

Correlation between cytokine profile, antibody titre and viral load during sub-clinical chicken anaemia virus infection

M.Y. WANI¹, K. DHAMA², SHYMA K. LATHEEF¹, S.D. SINGH², R. TIWARI³

¹Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

²Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

³Department of Veterinary Microbiology, Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwa Vidyalaya Evam Go-Anusandhan Sansthan, Mathura, Uttar Pradesh, India

ABSTRACT: The present work was carried out to investigate the correlation between viral load and the cytokine profile and virus specific antibody levels at five day intervals up to 25 days post infection (dpi) in various tissues of chicks infected with chicken infectious anaemia virus (CIAV) at six weeks of age (sub-clinical infection). For determining the viral load in the various tissues, recombinant plasmid VP2-pET32b was prepared and serially diluted tenfold for the generation of a standard curve using real time polymerase chain reaction (PCR). Cytokine fold changes in mRNA levels of IFN- γ , IL-1, IL-2, IL-12 in the spleen and IFN- γ , IL-12 in freshly collected whole blood were determined. The results showed that peak viral load occurs between 10–20 days post infection in the lymphoid tissues viz., thymus, spleen, liver, bone marrow, bursa and also blood, being highest in blood followed by thymus. A varied response in the expression levels of individual cytokines was observed during all the intervals post infection. The IFN- γ increased two to five fold in blood and spleen while IL-2 decreased in the splenic tissue during the same period. Peak IFN- γ coincided with peak viral load in the spleen at 10 dpi while in blood it peaked earlier at five days post infection and remained high during the peak viral load at 10–20 dpi. Virus specific antibodies were significantly higher at 15 dpi and were thereafter found to be strongly associated with viral load regression although higher concentrations of virus remained in the blood and thymic tissues until 25 dpi, indicating a role of other immune components in virus clearance. In conclusion, this study reports a negative correlation of viral load and the progression of the antibody response and IFN- γ cytokine expression in chicks infected with CIAV during their sub-clinical infectious stage. However, cytokine expression from the various individual immune cells like monocytes, heterophils, CD4⁺ T, CD8⁺ T cells and others during the pathogenesis of this immunosuppressive poultry pathogen remains to be elucidated.

Keywords: chicken infectious anaemia virus; cytokine; real time-PCR; viral load; antibody titre; immunosuppression

Chicken infectious anaemia virus (CIAV), the smallest avian pathogen, is responsible for severe anaemia, generalised lymphoid atrophy, reduced weight gain and immunosuppression and vaccine failures, especially in young chicks of less than three weeks of age (McNulty 1991; Pope 1991; Schat 2003, 2009; Dhama et al. 2008). The viral genome consists of a circular, non-enveloped, negative-sense, single stranded DNA of 2.3 kb that contains three partially overlapping major open reading frames (ORFs) encoding three proteins;

namely, VP1, VP2 and VP3 (Todd 2000; Miller et al. 2005; Natesan et al. 2006a). The VP1 (51 kD) acts as a major capsid protein, VP2 (24 kD) acts as a scaffold protein and is essential for the virus assembly, while VP3 (13 kD), also known as apoptin, is important for the disease pathogenesis and apoptosis (Noteborn 2004; Miller et al. 2005; Natesan et al. 2006b). The virus has been classified under the genus *Gyrovirus* of the family Circoviridae (Pringle 1999). Due to its unique structure CIAV has remarkably high physical, chemical and ther-

mal stability (Yuasa 1992). Also, the virus possesses other unique features like vertical transmission and a highly contagious nature due to which it is ubiquitously found in the wild and exhibits worldwide distribution (McNulty et al. 1988; Todd 2000; De Herdt et al. 2001; Dhama et al. 2008; Schat 2009; Oluwayelu 2010; Bhatt et al. 2011; Snoeck et al. 2012; Nayabian and Mardani 2013).

Infections with CIAV generally cause severe and acute clinical disease in young chicks up to 3–4 weeks of age, after which age resistance to clinical infection has been reported to develop. Sub-clinical and persistent latent viral infections occurring in adult broiler and layer chickens are associated with production losses and vaccination failures (Hagood et al. 2000; Senthilkumar et al. 2002; Miller and Schat 2004; Dhama et al. 2008; Hoerr 2010). Pathogenesis of the virus involves adsorption and penetration of the virus into haematopoietic precursor (haemocytoblasts) and thymic precursor (lymphoblasts) cells in the bone marrow and thymus cortex, respectively, at 6–8 days post infection (dpi), leading to transient severe anaemia and immunosuppression (Goryo et al. 1989a,b; Adair 2000; Noteborn 2004). CIAV has specific tropism for lymphoid tissues, particularly for the thymus cortex (T cells preferably), and affects lymphopoiesis so that there is depletion of thymic lymphoblastoid cells and lymphocytes in the thymus (precursor T cells), spleen (mature T cells) and other lymphoid tissues (Jeurissen et al. 1992; Smyth et al. 1993; Dhama 2002). Pathogenesis of the virus with regard to the role of viral load on body weight gain, haematocrit values and antibody titres has been previously studied in CIAV-infected embryos and young chicks (Tan and Tannock 2005). Also, it was found that CIAV can also replicate in susceptible adult chicks at higher concentrations in the liver, thymus and spleen (Kaffashi et al. 2006; Smyth et al. 2006). Limited reports are available regarding the effect of CIAV on cytokine production especially those involved in cell-mediated immunity (Adair et al. 1991; McConnell et al. 1993a,b; Markowski-Grimsrud and Schat 2003; Basaraddi et al. 2013). Development of virus-specific antibodies is regarded as essential for virus clearance in chicks and for the recovery from clinical signs; however, the correlation of antibody titre with viral load regression and/or clearance has not been studied. Therefore, the present study was carried out to determine the correlation between virus load, cell-mediating cytokines (IL-2, IFN- γ , IL-12, IL-1) and

virus-specific antibody titres in the serum during subclinical experimental CIAV infection of chicks.

MATERIAL AND METHODS

Virus and experimental chicks. An Indian field isolate of CIAV (CIAV-E strain; GenBank accession No. AY583757), maintained in the Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, was used for experimental infection purposes. Embryonated specific pathogen-free (SPF) eggs ($n = 40$), obtained from M/S Venkateshwara Hatcheries Private Limited (VHL), Pune, Maharashtra, were hatched in the Hatchery Unit of the Central Avian Research Institute, Izatnagar and reared in Experimental Sheds of the Avian Disease Section under strictly isolated conditions and fed feed and water *ad libitum*. All experimental procedures on animals were carried out according to the recommendations and with the 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).

Experimental design. Chicks ($n = 32$), at the age of six weeks were randomly and equally divided into two groups, Infected and Control. All the chicks of the Infected group were inoculated intramuscularly with a $10^{4.5}$ median tissue culture infective dose (TCID₅₀) of the MDCC-MSB1 passaged CIAV-E isolate, while the control chicks received uninfected cell lysate as described previously (Natesan et al. 2006a). All the chicks were regularly monitored for the development of clinical signs of the disease and weighed at five day intervals. Three birds from both groups were sacrificed humanely at 5, 10, 15, 20 and 25 days post infection (dpi) to assess the viral load in various tissues viz., thymus, spleen, bursa, liver, bone marrow and whole blood. Quantitative real time polymerase chain reaction (q real time-PCR) was employed to determine the viral copy number in the tissues of CIAV-infected chicks; the VP2-pET plasmid was prepared and used for making the standard curve. The mRNA expression levels of the cytokines viz., IL-1 β , IL-2, IL-12 β and IFN- γ were evaluated in splenic tissue samples, and IFN- γ and IL-12 levels were determined in freshly collected whole blood.

Preparation of VP2-pET plasmid for standard curve. The entire VP2 gene (Accession No. AY583757.1) of CIAV was PCR-amplified using

the VP2F (accaagaattcaatgcacgggaacggcgac) and VP2R (tcctctcgagtcacactatactaccggggcg) primers. The amplified PCR products were processed using a PCR purification kit and double-digested, along with the pET32b vector, using *EcoRI* and *XhoI* enzymes. Both the digested products were run on a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and ligated together using DNA ligase. The ligated product was amplified by transformation in *E. coli* (DH5α) and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The resulting purified plasmid was linearised by digestion with the *PvuI* restriction enzyme. The concentration of the digested DNA was calculated by measuring the absorbance at 260 nm (A₂₆₀) using a previously described formula (Kaffashi et al. 2006).

$$\text{Copy number of DNA}/\mu\text{l} = A_{260} \times 4.56 \times 10^{16}/N$$

Where:

N = the number of base pairs in the DNA molecule

For the generation of the standard curve, 10-fold serial dilutions of the linearised plasmid containing 10⁸ to 10 copies of plasmid were used in triplicates along with non-template controls.

Tissue DNA isolation. Tissue samples, 25 mg each, of spleen, liver, thymus, bursa and blood were used for isolation of whole DNA using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer’s recommendations. The isolated DNA samples were diluted to 1 : 100 in

order to avoid possible inhibitory effects on efficient PCR reaction by tissue inhibitory substances (Trichopad et al. 2004; Kaffashi et al. 2006).

Total RNA isolation and complementary-DNA synthesis. Total RNA was isolated from 100 μl of freshly collected blood using TRIzol Reagent (Invitrogen, USA) as per the single-step RNA isolation protocol prescribed by Invitrogen (Chomczynski and Sacchi 1987). The quality and quantity of extracted RNA was checked using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). RNA samples with A280/A260 ratios in the range of 1.8 to 2.0 and A260/A230 ratios in the range of 2.0 to 2.2 were considered pure and used for cDNA synthesis.

Complementary DNA (cDNA) synthesis from total RNA (1 μg per reaction) was carried out on a thermal cycler in 20 μl volumes using random hexamer primers and Revert Aid First-Strand cDNA Synthesis kit (Thermo Scientific, USA) as per the manufacturer’s recommendations.

Quantitative real time PCR. For quantification of the viral DNA in the various tissues and the mRNA expression levels of β actin, IL-1β, IL-2, IL-12β, and IFN-γ, isolated DNA samples and the cDNA samples of the respective blood and spleens were subjected to real time PCR analysis using a MX 3000P thermal cycler (Stratagene, CA, USA). Duplicate sets of each reaction sample were prepared using 10 μl of QuantiFast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 2.5 pmole each of gene-specific forward and reverse primers, 2 μl of 1 : 100 diluted DNA samples or 100 ng of

Table 1. Primers used in the quantitative real time PCR assay for various cytokine genes and CIAV

Sr. No.	Gene	Primer sequence (5'-3')	Amplicon size (bp)	Efficiency	R ²	Reference (if any)
1	IFN-γ	F, GCCGCACATCAAACACATATCTG R, GCGCTGGATTCTCAAGTCGTTC	127	98.2	0.996	self-designed
2	IL-1β	F, AACATCGCCACCTACAAG R, GACGGTAATGAAACATAAACG	185	94.2	0.995	Basaraddi et al. 2013
3	β-actin	F, GCACCACACTTTTCTACAATGAG R, ACGACCAGAGGCATACAGG	184	100.7	0.997	Basaraddi et al. 2013
4	IL-12β (p40)	F, TGCCCAGTGCCAGAAGGAAAAC R, CTCGGTGTGCTCCAGGTCTTG	214	104.2	0.963	Basaraddi et al. 2013
5	IL-2	F, CACACCAACTGAGACCCAGGA R, TCCGGTGTGATTTAGACCCGT	118	102.3	0.989	self-designed
6	VP2 (Q)*	F, ATGGCAAGACGAGCTCGC R, TCACACTATACGTACCGGGG	178	102.3	0.995	self-designed

*VP2 (Q) represents the quantitative real time PCR primer set for VP2 gene of CIAV

cDNA and nuclease-free water to make final reaction volume of 20 μ l. Non-template controls were also tested in the real-time quantification assay. The specificity of the target gene products was confirmed by analysis of the dissociation curve generated by the software at annealing temperatures through 95 °C. All the primers used in this study were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa USA) (Table 1).

Amplification conditions comprised an initial incubation at 95 °C/10 min for activation of hot start DNA polymerase, followed by 40 cycles of amplification with denaturation at 95 °C for 15 s, combined annealing and extension at 60 °C for 30 s for the VP2 gene and separate annealing and extension at 60 °C for 20 s and 72 °C for 30 s, respectively, for all other genes.

Enzyme-linked immunosorbant assay (ELISA). Serum samples were collected from both chicks of the control and virus-infected groups from 0–25 dpi at five day intervals to monitor the development of CIAV-specific antibodies using a commercially available CIAV Elisa Kit (IDEXX Laboratories, Maine, USA). Sera samples were diluted 100-fold and tested as per the manufacturer's recommended protocol.

Statistical analysis. All the values obtained in the study are represented as mean \pm SD. Viral copy numbers for each tissue sample were interpolated from the generated standard curve. For cytokine expression levels, values were normalised to the endogenous control (beta-actin) and the fold changes in the target genes were determined using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). The analysis of variance (ANOVA) followed by a post-hoc Tukey's test was used to determine significant differences between values using SPSS v.16.0 statistical software. *P*-values of less than 0.05 were considered to be significant.

RESULTS

Effect of CIAV infection on body weight and thymus : body weight ratio

Characteristic clinical signs of chicken infectious anaemia (CIA) were not observed in the chicks of the infected group but CIAV inoculation was found to reduce the body weight gain and significant differences were found between infected and control chicks at 15 and 25 dpi ($P < 0.05$). Also, significant

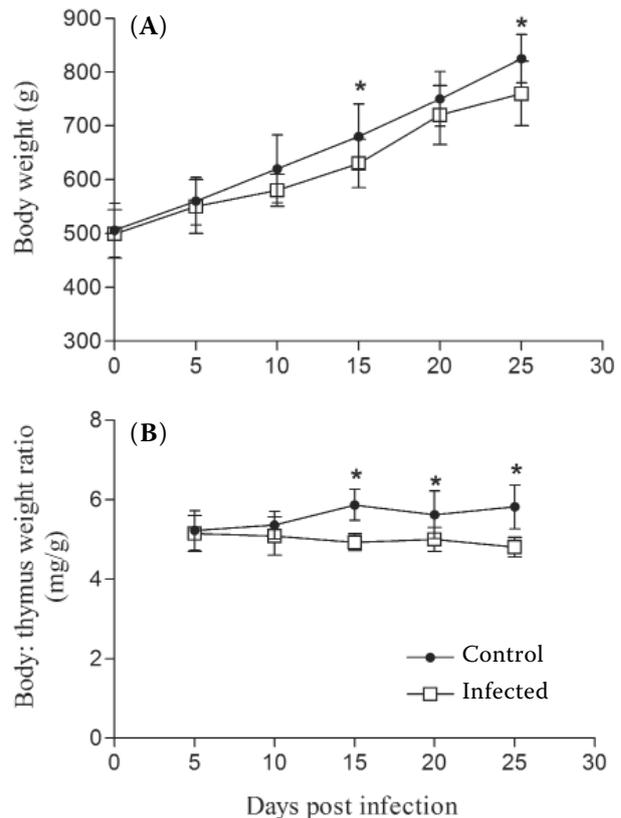


Figure 1. Effect of CIAV infection on (A) mean body weight (g) and (B) thymus : body weight ratio (mg/g tissue) in chicks on different post-infection days. Values are represented as mean \pm SD; * $P < 0.05$

differences ($P < 0.05$) in the thymus:body weight ratios were observed from 15 dpi onwards in the sacrificed chicks (Figure 1). The chicks of the control group remained healthy throughout the experiment.

Standardisation of quantitative PCR for viral load

Amplification of the entire VP2 gene of CIAV employing the VP2F and VP2R primer set resulted in the generation of a virus-specific product 680 bp in size. The recombinant VP2-pETb2(b) plasmid was confirmed by PCR, gene insert release and finally by sequencing. Amplification of the VP2 gene using the quantitative real-time PCR primer set produced the expected product size of 178 bp (Figure 2).

The standard curve generated for the quantification of viral load indicated a linear relationship between the amount of input plasmid DNA and the Ct values over eight \log_{10} dilutions. The equation for the standard curve and correlation coefficient

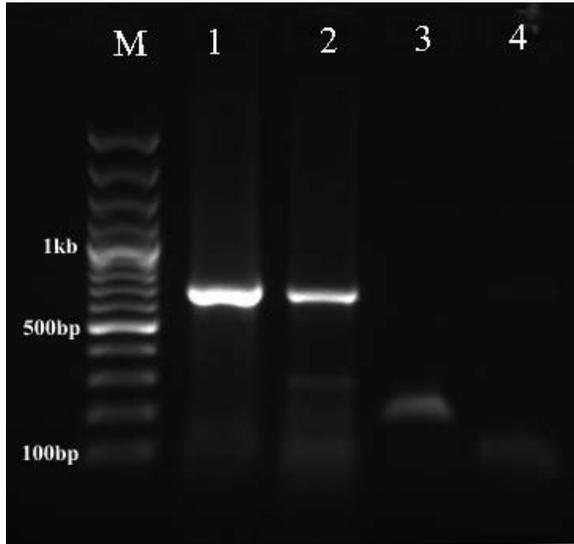


Figure 2. Amplification of the VP2 gene of CIAV using different primer sets as described in the Materials and Methods: M = 1 kb plus marker; 1 and 2 = amplification product of the entire VP2 gene using expression primer set along with recombinant plasmid and virus DNA respectively; 3 = amplified product of recombinant plasmid using q-real time PCR primer set; 4 = non-template control

(r^2) were $Y = -3.36X + 36.05$ and 0.995 , respectively. A single dissociation peak during melting curve analysis through annealing to melting points of the amplified product was observed (Figure 3).

Viral loads in various body organs

The CIAV genome was detected in all the chicks of the infected group sacrificed at five day intervals up to 25 days post infection. The highest copy number of the viral genome was found in the blood and thymus as compared to bone marrow, liver, bursa and spleen on all the post-infection days tested. Peak levels of CIAV genome were observed between 10 and 20 dpi in blood ($\log_{10} 7.63 \pm 1.21$ to 6.82 ± 0.78), thymus ($\log_{10} 6.29 \pm 0.64$ to 5.46 ± 0.74), bone marrow ($\log_{10} 5.21 \pm 0.23$ to 4.23 ± 0.44), bursa ($\log_{10} 5.49 \pm 0.23$ to 4.18 ± 0.64), spleen ($\log_{10} 5.01 \pm 0.32$ to 3.61 ± 0.23) and liver ($\log_{10} 4.41 \pm 0.81$ to 3.82 ± 0.12), respectively. A downward trend in the viral copy number was observed from 15 dpi onwards, although significantly higher levels were

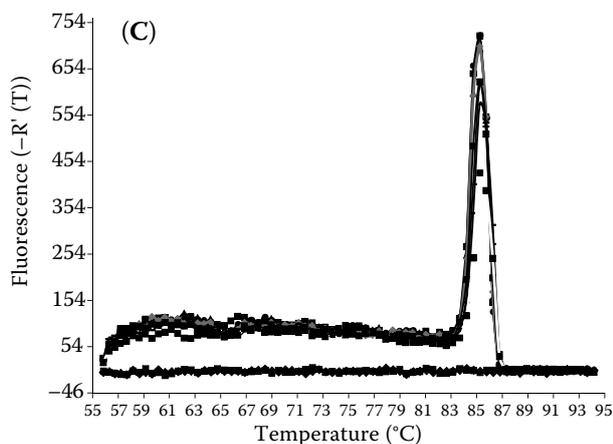
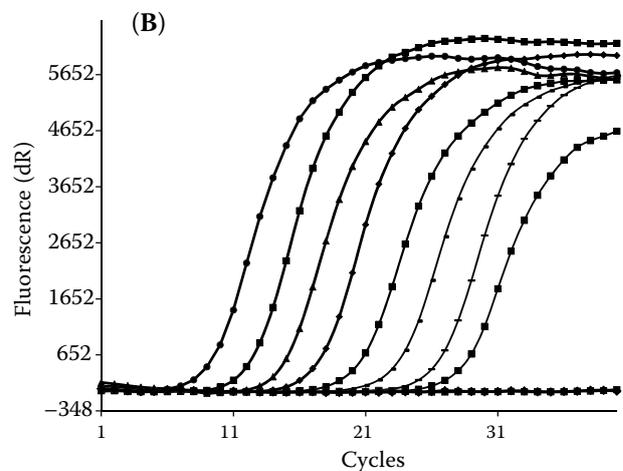
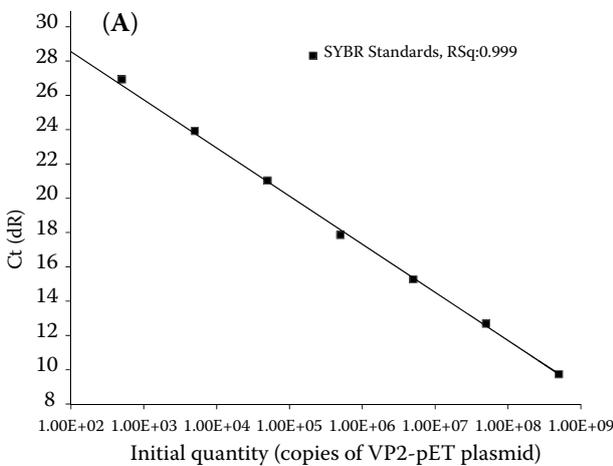


Figure 3. Standard curve generated during real time PCR using the VP2-pET32b recombinant plasmid, serially diluted ten-fold (A). Amplification plots of ten-fold serially diluted ($10^8 - 10$ copy number) VP2-Pet32(b) linearised recombinant plasmid (B). Dissociation curve of the amplified VP2 gene product (178bp size) during melting curve analysis (C)

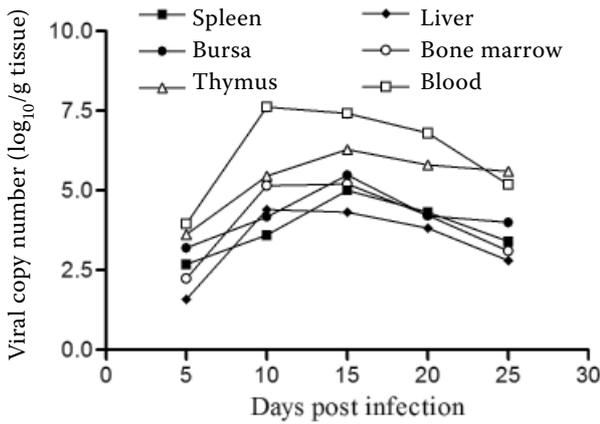


Figure 4. Viral load in different body organs of the chick inoculated using the intramuscular route on various post-infection days. Values are represented as mean CIAV copy number \log_{10}/g of tissue

observed in the thymus ($\log_{10} 5.61 \pm 0.43$) and the whole blood ($\log_{10} 5.21 \pm 0.45$) at 25 dpi (Figure 4).

Correlation between viral load and cytokine mRNA expression levels

In the first step, the efficiencies of various primers were determined using five-fold serially diluted cDNA samples. It was found that all the primers used in this study were highly efficient for amplifying the various target gene products (1.95–2.07) (Table 1). In the second step, the effect of CIAV infection on IL-1 β , IL-2, IL-12 β and IFN- γ transcript levels in spleen were determined at 5, 10 and 15 dpi. CIAV infection was found to significantly influence

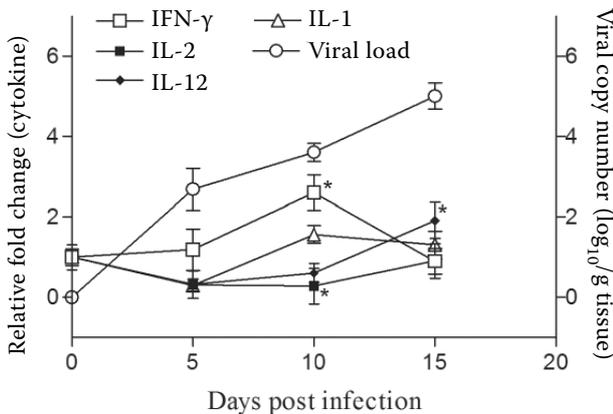


Figure 5. Effect of CIAV infection on cytokine expression and comparison with the virus load (\log_{10}/g) in the spleen of sacrificed birds on various post-infection days. Values are represented as mean \pm SD; * $P < 0.05$

the expression levels of all these cytokines. The relative mRNA expression levels of IFN- γ (2.60 ± 0.44) and IL-1 (1.57 ± 0.22) were significantly increased ($P < 0.05$) on 10 dpi compared to five and 15 dpi intervals. On the other hand, IL-2 and IL-12 levels were decreased on all the post-infection days tested except on 15 dpi at which time increased transcript levels of IL-12 were found. Comparing the splenic viral load with cytokine mRNA transcript levels revealed that IFN- γ levels peaked at five days before the peak viral load while IL-12 levels peaked together with that of the peak viral load (Figure 5).

Expression levels of IFN- γ and IL-12 were also determined in freshly collected whole blood. The levels of IFN- γ were significantly increased at all the post infection intervals in blood and peak levels (5.20 ± 1.34) were found at 5 dpi. The transcript levels of IL-12 were first increased (2.34 ± 0.33) at five dpi and then returned to basal levels. Comparison of the IFN- γ profile with viral load indicated that peak IFN- γ levels were achieved five days before the peak viral load in whole blood. As the peak viral load was maintained from 10 to 15 dpi, significantly higher concentrations (2.40 ± 0.62 to 2.34 ± 0.41) of IFN- γ were also observed in the whole blood during that period (Figure 6).

Anti-CIAV antibody levels in serum and viral load in blood

CIAV-specific antibody development started after 10 dpi in the chicks of the virus-infected group, reached its maximum level at 15 dpi at which point

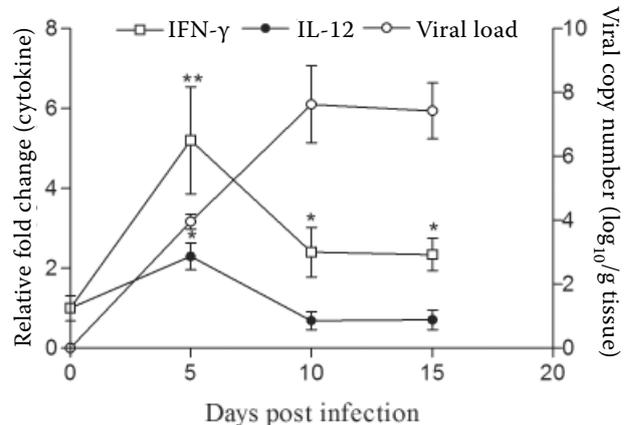


Figure 6. Effect of CIAV infection on cytokine expression and comparison with the virus load (\log_{10}/g) in freshly collected whole blood on various post-infection days. Values are represented as mean \pm SD, * $P < 0.05$, ** $P < 0.01$

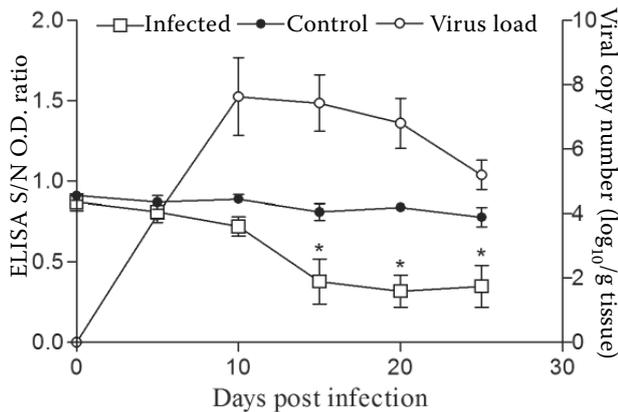


Figure 7. Comparison between the CIAV-specific antibody levels in the serum and the virus load in blood. The virus-specific antibodies were detected using ELISA and expressed as signal-to-noise ratio of optical densities (absorbance, A_{630}) and virus load as copy number (\log_{10}/g); * $P < 0.05$

the titres were maintained at relatively constant levels up to 25 dpi. The antibody titres in the infected chicks were significantly different from the chicks of the control group from 15 dpi onwards. Since competitive ELISA was used in the CIAV ELISA Kit, higher values indicate the absence of antibody and vice-versa. All the control chicks were found to be negative for CIAV antibodies throughout the experiment even when 1 : 10 serum dilutions were used during the ELISA testing. Comparison of the virus load in the whole blood with the serum anti-CIAV antibody levels was also made, which is presented in Figure 7. It was found that together with the increase in antibody levels in serum the virus load started decreasing from 15 dpi in the blood and was lowest at 25 dpi.

DISCUSSION

Infection of adult chicks with CIAV generally does not cause clinical disease albeit subclinical infections frequently occur which have been found to be associated with significant economic losses (McNulty et al. 1991; Hoop 1992; Hagood et al. 2000; Schat, 2003; Dhama et al. 2008). The transmission and spread of CIAV infections in adult birds occur through horizontal spread, which does not induce clinical disease (chicken infection anaemia). Clinical disease is generally observed in young chicks (one to three weeks) which are infected either vertically or horizontally. Development of age resistance to clinical disease has been found to oc-

cur after three to four weeks of age, though all ages are susceptible to virus infection. The present study reports the correlation between cytokine profile, antibody titre and viral load during sub-clinical infection of chicks with CIAV. Significant losses in body weight were observed along with marked a negative effect on thymus weight in the experimental CIAV-infected chicks inoculated intramuscularly at six weeks of age. However, as infection was subclinical clinical signs of CIA and mortality were not observed. Other factors which influence the severity of infection include the dose of inoculums, age of the bird, immunocompetence, concurrent immunosuppressive agents and management practices. Subclinical infections in older chickens have also been reported to cause substantial economic losses by affecting growth and production performance and the health status of birds.

The primary cells targeted during the pathogenesis of CIAV infection include haematopoietic precursor (haemocyto blasts) and thymic precursor (lymphoblasts) cells in the bone marrow and thymus cortex, respectively (Goryo et al. 1989a,b; Smyth et al. 1993; Adair 2000). Virus spreads by viraemia and CIAV antigen can be detected in cells of the bone marrow, thymus and spleen at three to four days post-infection and subsequently in other tissues like the liver, proventriculus, duodenum, lung, kidneys and heart, indicating its wide distribution throughout the body (Hoop and Reece 1991; Smyth et al. 1993). In this study, virus load in both the primary and secondary lymphoid tissues including the blood and liver was studied in a temporal fashion using SYBR Green quantitative-real time PCR, which is a rapid, highly sensitive, cost-effective, reproducible and reliable method for virus quantification (Niesters 2001; Ponchel et al. 2003; Abdul-Careem et al. 2006). It was found that the virus concentration increased in all the tissues (thymus, bone marrow, spleen, bursa, liver, blood) and was highest between 10 to 20 dpi. Though thymus and bone marrow are the primary targets of viral infection, the highest concentration was found in blood which may be due to the release of infected cells into the blood from the primary lymphoid organs. Increases in the thymic viral load were found to be associated with its tissue degeneration as lower thymus to body weight ratios were observed from 10 dpi onwards. Such effects were observed by others when virus was inoculated at a clinically susceptible age (Kaffashi et al. 2006; Smyth et al. 2006). Dissemination of the virus from

the primary lymphoid tissues to other organs may be the primary cause of the increase in viral load in other tissues. However, the virus can also replicate in secondary tissues as virus transcript levels were reported in these tissues (Markowski-Grimsrud and Schat 2003). The amount of viral load in all the tissues started regressing after 15 dpi though higher concentrations were observed even at 25 dpi in the thymus and blood.

Cell-mediated immunity (CMI) is regarded as an integral and essential component for viral elimination and is driven by various cytokines viz., IFN- γ , IL2, IL-1, IL-12 and others, and functions to induce the Th₁ type of immune response (Kaiser and Staheli 2008). In the present study, these cytokines were studied in the spleen and blood in a temporal fashion for the first time in comparison with respective viral loads in these organs. Transcript levels of IFN- γ in the whole blood were found to increase five-fold on five dpi and two-fold both on 10 and 15 dpi while a delayed response was observed in the spleen, where only a 2.5-fold increase was observed at 10 dpi. Markowski-Grimsrud and Schat (2003) also found that CIAV infection increased transcript levels of IFN- γ in the splenic tissue at seven dpi, though varying responses were observed depending on the age of the infected chicks. The transcript levels of IL-12, a driving inflammatory Th₁ cytokine, followed the same trend of IFN- γ in blood while in spleen its expression was decreased at five and 10 dpi and then increased by 15 dpi. In contrast to IFN and IL-12 responses, IL-2 expression levels in the spleen were significantly decreased both on five and 10 dpi and then came back to normal levels at 15 dpi. Such an inhibitory effect of CIAV on IL-2 in splenocytes, although higher as assessed indirectly in splenocyte cell culture from infected chicks, was observed by other researchers (Adair et al. 1991; McConnell et al. 1993a). IL-2 is a leukocytotropic hormone secreted mainly by CD4⁺ T cells and is instrumental in discriminating between foreign (non-self) and self-antigens by stimulating the growth, differentiation and survival of antigen-selected cytotoxic T cells (Beadling et al. 1993; Beadling and Smith 2002). Also, more recently, Vaziry et al. (2011) found that a CIAV vaccinal strain led to persistent viral presence in the spleen with a decreased CD4⁺ T cell population. The depression in the cytokine profile in our study, especially that of IL-2, may be due to diminished CD4⁺ T cell population and is one of the possible mechanisms/or at least an associated component

for the impairment in the generation of pathogen-specific cytotoxic T cells during concurrent infections and vaccination failures which generally occur during CIAV infections.

Recovery from CIAV infection is said to be mainly due to induction of the humoral immune response as B lymphocytes are resistant to CIAV infection (Adair 2000). In the present study, anti-CIAV antibodies in the serum of the infected chicks were observed from 10 dpi onwards and the values were significantly different from 15 dpi onwards from the respective control group. The appearance of antibodies in the serum was found to be associated with viral clearance as viral load after 15 dpi was lower (6.82 ± 0.78 and $\log_{10} 5.52 \pm 0.21$) at 20 and 25 dpi respectively, compared to the viral load at 10 dpi ($\log_{10} 7.63 \pm 1.21$) in blood. The regression in the viral load was found not only in the whole blood as shown in the Figure 4 but also in other organs from 15 dpi onwards. Although the significantly higher anti-CIAV antibody titres observed on 15 dpi were maintained up to end of the study (25 dpi), still higher viral concentrations in the blood and thymus were found. Such observations indicate other immune components are also responsible for complete recovery. IFN is regarded as vital and central during viral infection as it is involved both in induction and regulation of innate and adaptive antiviral mechanisms. In the present study we found that IFN levels increased both in the blood as well as in splenocytes at various post-infection intervals, although a higher effect was observed in the blood compared to spleen. Complete removal of virus-infected cells depends on the generation of virus-specific CD8⁺ T cells. A delay in virus clearance may be due to lower numbers of virus-specific cytotoxic cells as CIAV is said to deplete CD8⁺ T cells also (Markowski-Grimsrud and Schat 2003). Moreover, in adult birds CIAV has been found to be present as a latent and persistent infection in gonads (Cardona et al. 2000). Though not studied in detail, this type of CIAV association with its host is said to be a unique host-parasite relationship in which virus replication is influenced by hormones (Miller and Schat 2004; Schat 2009). The association and transmission of CIAV from Marek's disease-infected feather follicles and lymphoma was studied recently (Davidson et al. 2008; Haridy et al. 2012), but the relationship between Marek's disease virus and other oncogenic viruses which infect lymphoid organs and CIAV infection, especially in adult birds, remains to be addressed.

Elucidating the associations and interactions of different avian viral pathogens will be of importance in determining their coexistent nature, heterodynamic and evolutionary significance.

The present data indicate an immunosuppression of chicks during sub-clinical infection. It is thought that CIAV induces immunosuppression and can render adult birds susceptible to a variety of secondary infections (viral, bacterial or fungal origin); can depress vaccinal immunity against other poultry pathogens; may enhance vaccination reactions; aggravates the residual pathogenicity of attenuated vaccine viruses and increases the chances of the emergence of variant viruses thus leading to vaccine failure/outbreaks. Thus, the virus could play an important role both in the occurrence of various diseases in field and backyard rearing systems as well as in many of the diseases encountered in flocks raised under the high-density conditions of modern poultry production.

Taken together, these findings confirm that lymphoid tissues are the primary targets for CIAV infection and peak levels of virus were found in the blood followed by the thymus and other tissues on all post-infection days. Varying responses with regard to individual cytokines were observed indicating the immunosuppressive nature of the virus; levels of IL-2 and IL-1 decreased significantly. The increase in IFN- γ peaked before the actual viral peak in the blood at 10 dpi while IFN- γ and viral load peaked simultaneously in the spleen at 15 dpi. Virus-specific antibodies appeared at 15 dpi and were found to be strongly associated with viral load regression, although virus remained in the various tissues until the end of the experiment (25 dpi). A depression in the IL-2 levels was observed indicating immunosuppression and a delayed and inefficient virus clearance from the blood and other tissues. However, cytokine expression levels and their secretion from particular cell types like monocytes, heterophils, CD4⁺T, CD8⁺ T cells and others during CIAV infections remains to be elucidated. This would provide further insights into the immunopathological mechanisms underlying the establishment of virus pathogenesis. Also, the persistence of virus even after the appearance of significantly higher antibody levels needs to be examined further to understand the mechanisms of latent viral infection in the case of CIAV, its role both in the spread of the disease under field conditions as well as its association and interaction with other lymphoid oncogenic viruses like Marek's disease virus, in order to determine their evolutionary significance.

Acknowledgement

The authors are thankful to the Director of the IVRI, Izatnagar for providing the facilities necessary for carrying out this research work. Thanks are also due to ICAR-NAE and DBT Projects, New Delhi for strengthening facilities engaged in CIAV research at the IVRI, Izatnagar.

REFERENCES

- Abdul-Careem MF, Hunter BD, Nagy E, Reada LR, Sanei B, Spencer JL, Sharif S (2006): Development of a real-time PCR assay using SYBR Green chemistry for monitoring Marek's disease virus genome load in feather tips. *Journal of Virological Methods* 133, 34–40.
- Adair BM (2000): Immunopathogenesis of chicken anemia virus infection. *Developmental and Comparative Immunology* 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 Diseases* 35, 783–792.
- Basaraddi MS, Dhama K, Wani MY, Sawant PM, Tiwari R, Kumar D, Singh SD, Singh R (2013): Downregulation in cytokines profiles and immunopathological changes in chicks infected with chicken infectious anaemia virus. *African Journal of Microbiology Research* 7, 2464–2474.
- Beadling CB, Smith KA (2002): DNA array analysis of interleukin-2-regulated immediate/early genes. *Medical Immunology* 1, 2.
- Beadling C, Johnson KW, Smith KA (1993): Isolation of interleukin 2-induced immediate-early genes. *Proceedings of National Academy of Sciences, U.S.A.*, 90, 2719–2723.
- 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. *Transboundary and Emerging Diseases* 58, 458–460.
- Cardona CJ, Oswald WB, Schat KA (2000): Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. *Journal of General Virology* 81, 2067–2075.
- Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162, 156–159.

- Davidson I, Artzi N, Shkoda I, Lublin A, Loeb E, Schat KA (2008): The contribution of feathers in the spread of chicken anemia virus. *Virus Research* 132, 152–159.
- De Herdt P, Van Den BG, Ducatelle R, Uyttebroeck E, Schrier C (2001): Epidemiology and significance of chicken infectious anemia virus infections in broilers and broiler parents under non-vaccinated European circumstances. *Avian Diseases* 45, 706–708.
- Dhama K (2002): Pathogenicity 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. *Indian Journal of Veterinary Pathology* 32, 158–167.
- Goryo M, Suwa T, Umemura T, Itakura C, Yamashiro S (1989a): Ultrastructure of bone marrow in chicks inoculated with chicken anemia agent (MSB1-TK5803 strain). *Avian Pathology* 18, 329–343.
- Goryo M, Hayashi S, Yoshizawa K, Umemura T, Itakura C, Yamashiro S (1989b): Ultrastructure of the thymus in chicks inoculated with chicken anaemia agent (MSB1-TK5803 strain). *Avian Pathology* 18, 605–617.
- 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 Diseases* 44, 803–808.
- Haridy M, Sasaki J, Okada K, Goryo M (2012): Persistence of inclusions and antigens of chicken anemia virus in Marek's disease lymphoma. *Research in Veterinary Science* 93, 1353–1360.
- Hoerr FJ (2010): Clinical aspects of immunosuppression in poultry. *Avian Diseases* 54, 2–15.
- Hoop RK (1992): Persistence and vertical transmission of chicken anaemia agent (CAA) in experimentally infected laying hens. *Avian Pathology* 21, 493–501.
- Hoop RK, Reece RL (1991): The use of immunofluorescence and immunoperoxidase staining in studying the pathogenesis of chicken anaemia agent (CAA) in experimentally infected chickens. *Avian Pathology* 20, 349–355.
- 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. *Journal of Virology* 66, 7383–7388.
- Kaffashi A, Noormohammadi AJ, Allott ML, Browning GF (2006): Virus load in 1-day-old and 6-week-old chickens infected with chicken anaemia virus by the intraocular route. *Avian Pathology* 35, 471–474.
- Kaiser P, Staheli P (2008): Avian cytokines and chemokines. In: Davison F, Kaspers B, Schat KA (eds.): *Avian Immunology*. Academic Press, Elsevier Ltd., New York. 203 pp.
- Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using realtime quantitative PCR and the 2^{-CT} method. *Methods* 25, 402–408.
- Markowski-Grimsrud CJ, Schat KA (2003): Infection with chicken anemia virus impairs the generation of antigen-specific cytotoxic T lymphocytes. *Immunology* 109, 283–294.
- McConnell CD, Adair BM, McNulty MS (1993a): Effects of chicken anemia virus on cell-mediated immune function in chickens exposed to the virus by a natural route. *Avian Diseases* 37, 366–374.
- McConnell CD, Adair BM, McNulty MS (1993b): Effects of chicken anemia virus on macrophage function in chickens. *Avian Diseases* 37, 358–365.
- McNulty MS (1991): Chicken anemia agent: a review. *Avian Pathology* 20, 187–203.
- McNulty MS, Connor TJ, McNeilly F, Kirkpatrick KS, McFerran JB (1988): A serological survey of domestic poultry in the United Kingdom for antibody to chicken anemia agent. *Avian Pathology* 17, 315–324.
- McNulty MS, McIlroy SG, Bruce DW, Todd D (1991): Economic effects of subclinical chicken anemia agent infection in broiler chickens. *Avian Diseases* 35, 263–268.
- Miller MM, Schat KA (2004): Chicken infectious anaemia virus: an example of the ultimate host-parasite relationship. *Avian Diseases* 48, 734–745.
- Miller M M, Jarosinski K W, Schat KA (2005): Positive and negative regulation of chicken anemia virus transcription. *Journal of Virology* 79, 2859–2868.
- Natesan S, Kataria JM, Dhama K, Rahul S, Baradhvaj N (2006a): Biological and molecular characterization of chicken anaemia virus isolates from Indian origin. *Virus Research* 118, 78–86.
- Natesan S, Kataria JM, Dhama K, Bhardwaj N, Sylvester A (2006b): Anti-neoplastic effect of chicken anemia virus VP3 protein (apoptin) in Rous sarcoma virus-induced tumours in chicken. *Journal of General Virology* 87, 2933–2940.
- Nayabian H, Mardani K (2013): Molecular characterisation of the chicken anemia viruses isolated from broiler farms of west Azerbaijan, Iran. *Avian Pathology*. DOI:10.1080/03079457.2013.766668.
- Niesters HG (2001): Quantitation of viral load using realtime amplification techniques. *Methods* 25, 419–429.
- Noteborn MHM (2004): Chicken anaemia virus induced apoptosis: underlying molecular mechanisms. *Veterinary Microbiology* 98, 89–94.

- Oluwayelu DO (2010): Diagnosis and epidemiology of chicken infectious anemia in Africa. *African Journal of Biotechnology* 9, 2043–2049.
- Ponchel F, Toomes C, Bransfield K, Leong FT, Douglas SH, Field SL, Bell SM, Combaret V, Puisieux A, Mighell AJ, Robinson PA, Inglehearn CF, Isaacs JD, Markham AF (2003): Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnology* 13, 18.
- Pope CR (1991): Chicken anaemia agent. *Veterinary Immunology and Immunopathology* 30, 51–65.
- Pringle CR (1999): Virus Taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Archives in Virology* 144, 2065–2070.
- Schat KA (2003): Chicken infectious anemia. In: Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (eds.): *Diseases of Poultry*. 11th ed. Iowa State University Press, Ames, USA. 182–202.
- Schat KA (2009): Chicken infectious anaemia. *Current Topics in Microbiology and Immunology* 331, 151–183.
- Senthilkumar N, Kataria JM, Dhama K, Rahul S (2002): Day old specific pathogen free chick infected with chicken anaemia virus become carriers during the growing period. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* 23, 137–140.
- 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 Diseases* 37, 324–338.
- Smyth JA, Moffett DA, Connor TJ, McNulty MS (2006): Chicken anaemia virus inoculated by the oral route causes lymphocyte depletion in the thymus in 3-week-old and 6-week-old chickens. *Avian Pathology* 35(3), 254–259.
- Snoeck CJ, Komoyo GF, Mbee BP, Nakoune E, Le Faou A, Okwen MP, Muller CP (2012): Epidemiology of chicken anaemia virus in Central African Republic and Cameroon. *Virology Journal* 9, 189. DOI:10.1186/1743-422X-9-189.
- Tan J, Tannock GA (2005): Role of viral load in the pathogenesis of chicken anaemia virus. *Journal of General Virology* 86, 1327–1333.
- Tichopad A, Didier A, Pfaffl MW (2004): Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. *Molecular and Cellular Probes* 18, 45–50.
- Todd D (2000): Circoviruses: immunosuppressive threats to avian species: a review. *Avian Pathology* 29, 373–394.
- Vaziry A, Silim A, Bleau C, Frenette D, Lamontagne L (2011): Chicken infectious anaemia vaccinal strain persists in the spleen and thymus of young chicks and induces thymic lymphoid cell disorders. *Avian Pathology* 40, 377–385.
- Yuasa N (1992): Effect of chemicals on the infectivity of chicken anaemia virus. *Avian Pathology* 21, 315–319.

Received: 2013–05–17

Accepted after corrections: 2014–01–28

Corresponding Author:

Kuldeep Dhama, Principal Scientist, Indian Veterinary Research Institute, Division of Pathology, Avian Diseases Section, Izatnagar, Bareilly (Uttar Pradesh), 243 122 India

Tel.: +91 581 2310074 (off.), +919837654996, Fax +91 581 2303284, +91 581 2302179, E-mail: kdharma@rediffmail.com