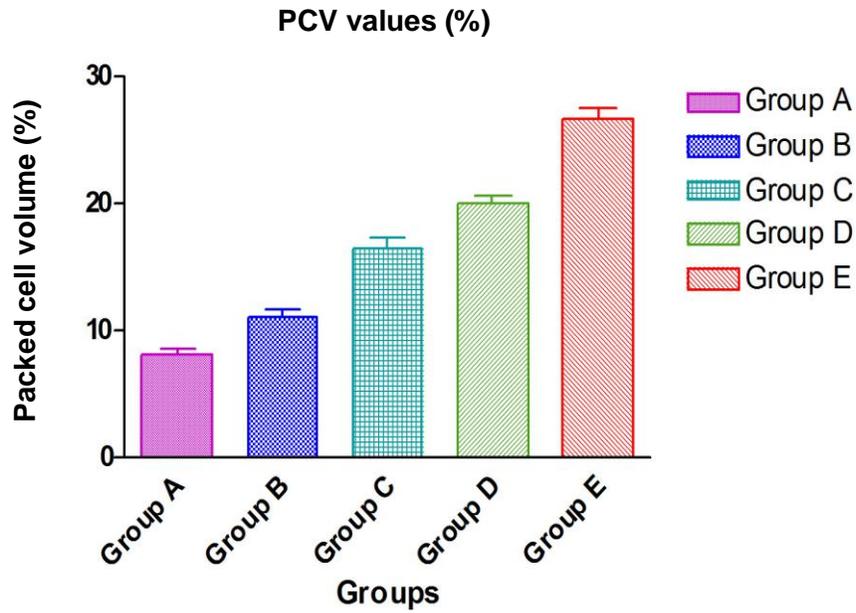
PCV values (%)

Figure S1. Effect of CIAV infection on %PCV values in chicks inoculated at 1st (Group A), 14th (Group B), 21st (Group C) and 28th (Group D) days of age.
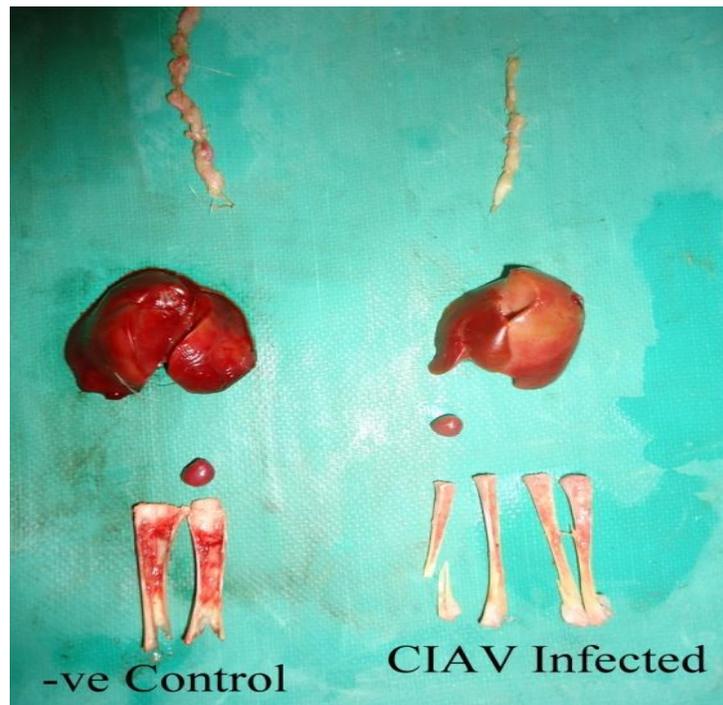
Figure S2. Gross lesions in thymus, spleen, liver and bone marrow in CIAV infected chicks on 12 dpi indicating atrophied thymus and spleen, pale liver and whitish pale bone marrow.

IL-1, IL-2, IFN and other cytokine production, it adversely affects the inflammatory and immunoregulatory responses, the expression of surface receptors and the cytotoxic activities of pool of cells including macrophages and T lymphocyte (CTL) (McNulty, 1991; Todd, 2000; Schat, 2003). These detrimental effects on T-cell

mediated functions adversely affects lymphocyte transformation response to mitogens, cytokine production; reduces macrophage functions namely, IL-1, FcR expression, phagocytosis and bactericidal activities (Cloud et al., 1992; McConnell et al., 1993a, 1993b). All of such changes have substantial negative effects on immune response leading to enhancement of the concurrent infection with other pathogens and vaccination failure.

To confirm that the establishment of CIAV infection in experimentally infected chicks and the changes in the immune profile were indeed caused by CIAV, pathology, haematology and PCR testing of various tissues of the virus infected chicks was carried out. Generally normochromic anemia with watery blood, paler plasma and increased clotting time and associated symptoms are recorded in CIAV infections (Taniguchi et al., 1982; Ramadan et al., 1998). Accordingly, in the present study, PCV was decreased in all the infected groups at 12 DPI, but the decrease was more pronounced in groups infected at younger ages (group A and B), suggesting the more severity and susceptibility of young chicks to the infection as compared to that at later stages (Groups C and D). CIAV VP2 specific PCR assay yielded desired virus specific amplicon of 651 bp indicating that CIAV replicated in the tissues causing associated signs and lesions. The pooled tissue (thymus, bone marrow, spleen and bursa) samples were found positive on PCR assay at 12 dpi from all the infected groups. PCR has several advantages over conventional diagnostic tests and previous reports indicated that it is very useful for detecting CIAV infection (Dhama, 2002; Hussein et al., 2002; Yassir et al., 2011; Snoeck et al., 2012). Clinical signs and gross lesions were more pronounced in chicks infected at young age (1 and 14 day of age) as compared to that of chicks infected at later stages (21 and 28 day of age). Similarly, histopatholgical alterations in tissues of the CIAV infected chicks were more pronounced in chicks infected at younger ages. The histopathology of thymus and bursa showed mild to moderate atrophy and mild depletion of lymphocytes. In liver, mild degeneration of hepatocytes, dilated blood capillaries and focal lymphoid aggregation were observed. These observations were in agreement with the earlier reports (Goryo et al., 1989; McNulty, 1991; Sakr and Talaat, 1991; Smyth et al., 1993; Dhama, 2002).

Immunodepression caused by CIAV infection has dramatic effects on the poultry birds as it increases their susceptibility to secondary infections (viral, bacterial or fungal origin), depresses vaccinal immunity against other pathogens, complications like vaccinal reactions, aggravates residual pathogenicity of attenuated vaccine viruses and emergence of variant virus and most importantly leads to vaccination failure (Liu et al., 1997; Pascucci, 1997; Todd, 2000; Dhama et al., 2008). The important concurrent infections associated with CIAV infection include MD, ND, FP, ILT, IBD, etc. In the field,

CIAV infection seems to cause few clinical signs of disease; however, the production losses both in terms of weight gain and due to concurrent infections are very high. Sub-clinical CIAV infections in older chickens have also been reported to cause substantial economic losses by affecting growth and health of birds and production of commercial SPF eggs.

In conclusion the present study provide supportive evidence that CIAV infection has profound effect on immunological profile of the young chicks, and reports the downregulatory changes in cytokines (IL-1, IL-12, IL-10 and GM-CSF) in chicks infected with CIAV at various intervals of clinically susceptible period, with younger ages of upto 2 weeks being more vulnerable. The cytokine down regulation correlated to some extent with apoptotic pattern in the thymus. CMI responses were also depressed in CIAV infected chicks as assayed by LTT, with a similar pattern as with cytokine downregulation. This has further provided evidence regarding the apoptotic involvement of lymphocytes in thymus due to which there was decreased peripheral pool and the decreased blastogenic responses in infected chicks. The observations of clinical signs and disease (CIA), anaemia, gross lesions and histopathological studies and PCR testing confirmed the establishment of the CIAV infection in experimental chicks. However, further studies are required regarding the role of other cytokines and the impact of viral load on the cytokine profile and immunopathogenesis both in young and adult birds.

ACKNOWLEDGEMENTS

REFERENCES

Adair BM (2000). Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24:247-255.

Adair BM, McNeilly F, McConnell CDG, Todd T, Nelson RT, McNulty MS (1991). Effects of chicken anemia agent on lymphokine production and lymphocyte transformation in experimentally infected chickens. Avian Dis. 35:783-792.

Bhatt P, Shukla SK, Mahendran M, Dhama K, Chawak MM, Kataria, JM (2011). Prevalence of Chicken Infectious Anaemia Virus (CIAV) in Commercial Poultry Flocks of Northern India: A Serological Survey. Transbound. Emerg. Dis. 58(5):458-60.

Bounous DI, Campagnoli RP, Brown J (1992). Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assay using chicken splenocytes. Avian Dis. 36:1022-1027.

Bülow VV, Rudolph R, Fuchs B (1986). Enhanced pathogenicity of chicken anemia agent (CAA) in dual infection with Marek's disease virus (MDV) and infectious bursal disease virus (IBDV) or Reticuloendotheliosis virus (REV). J. Vet. Med. B 33:93-116.

Cloud SS, Rosenberger JK, Lillehoj HS (1992). Immune dysfunction following infection with chicken anemia agent and infectious bursal

disease virus. 2. Alterations of *in vitro* lymphoproliferation and *in vivo* immune responses. Vet. Immunol. Immunopathol. 34:353-366.

Dhama K (2002). Pathogenecity and immunosuppressive effects of chicken infectious anaemia virus (CIAV) in chicks and evaluation of diagnostic tests for its detection. Ph.D. Thesis, Deemed University, Indian Veterinary Research Institute (IVRI), Izatnagar, (U.P), India.

Dhama K, Mahendran M, Somavanshi R, Chawak MM (2008). Chicken infectious anaemia virus: An immunosuppressive pathogen of poultry – A review. Ind. J. Vet. Path. 32(2):158-167.

Gholami-Ahangaran M, Zia-Jahromi N (2012). Serological and molecular identification of subclinical chicken anaemia virus infection in broiler chickens in Iran. Afr. J. Microbiol. Res. 6(21):4471-4474.

Goryo M, Sugimura H, Matsumoto S, Umemura T, Itakura C (1985). Isolation of an agent inducing chicken anaemia. Avian Pathol. 14:483-496.

Goryo M, Suwa T, Umeura T, Itakura C, Yamashiro S (1989). Histopathology of chicks inoculated with chicken anaemia agent (MSB1-TK 5803 strain). Avian Pathol. 18:73-89.

Hagood LT, Kelly TF, Wright JC, Hoerr, FJ (2000). Evaluation of chicken infectious anaemia virus and associated risk factors with disease and production losses in broilers. Avian Dis. 44:803-808.

Hegazy AM, Abdallah FM, Abd-El Samie LK, Nazim AA (2010). Chicken infectious anemia virus (CIAV) in broilers and laying hens in Sharkia Province, Egypt. J. Anim. Sci. 6(9):752-761.

Hoerr FJ (2010). Clinical aspects of immunosuppression in poultry. Avian Dis. 54(1):2-15.

Hu LB, Lucio B, Schat KA (1993). Depletion of CD4$^+$ and CD8$^+$ T lymphocyte subpopulation by CIA-1, a chicken infectious anemia virus. Avian Dis. 37:492-500.

Hussein HA, Sabry MZ, El-Ebiary EA, El-Safty M, Abdel-Hady AL (2002). Chicken infectious anemia virus in Egypt: Molecular diagnosis by PCR and isolation of the virus from infected flocks. Ar. J. Biotech. 5(2):263-274.

Jeurissen SHM, Wagenaar F, Pol JMA, Van Der Eb AJ, Noteborn MHM (1992). Chicken anaemia virus causes apoptosis of thymocytes after *in vivo* infection and of cell lines after *in vitro* infection. J. Virol. 66(12):7383-7388.

Kaiser MG, Cheeseman JH, Kaiser P, Lamont SJ (2006). Cytokine expression in chicken peripheral blood mononuclear cells after *in vitro* exposure to salmonella enterica serovar Enteritidis. Poult. Sci. 85:1907-1911.

Liu ZG, Zheng SM, Xu YB, Zhou ZY, Wang XR, Yang LP (1997). Immunopathological changes of chicken infectious anaemia. Scientia Agricultura Sinica. 30:74-82.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408.

Markowski GCJ, Schat KA (2003). Infection with chicken anaemia virus impairs the generation of pathogen-specific cytotoxic T lymphocytes. Immunology 109(2):283-294.

McConnell CD, Adair BM, McNulty MS (1993a). Effects of chicken anemia virus on macrophage function in chickens. Avian Dis. 37:358-65.

McConnell CD, Adair BM, McNulty MS (1993b). Effects of chicken anemia virus on cell-mediated immune function in chickens exposed to the virus by a natural route. Avian Dis. 37:366-74.

McNulty MS (1991). Chicken anemia agent: a review. Avian Pathol. 20:187-203.

Natesan S, Kataria JM, Dhama K, Rahul S, Baradhwaj N (2006). Biological and molecular characterization of chicken anemia virus isolates of Indian origin. Virus Res. 118:78-86.

Noteborn MHM, Koch G (1995). Chicken anaemia virus infection: molecular basis of pathogenicity. Avian Pathol. 24:11-31.

Oluwayelu DO (2010). Diagnosis and epidemiology of chicken infectious anemia in Africa. Afr. J. Biotech. 9(14):2043-2049.

Otaki Y, Nunoya T, Tajima M, Kato A, Nomura Y (1988a). Depression of vaccinal immunity to Marek's disease by infection with chicken anemia agent. Avian Pathol. 17:333-347.

Otaki Y, Tajima M, Saito K, Nomura Y (1988b). Immune response of chicks inoculated with chicken anemia agent alone or in combination with Marek's disease or turkey herpes virus. Jap. J. Vet. Sci. 50:1040-1047.

Pascucci S (1997). Immunosuppressive effect of chicken anaemia virus. Selezione Veterinaria 8-9:599-609.

Pringle CR (1999). Virus Taxonomy at the Xlth International Congress of Virologv, Sydney, Australia, 1999. Archiv. Virol. 144:2065-2070.

Ragland WL, Novak R, Savic EAV, Ester K (2002). Chicken anemia virus and infectious bursal disease virus interfere with transcription of chicken IFN-α and IFN-γ mRNA. J. Interferon Cytokine Res. 22:437-441.

Ramadan G, El-Hussini H, Bekhit A (1998). Biochemical and hematological characterization of chicken anemia virus (CAV). Assuit Vet. Med. J. 38(76):209-222.

Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, Smith AL, Kaiser P (2004). Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173(4):2675-2682.

Sakr EA, Talaat AM (1991). Pathological changes in chicks induced by chicken anemia agent, isolated for the first time in Egypt. Benha Vet. Med. J. 2(1):106-117.

Schat KA (2003). Chicken infectious anaemia. In: Diseases of Poultry, 11th edn. Y.M. Saif et al., (Editors), Iowa State University Press, Ames, IA, Ch. 7:182-202.

Schat KA (2009). Chicken infectious anaemia. Curr. Topics in Micro. Immunol. 331:151-183.

Scott ANJ, Connor TJ, Creelan JL, McNulty MS, Todd D (1999). Antigenicity and pathogenicity characteristics of molecularly cloned chicken anaemia virus isolates obtained after multiple cell culture passages. Arch. Virol. 144:1961-1975.

Singh B, Oberoi MS, Jand SK, Singh A (1996). Emerging diseases of poultry in India. J. Res. 33:391-410.

Smyth JA, Moffett DA, McNulty MS, Todd D, Mackie DP (1993). A sequential histopathologic and immunocytochemical study of chicken anaemia virus infection at one day of age. Avian Dis. 37:324-338.

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.

Taniguchi T, Yuasa N, Maeda M, Horiuchi T (1982). Haematopathological changes in dead and moribund chicks induced by chicken anaemia agent. Natl. Instt. Anim. Hlth. Q. (Japan) 22:61-69.

Todd D (2000). Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol. 29:373-394.

Wintrobe (1967). Clinical hematology. Lea and Febiger Pub. Philadelphia. pp. 414-420.

Yassir ME, Kun Q, Wenjie J, Pingping W, Aijian Q (2011). Molecular epidemiology of chicken anemia virus in commercial farms in China. Virol. J. 8:145.