Pathogenesis of Two Chicken Infectious Anaemia Virus (CIAV) Vaccines in Lymphoid and Non-lymphoid Organs

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1. Abstract

Chicken infectious anaemia virus (CIAV) is one of the most important ubiquitous immunosuppressive pathogen in chicken, which leads to great economic loss in poultry industry worldwide. Here we experimentally investigated the pathogenesis of two live attenuated chicken anaemia virus vaccines. One hundred twenty five 1-day-old specific pathogen-free (SPF) chicks were allocated into five groups (25 chicks each). Group 1 was orally vaccinated with attenuated CIAV vaccine strain in drinking water. Group 2 was kept as a contact non-vaccinated group to group 1. Group 3 was intramuscularly vaccinated with attenuated CIAV vaccine strain. Group 4 was kept as a contact non-vaccinated group to group 3, and group 5 served as a control group. Three chickens from each group were sacrificed at 3rd, 7th, 15th, 22nd, 29th, 36th and 41st days post vaccination (dpv). Tissue specimens from bone marrow, thymus, spleen, cecal tonsils, bursa of Fabricius and liver were collected for histopathology, lesion scoring and immunohistochemistry fluorescence detection of CIAV antigen. Liver specimens were also collected at 7th, 15th, 21st dpv for real time-PCR (qPCR), to quantitate the CAV genomic DNA. Mortalities occurred in both vaccinated groups and their contact groups. Group 4 had the highest no. of mortalities. Frothy diarrhea, ulcer, S/C hemorrhages, cannibalism and signs of mild depression were the most developed clinical signs in group 1, 2, 3 and 4. Pale bone marrow and atrophy of thymus were the most characteristic post-mortem changes in group 1, 2, 3 and 4. Microscopically, changes characteristic for CIAV were observed, and the most affected organs were bone marrow, thymus, liver and spleen; respectively. The demonstrated histopathological lesions started as early as the 3rd dpv in almost all organ of concern in all 4 groups. Immunofluorescence labelling of CIAV antigen demonstrates virus in all examined organs in both vaccinated and contact groups, with the bone marrow as the strongest positive. CIAV genome detected in livers of vaccinated and contact groups at concerned days. Indeed, live attenuated CIAV vaccines (either by oral or parenteral route) are pathogenic to 1-day old chicks and they could spread horizontally and transmitted to contact chicks, inducing mortalities and immunosuppression.

Key words: *Chicken infectious anemia, CIAV, CAV vaccines, Histopathology, Immunofluorescence, Immunosuppression, Contact chicks, qPCR (real time).*

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2. Introduction

Chicken infectious anaemia (CIA) is a contagious immunosuppressive disease with a ubiquitous nature virtually all over the world. The disease is caused by highly resistant chicken infectious anaemia virus (CIAV) [1, 2, 3], which is a small, non-enveloped, icosahedral virus with single stranded covalently closed circular DNA [4, 5, 6, 7].

The virus first isolation was in Japan by Yuasa *et al.* [8]. A condition of hematopoietic destructive lesions with drastic reduction in packed cell volume and lack of hematopoiesis in chickens was described, suggesting that CIAV was present in chickens at least as early as 1970 [9].

It belongs to genus *Gyrovirus*, which was reassigned lately to the family *Anelloviridae* [10, 11]. Anaemia and acquired immunosuppression following CIAV infection are known to be due to apoptotic effect of VP3 viral protein (apoptosis-inducing protein during virus replication) on haematopoietic tissues and precursor lymphocytes of the thymus led to aplasia of the bone marrow and generalized lymphoid atrophy [12, 13, 14].

Until recently, the chicken has been considered the only recognized natural host and the main host, but a serological survey has revealed the pervasion of CIAV in domestic and wild birds [15]. Also, ostrich could be considered a reservoir for CIAV after its seromolecular detection [16]. Batheja *et al.* [17] suggested that CIAV could be a potential threat to humans.

Chicken anaemia pathogen has two modes of infection, vertically from infected breeder to their progeny and horizontally by contact exposure with infected chickens or fomites [18].

CIAV induce a clinical disease in progeny around 2 to 4 weeks of age (that lack maternal antibodies) and subclinical disease after 3 weeks of age [19] threatening the poultry industry worldwide, directly due to high mortalities with anaemia, atrophied thymus, replacement of haematopoietic cells with adipose tissue in bone marrow, reduced weight gain, subcutaneous and intramuscular haemorrhages (gangrenous dermatitis) in clinical form [20, 21] or indirectly as a sequel to immunosuppression associated with subclinical form that predisposing the host to secondary bacterial, viral or fungal infections (CIAV impairs the generation of pathogen-specific cytotoxic T lymphocytes (CLT) that have important role in adaptive cell mediated immunity to several secondary pathogens), growth retardation, vaccination failures [22, 23, 24, 25].

There are also other significant risks from using SPF eggs contaminated with CIAV genome (Specific-pathogen free flocks can be positive for viral DNA even in the absence of antibodies and virus can be reactivated and disseminated in eggs) in production of all poultry vaccines for administration in birds less than seven days of age [26, 27].

Previous studies showed that CIAV can enhance the pathogenicity of other viruses such as Marek's disease virus and IBDV [28, 29, 30, 31, 32, 2]. Aly and Hussein *et al.* [33, 34] reported CIAV first isolation in Egypt.

The most reliable control strategy against CIAV is good poultry management practice concomitant with vaccination of breeder hens before production time to protect progeny from clinical symptoms of disease through maternal antibodies [35].

Despite of widespread vaccination of breeders with available vaccines (inactivated and live attenuated) diminishing clinical disease, chickens are still susceptible to CIAV infection with subclinical symptoms [28].

Several reports have been published in the last few years documenting novel CIAV strains despite broad spectrum vaccination of breeder chicken [36, 37, 38, 3]. Also commercially available attenuated CAV vaccines were reported to have an adverse effect by inducing anaemia and lymphoid lesions which could be attributed to

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reversion to virulence [39, 40, 41, 42]. Irreversible attenuation of CIAV has proven to be difficult [43, 44].
Therefore, studying pathogenesis and pathogenicity of available attenuated vaccines against CIAV by oral and parenteral route were discussed in the present paper in one-day-old SPF chicks.

3. Materials and Methods

Vaccines used in the experiment: two different types of commercially available live attenuated CIAV vaccines were used:
AviPro® Thymovac Cux-1: Lyophilized live vaccine, for oral administration through drinking water, 2500 doses, one dose contains minimum $10^{4.5}$ $TCID_{50}$ of live chicken anaemia virus (CIAV), strain Cux-1, Lohmann animal health, Cuxhaven Germany.
Nobilis® CAV-P4: A freeze-dried pellet, for parenteral route administration (intramuscular or subcutaneous route), 1000 doses, active components per dose, live CIAV strain 26P4: ≥ 3.0 $\log^{10}$ $TCID_{50}$, Intervet international, Boxmeer, Netherlands.
Experimental design: An entire number of 125 one-day-old Specific-pathogen-free (SPF) chicks (were obtained from Nile SPF, National Project for production of Specific Pathogen Free Eggs, agriculture research center, Ministry of agriculture, Kom-oshem, Fayom, Egypt) were used in this study.
Chicks were floor raised (in a previously cleaned, disinfected, strictly isolated, 3 separated rooms with fresh pine sawdust was used as bedding inside rooms) under hygienic condition in The Department of Pathology, Faculty of Veterinary Medicine, Cairo University. Chicks were supplied with water and ration *ad libitum*.
Before the experimental study, chicks were tested using a commercially available CIAV ELISA kit (BioChek, Reeuwijk, The Netherlands) to ensure that they were devoid of maternal antibodies against CIAV [26], ELISA was used following the manufacturing instructions.
Chicks were set into five equal groups; 25 chicks per each. **Group 1** was vaccinated directly in the oral cavity with one dose attenuated CAV-Cux-1 vaccine strain (in a total volume of 1 cm drinking water) at one day old age. **Group 2** was served as a contact, non-vaccinated group, kept commingled with group 1. **Group 3** was vaccinated with 0.2 ml, one dose attenuated CAV-26P4 vaccine strain intramuscularly at one day old age. **Group 4** was served as a contact, non-vaccinated group, kept commingled with group 3. **Group 5** served as a control untreated group (negative control).
Clinical signs and mortalities were recorded daily during the experiment period. Three chicks were sacrificed humanely from each group at 3rd, 7th, 15th, 22nd, 29th, 36th and 41st days-post-vaccination (dpv) to collect tissue specimens from bone marrow, thymus, spleen, cecal tonsils, bursa of Fabricius and liver for histopathology and immunohistochemistry (IHC-fluorescence).
Dead chick's organs (bone marrow, thymus, spleen, cecal tonsils, bursa of Fabricius, liver, pancreas, proventriculus, duodenum, lung, kidney and heart) were also collected and subjected to paraffin IHC-fluorescence staining.
Liver specimens from three chicks were collected aseptically, stored at -80°C, at 7th, 15th, 21st dpv for subsequent nucleic acid extraction and real time-PCR (qPCR) viral quantification.
Histopathology : 10% neutral buffered formalin was used to fix collected tissue specimens, then processed routinely, dehydrated and embedded in paraffin wax, sectioned 5-µm-thick and stained with hematoxylin and eosin H&E [45]. Slides were examined by Carl Zeiss Axio Lab. A1, GmbH Microscopy and figures captured by AxioCam MRc5 camera using ZEN 2012 (blue edition) imaging software for microscopy.

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Histopathological score lesions for all examined organs were done as described by Gibson-Corley *et al.* [46]. Ordinal scoring system showing an ordered progression in lesion severity, lesions were scored as 0 (normal), 1 (mild), (2 moderate), 3 (severe), 4 (very severe). Three random optical fields were examined and scored and then the mean of the three fields was calculated.

Immunohistochemistry (IHC-fluorescence technique):

Using vaccine strain (Nobilis®, CAV P4), Hyperimmune serum against CIAV was raised in albino rabbit following the Laboratory Manual for the Isolation and Identification of Avian Pathogens [47], after series of injections, and the serum was collected when titers were high. Inactivation was done at 56 C˚ water bath for 30 minutes.

For IHC-fluorescent, tissue sections on poly-L-Lysine coated slides were dewaxed and rehydrated following conventional procedures, heat induced antigen retrieval was done, blocking of non-specific protein binding and endogenous peroxide (to block non-specific staining between the primary antibodies and the tissue) was followed by overnight incubation in primary antibody (Rabbit anti CIAV Ig polyclonal serum already mentioned above). It was followed by incubation with secondary antibody (Fluorescein isothiocyanate - conjugated Antibody to Rabbit IgG (H+L) produced in Goat Catalog No. 172-1506 (KPL), Gaithersburg, USA) for 30 min in dark place. Counterstaining was done using diamidino phenylindole solution (DAPI), DAPI binds to DNA and is a convenient nuclear counterstain (5 mg/ml, Sigma) for 10 min, after washing, aqueous glycerol was used as a mounting media. For negative control, all steps were followed with omission of primary antibodies. The slides were examined under fluorescent microscope (Carl Zeiss Axio Lab. A1 with filter set 09). Antibodies were standardized at the dilution of 1:200, 1:400 respectively for the above technique. The applied protocol was adapted from the previously reported by [48, 49].

Real-time-PCR (qPCR):

Nucleic acid extractions: CIAV DNA was extracted from liver samples which were stored at -80°C. The frozen samples were thawed and homogenized in saline containing 200 mcg/ml Streptomycin and 2000 IU/ml Penicillin making ground homogenized, 20% W/V, and centrifuged at 3000 rpm for 15 minutes [47].

Total DNA from tissue suspensions supernatants were extracted using the PathoGene-spin™ DNA/RNA Extraction Kit using their recommended protocol (iNtRON Biotechnology, Seongnam, Korea). The eluted DNA was measured in a spectrophotometer (the NanoDrop ND-1000 (NanoDrop, Wilmington, DE).

qPCR protocol: Primers and probe (Table 1) used for the detection of CIAV were custom made (Sigma) as described before [50]. Amplification and data acquisition was carried out using AB Applied Biosystems real-time PCR machine.

Under the following cycling conditions, 95°C for 10 min followed by 45 cycles at 95°C for 15 sec (consisting of denaturation) and 60°C for 1 min (annealing / extension). Results were analyzed using an Analysis computer system, V 2.2.2 software supplied with AB Applied Biosystems [50].

To calculate the copy number of viral nucleic acid, a standard curve was generated using **Nobilis® CAV-P4** CIAV vaccine (fig. 1), and dilutions with a known number of virus copies (copy number $TCID_{50}$ log^{10} / µl) were prepared. Then dilutions were run and the results were used to plot a standard curve. A linear equation was obtained from the standard curve ($y = -4.5929x + 37.79$ with $R^2 = 0.9751$), which was used to quantify the viral target in each sample.

Statistical analysis: was conducted with the Statistical Package for Social Science [51] to determine if variables differed between groups, according to Snedecor and Cochran [52]. The Shapiro-Willk test was used to test the normal distribution of the

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data before statistical analysis was performed. A non-parametric test called Kruskal Wallis Test was used to determine the statistical differences between histopathological lesion scores. Probability values of less than 5% ($P < 0.05$) were considered significant.

Two-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.0.0 for Windows, GraphPad Software, San Diego, California USA. A two-way ANOVA was performed to analyze the effect of different groups and dpv on $TCID_{50}$ of CIA virus of liver samples.

4. Results

Clinical signs and gross pathology: No mortalities, clinical signs, cannibalism or gross lesions were recorded in negative control (group 5) allover the whole experimental times.

Mortalities were recorded at 22 dpv at group 1 (attenuated Cux-1 strain) and at 6 dpv at group 2 (contact group for group1) in one chick; respectively. Whereas two chicks were died in group 3 (attenuated CAV-26P4 strain) at 12 and 15 dpv. Four chicks died in group 4 (contact group for group 3) at 13, 15 and 18 dpv.

Chicks in groups 1, 2, 3 and 4 exhibited depression, reluctance to move and pallor of combs (Fig. 2a). Frothy diarrhea appeared in few chicks at 6 and 8 dpv in group 1, 2, 3 and 4, then became more severe at 9 dpv and then continued until the end of the experiment. Uneven sizes of chicks were also observed. Vent, wing and toe cannibalism (Fig. 2b, 2c) was observed at 16 and 4 dpv and continued until 44 and 21 dpv in group 1 and 2; respectively. While group 3 and 4, cannibalism began at 7 dpv and ended at 32 dpv.

Hemorrhagic streaks on wings, breast and thigh muscles (Fig. 2d) at 36 and 41 dpv were noticed in group 1, 2, 3 and 4 but more severe in group 4.

Congested thymus with dark reddish colour (Fig. 2e) were noticed at 3 dpv in nearly all groups and started to become atrophied in some chicks (Fig. 2f) at 15 dpv in all groups. Sometimes thymic lobes showed petechial hemorrhages.

The bone marrow appeared fatty, pale, and yellow in colour and sometimes between pink and dark red (Fig. 2g, 2h). Bone marrow gross lesions began at 15 dpv in group 1, 2, 3 and 4.

The liver appeared predominantly engorged in all groups, ranging in colour from normal, mottled with dark red to red or pale and occasionally showing leopard hemorrhages (Fig. 2i).

The bursa of Fabricius showed no gross lesions except for a transparent exudate at 36 and 41 dpv with the bursa swollen in few chicks, as well as at 15 dpv in all groups (Fig. 2k). Hemorrhagic petechiae were observed on the lining mucosa of proventriculus (Fig. 2L), duodenal wall and cecal tonsils (Fig. 2j) in all groups starting at 36 dpv.

Histopathology, score and immunofluorescence:

Examined organs of negative control (group 5) at all sampling time-points were apparently normal (Fig. 3a, 4a, 5a, 6a, 7a, 8a), with negative expression of CIAV antigen was observed by IHC-fluorescence (Fig. 3f).

Kruskal Wallis test revealed a statistically significant difference (Asymp. Sig. = 0.013, 0.041, 0.039 for bone marrow at 3, 29 and 36 dpv; respectively **(Table (2), Fig. (9A))**, Asymp. Sig. = 0.027 for thymus at 29 dpv **(Table (3), Fig. (9B))**, Asymp. Sig. = 0.031 for spleen at 36 dpv **(Table (4), Fig. (9C))**, Asymp. Sig. = 0.027, 0.012 for bursa of Fabricius at 36 and 41 dpv; respectively **(Table (5), Fig. (9D))**, Asymp. Sig. = 0.012, 0.012, 0.012 for cecal tonsils at 7, 15 and 41 dpv; respectively **(Table (6), Fig. (9E))** and Asymp. Sig. = 0.054 for liver at 36 dpv **(Table (7), Fig. (9F))**) histopathological scores. The test revealed insignificant difference (Asymp. Sig. ≥ 0.055 for other dpv in all organs) histopathological scores.

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Bone marrow at 3 dpv, lesions started to appear in group 2 as moderate depletion of haemopoietic cells (pancytopenia).

At 7 dpv, nearly all groups showed diffuse depletion of erethropoeitic and granulopoeitic cells, replaced by adipocytes (Fig. 3d).

From 15 dpv extended to 41 dpv, bone marrow showed moderate to very severe hypohemopoietic cells replaced by lipocytes in all groups (Fig. 3b, 3c, 3e).

Moreover group 1 and 2 at 22, 29 and 36 dpv, showed multifocal reticular cells aggregation in addition to congested blood vessels with hemorrhage and focal bony spicules degeneration. Group 1 at 41 dpv showed hyperplasia of erethropoeitic cells.

At 29 dpv, group 1 was significantly different from group 4, as group 1 showed very severe depletion of haemopoietic cells while group 4 was moderate. At 36 dpv group 4 was apparently normal.

Bone marrow have the strongest viral antigen expression, demonstrating a granular pattern green-apple staining mainly in nuclei of haemopoietic cells and reticular cells (Fig. 3g, 3h, 3i, 3J).

Thymus of group 1, 2, 3 and 4 at 3, 7, 15, 22 and 41 dpv, exhibited mild to severe depletion of lymphocytes with congested blood vessels, pronounced hemorrhage and mild degeneration of thymocytes (Fig. 4b, 4c, 4d, 4e).

Concerning 29 dpv, severe depletion of lymphocytes in cortex and medulla with degeneration and necrosis of Hassal's corpuscles cells in group 4 (group 4 showed a significant differences with group 2, 3 and 5 and lesion scoring was the highest). The same lesions were also observed in group 3 and 4 at 36 dpv.

Thymus exhibited moderate expression of green-apple stained CIAV in both cortical zone and medulla mainly within lymphoid elements (Fig. 4f).

Spleen: splenic tissue showed mild to moderate depletion of lymphoid follicles with mild focal hemorrhage from 3 extended to 41 dpv (Fig. 5b, 5c, 5d, 5e).

All groups at 22 dpv showed mild hemorrhage and thickening of splenic capsule. Concerning 29 dpv, group 1 and 4 showed proliferation of sheathed capillaries in addition to depletion of spleenocytes and focal hemorrhage in group 2 and 3.

At 36 dpv, mild to moderate depletion of lymphocytes in spleen, in group 3 and 4 (group 4 showed a significant differences with group 1, 2 and 5 with a moderate lesion scoring) and thickening in the wall of some blood vessels were detected in group 1.

Marked diffuse expression of green-apple stained viral antigen was detected in spleens mainly within lymphocytes (Fig. 5f).

Bursa of Fabracious exhibited lesions such as, mild to moderate depletion of lymphocytes with interfollicular oedema and corrugated hyperplasia of lining epithelium in addition to epithelization in almost all groups, some follicles had few heterophils infiltration, at 3 and 7 dpv (Fig. 6b, 6d).

Nearly all bursae of all groups at 15 dpv exhibited mild to moderate depletion of lymphocytes with hyperplasia of plical lining epithelium, moreover mild interfollicular oedema were shown in group 2 and 4 (Fig. 6c). At 22 dpv mild depletion of lymphocytes with focal hyperplasia of lining epithelium in few examined bursae in all groups.

All groups at 29 dpv, bursa had mild hyperplasia of lining epithelium with mild depletion of lymphocytes and proliferation of corticomedullary lining epithelium (epithelization) (Fig. 6e). At 36 dpv, group 1, 3 and 4 showed mild hyperplasia of the lining epithelium with moderate depletion of lymphocytes (group 2 was apparently normal, showed significant differences with group 3 and 4 which have moderate lesion scoring). At 41 dpv, group 4 showed mild focal hyperplasia of lining epithelium giving it a significant differences with other apparently normal groups (1, 2, 3 and 5).

Mild expression of green-apple stained viral antigen was detected in bursae and

mainly observed within lymphocytes in follicular cortex area (Fig. 6f).

Cecal tonsils at 3 and 7 dpv exhibited hyperplasia of the lining epithelium with activation of goblet cells and mild extravasated RBCs in lamina propria.

Mucous glands activation admixes with necrosis of the tips of few villi (group 4 at 7 dpv showed significant differences with group 1, 2, 3 and 5, exhibited mild lesion scoring against apparently normal other groups).

At 15 dpv group 1 and 4 showed mild proliferation of lymphocytes with shortening of few villi, showing significant differences with other apparently normal groups. All groups' cecal tonsils at 22 dpv, showed hyperplasia of lining epithelium, with some showed activation of mucous glands and others showed its focal atrophy (Fig. 7e), with pronounced hemorrhage in lamina propria.

Concerning 29 dpv group 1, 2 and 4 demonstrated hyperplasia of the lining epithelium with activation of mucous glands and extravasated RBCS in lamina propria in addition to depletion and degeneration of lymphocytes.

Concerning 36 dpv group 2, 3 and 4 exhibited mild to moderate hyperplasia with vacuolar degeneration of lining epithelium and pronounced hemorrhage in lamina propria (Fig. 7d), group 1 characterized by necrosis of the lining epithelium. Group 3 and 4 exhibited apparently normal architecture at 41 dpv, while group 1 and 2 showed depletion of lymphocytes with disseminated submucosal hemorrhage (Fig. 7b, 7c) giving them a significant differences with group 3 and 4.

Mild expression of green-apple stained viral antigen was detected in cecal tonsils and mainly observed within lymphocytes and RBCs in lamina propria (Fig. 7f).

Liver at 3 and 7 dpv exhibited mild to moderate congested blood vessels associated with pronounced vacuolar degeneration of hepatocytes and necrosis around blood vessels (Fig. 8b).

Livers of all groups at 15 dpv, exhibited mild to moderate congested blood vessels and sinusoids associated with perivascular aggregation of mononuclear cells, Focal vacuolar degeneration of hepatocytes were also observed. Liver in all groups at 22 dpv showed mild congestion with thickening of some blood vessels wall. Moreover thrombosis of some veins and microthrombi within sinusoids were observed.

Mild degeneration of hepatocytes, hyperplasia of bile ductules, focal fibrous connective tissue proliferation and focal infiltration with mononuclear cells (Fig. 8c) were showed in group 1.

Concerning livers of group 2, 3 and 4 at 29 dpv, exhibited congested blood vessels with thrombus formation and interstitial hemorrhage.

At 36 dpv, livers showed dilated blood vessels with large thrombus and microthrombi within sinusoids in group 3 and 4. Focal hepatocellular necrosis near blood vessels were observed also in group 2 and 3. Previous lesions were observed also in group 1 in addition to hyperplasia of bile ductules and focal aggregation of mononuclear cells (group 1 and 4 showed a significant difference with group 5 with a high moderate score lesion).

At 41 dpv, groups exhibited congested blood vessels containing large thrombus with thickened wall, interstitial hemorrhage, and aggregation of mononuclear cells in addition to proliferation of bile ductules (Fig. 8d).

Intranuclear inclusion bodies were observed in hepatocytes of group 1 at 7, 15 and 22 dpv, in group 2 at 7 dpv and in group 4 at 22 dpv (Fig. 8e).

Moderate expression of green-apple stained viral antigen was detected in liver and mainly observed within blood vessels and sinusoids (Fig. 8f).

All lymphoid and non-lymphoid organs of groups 1, 2, 3 and 4 exhibited CIAV antigen by IHC-fluorescence.

Proventriculus showed great expression of green-apple stained viral antigen, was

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detected in mucosa, mainly observed within lymphocytes (Fig. 10A).
Pancreas showed mild aggregation of green-apple stained viral antigen in pancreatic acini (Fig. 10B).
Duodenum showed pronounced expression of green-apple stained viral antigen, was detected within lymphocytes constituting the lamina propria of villi (Fig. 10C).
A very mild expression was detected in kidneys (Fig. 10D). Pronounced severe expression of green-apple stained viral antigen was detected within lymphocytes and RBCs in lung tissue of dead chicks by IHC-fluorescence (Fig. 10E). While heart showed mild expression (Fig. 10F).

Viral loads in liver by qPCR: CIAV genome was detected and quantified (in three liver samples from each group) in all vaccinated groups, group 1 (attenuated Cux-1 strain) and group 3 (attenuated CAV-26P4 strain), and contact groups, group 2 (contact group for group 1) and group 4 (contact group for group 3) and the results of qPCR were as shown in table (8) and (Fig. 11). Expressed as mean Log_{10} number of viral $TCID_{50}$ at 7, 15 and 21 day-post-vaccination (dpv).
A two-way ANOVA revealed that, there was no statistically significant interaction between the effects of groups and dpv ($F(6, 24) = 0.1276$, $p = 0.9917$). Simple main effects analysis showed that groups did not have a statistically significant effect on $TCID_{50}$ of CIA virus of liver samples ($p = 0.6985$). Simple main effects analysis showed that dpv did not have a statistically significant effect on $TCID_{50}$ of CIA virus of liver samples ($p = 0.3288$).

5. Discussion

Chicken infectious anaemia is an important, immunosuppressive, ubiquitous and highly resistant viral disease of chickens characterized clinically by anaemia and immunosuppression. It can be transmitted horizontally and vertically. It is from the transovarian diseases.
Vaccination of the parent flock before laying was recommended to prevent clinical form of disease in newly hatched chicks via transferred maternal antibodies until 3 weeks of age preventing vertical transmission to progeny, however, viral DNA can still be transmitted [53, 19, 54, 55], also it can't prevent subclinical infection in elder chickens, virus horizontal transmission and immunosuppression after maternal antibodies wane [56].
The virus can persist within the infected body for a long period of time [57]. It was also found that CIAV persist dormant in gonads and could be reactivated at sexual maturity and disseminated in eggs which is considered an obstacle in SPF eggs industry, and some vaccines production for animal, birds and human that required eggs free from CIAV [26, 27].
Despite maternal immunity and vaccination, recent epidemiological data showed that CIAV causes problems in commercial poultry farms with novel CIAV strains [29, 36, 38, 3, 58].
Vaccination against CIAV is achieved by either live or inactivated vaccines [59, 17].
Attenuated CIAV was reported to induce post inoculation histopathological alterations, anaemia, thrombocytopenia and liver affection [40, 42].
Hence, the present study compare the pathogenesis of two CIAV live attenuated vaccines in lymphoid and non-lymphoid organs in order to know the ability of the two vaccines to induce lesions in different organs, the ability to disease-causing potential of contact chicks, Correlate between histopathology, immunohistochemistry and real-time qPCR and to detect the virus distribution in different organs (antigenic intensities in tissues).
In this study mortalities occurred in all groups, with group 4 had the highest number, (4 chicks). Mortalities occurred between 6 and 22 dpv. Previously deaths because of CIAV were reported around 3 to

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4 weeks of age [60, 61, 62]. While mortality between day 1 and 6 of onset of clinical signs was recorded by Adedeji *et al.* [2].

Contact non-vaccinated birds (group 2 and 4) attracted the excreted virus from vaccinated chickens (group 1 and 3) and consequently exhibited the characteristic clinical, necropsy and histopathological lesions of CIAV as well as the vaccinated groups with more severe clinical and post-mortem lesions, Commercial live attenuated vaccines has the risk of being transmitted both horizontally and vertically [17], can revert back to its virulence [63].

The characteristic clinical signs and necropsy findings of CIAV in this study were previously reported for CIAV infection in one-day-old chicks [64] and also reported for live attenuated CIAV-26P4 strain inoculated by I/M. route [40, 42]. On the contrary to the present results Allam *et al.* [42] reported no clinical signs and mild p.m. lesions for live attenuated CAV-cux-1 strain inoculated orally.

Also, clinical signs in groups 1 and 3 appeared at about the same time except for the onset of more severe depression in group 3 which was in lower similarity to Tan and Tannock [65], who reported earlier onset of clinical signs in virus-infected birds intramuscularly than those infected orally. Occurrence of virus was confirmed in dead birds by IHC-fluorescence.

Vent, wing and toe cannibalism seen in chicks in this study may, in most instances, be a sequel of ulcers, subcutaneous, intracutaneous and muscular hemorrhages rather than other causes. CIAV mainly infects hemocytoblast cells of the bone marrow and precursor lymphocytes in cortex of the thymus, since hematopoietic stem cells are precursors of thrombocytes, their depletion leads to thrombocytopenia which causes hemorrhages seen on muscles and different organs [66, 61]. The bursa showed no gross lesions except for a transparent exudate at 36 and 41 dpv with the bursa swollen in few chicks, whilst Toro *et al.* [67] reported pale atrophic bursa in similar cases.

The two attenuated CIAV vaccines in this study, induced histopathological changes in the lymphoid and non-lymphoid organs with the most affected organs being bone marrow, thymus and liver in the four concerned groups. Whereas, the spleen, bursa of Fabracious and cecal tonsils were less affected.

The histopathological changes in almost both lymphoid and non-lymphoid organs were seen on 3 dpv and that is concordance with Smyth *et al.* [68], who reported in a sequential studies that CIAV lesions were first detected in bone marrow, thymus and spleen at 3-4 dpv.

Taniguchi *et al.* [69], recorded depletion in hematopoietic cells from 4-6 day after CIA virus inoculation. While Kuscu and Gurel [66], determined that histopathological alterations in the thymus and bone marrow started 10 days post inoculation. The histopathological changes in both lymphoid and non-lymphoid organs were observed on 7th dpi by Nadeem *et al.* [70].

Histopathological alterations observed in this study, in both lymphoid, (with lymphocytic depletion and bone marrow hypoplasia and aplasia of hematopoietic cells), and non-lymphoid organs, are fully in agreement with the studies of [69, 68, 66, 36, 58, 70].

Previously, Hussein *et al.* [40] reported histopathological alteration in liver, spleen and thymus after inoculation of attenuated vaccinal strain 26P4 in one-day-old chicks. There were a direct correlation between virus expression in tissues and the exhibited histopathological lesions, as during replication, CIA virus induces cell apoptosis due to VP3 (apoptine).

Histological and immunohistochemical studies in the present experiments suggest that attenuated CIAV vaccines following vaccination at one-day-old chicks, replicates in the bone marrow and severely damage the haemopiotic tissues, infect mature T lymphocytes in cortex of the thymus, disseminating the virus to other lymphoid and non-lymphoid organs

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inducing their histopathological lesions [61].

In this study, the observed intranuclear inclusion bodies in hepatocytes were previously reported (but intracytoplasmic) by Engström and Luthman [71].

Intranuclear inclusion is due to the presence of virus antigen in the nucleus during viral replication [68].

Green-apple stained CIAV antigen by IHC-fluorescence, in this bioassay, was expressed in all examined lymphoid and non-lymphoid organs and widely distributed in chick's body shortly after infection. This results come in agreement with the study of Smyth *et al.* [68], who reported antigen expression in nearly all organs and described the presence of antigen in the pancreas. Tan and Tannock [65], reported high CIAV load in the pancreas suggested that it is a primary target organ.

High expression of green-apple stained CIAV antigen within lymphocytes and RBCs in lung tissues from dead chicks of group 2 and 4 by IHC-fluorescence, suggesting that CIAV could be airborne transmitted, infecting precursor lymphocytes in lung tissues.

Lateral transmission via inhalation of contaminated air has been confirmed previously by Islam *et al.* [72]. Further studies are required to determine if the lung is one of the main target organs for infection. While, Tan and Tannock [65] bring the presence of CAV-DNA in most organs back to viraemia after replication in the bone marrow, and this is not an indication that the virus replicate in organs other than tropism tissues.

And finally in order to confirm presence of viral DNA in concerned groups and to detect level of replication, qPCR was employed to quantify CIAV genome copies in liver, the liver has been an excellent site for CIAV recovery from naturally and experimentally induced CIAV infection [40, 73], and the results revealed that viral DNA was detected in livers of all groups 1, 2, 3, and 4 with no significant difference, meaning that, both attenuated vaccinal strains transmitted horizontally to contact groups. Hussein *et al.* [40], previously indicated virus genome in liver after experimental inoculation of attenuated vaccinal strain 26P4.

6. Conclusion

Since CIAV live attenuated vaccine contain living organisms, there is a degree of unpredictability raising some safety and stability concerns.

In the present study, the two live attenuated CIAV vaccines (either by oral or parenteral route) produced pronounced clinical signs and histopathological changes in examined organs, especially in lymphohematopoietic organs, are pathogenic to 1-day-old chicks and they could reverse to virulence, horizontally transmitted to in-contact not treated chicks inducing mortalities and immunosuppression.

Moreover, the lesions of the in-contact groups of the two vaccinal strains showed nearly the same severity as vaccinated or may be more and that CIAV antigen is widely distributed in the host body shortly after infection.

Therefore, recommendation for safer vaccine against CIA virus is needed. Studying ability of vaccinal strain persistence in organs and its ability to produce subclinical infections. Finally, it is not recommended to use CIAV attenuated vaccines in endemic areas.

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7. References

1. Schat, K.A., and V. Van Santen. (2008). Chicken infectious anemia. In: Diseases of Poultry, 12, Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, and D.E. Swayne, eds. Wiley-Blackwell. 211–235.
2. Adedeji AJ, Sati NM, Pewan SB, Ogbu KI, Adole JA, Lazarus DD, IjiwoSJ, Okpanachi A, Nwagbo IO, Joannis TM, Abdu PA (2016). Concurrent infections of chicken infectious anemia and infectious bursal disease in 5 weeks old pullets in Jos, Plateau State, Nigeria.Vet Sci 2(3):60–65.
3. Li, Y., Yan, N., Wang, Y., Liu, A., Liu, C., Lan, X., Yang, B., Gao, Y., Gao, H., Qi, X. and Cui, H., (2021). Molecular evolution and pathogenicity of chicken anemia virus isolates in China. Archives of Virology, 166(2), pp.439-449.
4. Dhama, K., Kataria, J. M., Senthilkumar, N., and Tomar, S. (2004). Differentiation of Indian isolates of chicken anaemia virus by polymerase chain reaction-restriction enzyme analysis. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases, 25(2), 75-79.
5. Dhama, K., Kataria, J. M., and Tomar, S. (2006). Chicken anaemia virus: An Overview. Poultry Pioneer and Guide, 2(6), 17-26.
6. Natesan, S., Kataria, J. M., Dhama, K., Rahul, S., and Baradhwaj, N. (2006). Biological and molecular characterization of chicken anaemia virus isolates of Indian origin. Virus research, 118(1-2), 78-86.
7. Pattison, M., McMullin, P.F., Bradbury, J. M. and Alexander, D. J. (2008). Poultry Diseases. Elsevier, UK.
8. Yuasa, N., Taniguchi, T., and Yoshida, I. (1979). Isolation and some characteristics of an agent inducing anemia in chicks. Avian Diseases, 366-385.
9. Jakowski, R. M., Fredrickson, T. N., Chomiak, T. W., and Luginbuhl, R. E. (1970). Hematopoietic destruction in Marek's disease. Avian diseases, 374-385.
10. Adams, M. J., Lefkowitz, E. J., King, A. M., Harrach, B., Harrison, R. L., Knowles, N. J., ...and Nibert, M. (2016). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). Archives of virology, 161(10), 2921-2949.
11. Rosario, K., Breitbart, M. and Harrach, B., Segalé s J, Delwart E, Biagini P, Varsani A. (2017). Revisiting the taxonomy of the family *Circoviridae*: establishment of the genus *Cyclovirus* and removal of the genus *Gyrovirus*. Archives of Virology, 162, pp.1447-1463.
12. Jeurissen SHM, Wagenaar F, Pol JMA, van der Eb AJ, Noteborn MHM. Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. J Virol. (1992); 66:7383–8.
13. Markowski-Grimsrud, C.J. and Schat, K.A., (2003). Infection with chicken anaemia virus impairs the generation of pathogen-specific cytotoxic T lymphocytes. Immunology, 109(2), pp.283-294.
14. De Smit, M.H. and Noteborn, M.H.M., (2009). Apoptosis-inducing proteins in chicken anemia virus and TT virus. In: de Villiers EM., Hausen H.. (eds) TT Viruses, Current Topics in Microbiology and Immunology, vol 331. Pp.131-149.
15. Farkas, T., Maeda, K., Sugiura, H., Kai, K., Hirai, K., Otsuki, K. and Hayashi, T., (1998). A serological survey of chickens, Japanese quail, pigeons, ducks and crows for antibodies to chicken anaemia virus (CAV) in Japan. Avian Pathology, 27(3), pp.316-320.
16. Gholami-Ahangaran, M., Fathi-Hafshejani, E. and Seyed-Hosseini, R., (2013). Seromolecular study of chicken

Online ISSN: 2537-1045
Print ISSN: 1110-1423

infectious anemia in chickens, ostriches, and turkeys in Iran. Journal of Applied Poultry Research, 22(3), pp.404-409.
17. Batheja, R., Ramamurthy, N., Silva, A.L.D., Pathak, D.C., Singh, A., Marriappan, A.K., Dey, S. and Chellappa, M.M., (2021). Chicken anemia virus infection of poultry and its control by vaccines. The Pharma Innovation Journal; 10(8), pp.773-778
18. Fatoba, A.J. and Adeleke, M.A., (2019). Chicken anemia virus: A deadly pathogen of poultry. Acta Virol, 63, pp.19-25.
19. Miller, M.M., Ealey, K.A., Oswald, W.B. and Schat, K.A., (2003). Detection of chicken anemia virus DNA in embryonal tissues and eggshell membranes. Avian diseases, 47(3), pp.662-671.
20. McIlroy, S.G., M.S. McNulty, D.W. Bruce, J.A. Smyth, E.A. Goodall, and M.J. Alcorn. (1992). Economic effects of clinical chicken anemia agent infection on profitable broiler production. Avian Dis. 36:566–574.
21. Schat KA (2009) Chicken anemia virus. Current Topics in Microbiology and Immunology, 331, 151e183.
22. McNulty, M.S., S.G. McIlroy, D.W. Bruce, and D. Todd. (1991). Economic effects of subclinical chicken anemia agent infection in broiler chickens. Avian Dis. 35:263–268.
23. De Herdt, P., G. Van den Bosch, R. Ducatelle, E. Uyttebroek, and C. Schrier. (2001). Epidemiology and significance of chicken infectious anemia virus infections in broilers and broiler parents under nonvaccinated European circumstances. Avian Dis. 45:706–708.
24. Zhang, X., Wu, B., Liu, Y., Chen, W., Dai, Z., Bi, Y. and Xie, Q., (2015). Assessing the efficacy of an inactivated chicken anemia virus vaccine. Vaccine, 33(16), pp.1916-1922.
25. Chandrashekaraiah, G.B., Karamala, S., Doddappaiah, N.H., Karumuri, N.K. and Kumar, V., (2020). Pathology of chicken infectious anemia (CIA) with concurrent infections. Journal of Entomology and Zoology Studies; 8(2), pp.519-524
26. Cardona, C.J., Oswald, W.B. and Schat, K.A., (2000). Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. Journal of General Virology, 81(8), pp.2067-2075.
27. Li, Y., Y. Hu, S. Cui, J. Fu, Y. Wang, Z. Cui, L. Fang, S. Chang, and P. Zhao. (2017). Molecular characterization of chicken infectious anemia virus from contaminated live-virus vaccines. Poult Sci. 96:1045–1501.
28. Hoerr, F.J., (2010). Clinical aspects of immunosuppression in poultry. Avian diseases, 54(1), pp.2-15.
29. Toro, H., Van Santen, V.L., Hoerr, F.J. and Breedlove, C., (2009). Effects of chicken anemia virus and infectious bursal disease virus in commercial chickens. Avian diseases, 53(1), pp.94-102.
30. Haridy, M., Goryo, M., Sasaki, J. and Okada, K., (2009). Pathological and immunohistochemical study of chickens with co-infection of Marek's disease virus and chicken anaemia virus. Avian Pathology, 38(6), pp.469-483.
31. Haridy, M., Sasaki, J., Okada, K. and Goryo, M., (2012). Persistence of inclusions and antigens of chicken anemia virus in Marek's disease lymphoma. Research in veterinary science, 93(3), pp.1353-1360.
32. Krishan, G., Shukla, S.K., Bhatt, P., Wani, M.Y., Dhama, K., Malik, Y.P.S. and Kumar, R., (2015). Clinical association of chicken anaemia virus with other infectious poultry diseases in North India and Nepal: Its pathological studies, molecular epidemiology and RFLP pattern of PCR amplified full length viral genome. Adv. Anim. Vet. Sci, 3(7), pp.395-405.

Online ISSN: 2537-1045
Print ISSN: 1110-1423

33. Aly, M. M. (2001). Isolation of chicken infectious anemia virus from outbreaks in broilers chickens in Egypt. J. Egypt. Vet. Med. Ass, 61(6), 137-147.
34. Hussein HA, Sabry MZ, El-Ebiary EA, El-Safty M, Abdel-Hady AL. Chicken infectious anemia virus in Egypt: molecular diagnosis by PCR and isolation of the virus from infected flocks. Ar J Biotechnol. (2002); 5 (2):263–74.
35. Todd, D., (2000). Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathology, 29(5), pp.373-394.
36. Hussein, E., Arafa, A.E., Anwar, N. and Khafaga, A., (2016). Molecular and pathological analysis of chicken anemia virus isolated from field infection in three Egyptian Provinces. Adv. Anim. Vet. Sci, 4(5), pp.218-229.
37. Abdel-Mawgod, S., Adel, A., Arafa, A.S. and Hussein, H.A., (2018). Full genome sequences of chicken anemia virus demonstrate mutations associated with pathogenicity in two different field isolates in Egypt. Virus disease, 29(3), pp.333-341.
38. Elsamadony, H.A., Tantawy, L.A., Omar, S.E. and Abd Alah, H.A., (2019).Molecular and pathological study on Chicken Anemia Virus. Journal of Virological Science, Vol. 5: 22- 34
39. Pagès-Manté, A., Saubi, N., Artigas, C. and Espuna, E., (1997). Experimental evaluation of an inactivated vaccine against chicken anaemia virus. Avian Pathology, 26(4), pp.721-729.
40. Hussein, H.A., Youssef, M.M., Osman, A., El-Ebiary, E.A. and Shalaby, M.A., (2003). Immunopathogenesis of attenuated strain of chicken infectious anemia virus in one-day-old specific-pathogen-free chicks. The Egyptian journal of immunology, 10(1), pp.89-102.
41. El-Bagoury, G.F., Samah, S., Suzan, K. and Khodeir, M.H., (2013) comparative study on live attenuated and inactivated chicken anemia virus vaccines. Benha Veterinary Medical Journal, vol. 25, no. 2:46-54.
42. Allam, T., Salah, N., Elballal, S. and Sultan, H., (2015). Clinicopathological studies on chicken infectious anemia disease live virus vaccines. Journal of Current Veterinary Research, 9(1), pp.40-57.
43. Todd, D., Connor, T.J., Calvert, V.M., Creelan, J.L., Meehan, B.M. and McNulty, M.S., (1995). Molecular cloning of an attenuated chicken anaemia virus isolate following repeated cell culture passage. Avian Pathology, 24(1), pp.171-187.
44. Todd, D., Connor, T.J., Creelan, J.L., Borghmans, B.J., Calvert, V.M. and McNulty, M.S., (1998). Effect of multiple cell culture passages on the biological behaviour of chicken anaemia virus. Avian Pathology, 27(1), pp.74-79.
45. Suvarna, K.S., Layton, C. and Bancroft, J.D. eds., (2018). Bancroft's theory and practice of histological techniques E-Book. Elsevier Health Sciences.
46. Gibson-Corley, K.N., Olivier, A.K. and Meyerholz, D.K., (2013). Principles for valid histopathologic scoring in research. Veterinary pathology, 50(6), pp.1007-1015.
47. Laboratory Manual for the Isolation and Identification of Avian Pathogens (1998): Fourth Edition, Chicken Anemia Virus, 146-149.
48. Singh, J., Banga, H.S., Brar, R.S., Singh, N.D., Sodhi, S. and Leishangthem, G.D., (2015). Histopathological and immunohistochemical diagnosis of infectious bursal disease in poultry birds. Veterinary World, 8(11), p.1331.
49. Zaqout, S., Becker, L.L. and Kaindl, A.M., (2020). Immunofluorescence staining of paraffin sections step by step. Frontiers in Neuroanatomy, 14, p.83.
50. Markowski-Grimsrud, C.J., Miller, M.M. and Schat, K.A., (2002).

Online ISSN: 2537-1045
Print ISSN: 1110-1423

Development of strain-specific real-time PCR and RT-PCR assays for quantitation of chicken anemia virus. Journal of virological methods, 101(1-2), pp.135-147.
51. SPSS Inc. Released (2009): PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.
52. Snedecor, G.W. and Cochran, W.C. (1989): Statistical methods. The eighth. Edition, Iowa University Press, Ames, Iowa, USA.
53. Yuasa, N., Noguchi, T., Furuta, K. and Yoshida, I., (1980). Maternal antibody and its effect on the susceptibility of chicks to chicken anemia agent. Avian Diseases, pp.197-201.
54. Hailemariam, Z., Omar, A.R., Hair-Bejo, M. and Giap, T.C., (2008). Detection and characterization of chicken anemia virus from commercial broiler breeder chickens. Virology journal, 5(1), pp.1-11.
55. Hadimli, H.H., ERGANİŞ, O., Güler, L. and UÇAN, U.S., (2008). Investigation of chicken infectious anemia virus infection by PCR and ELISA in chicken flocks. Turkish Journal of Veterinary and Animal Sciences, 32(2), pp.79-84.
56. Sommer, F. and Cardona, C., (2003). Chicken anemia virus in broilers: dynamics of the infection in two commercial broiler flocks. Avian diseases, 47(4), pp.1466-1473.
57. Hoop, R.K., (1992). Persistence and vertical transmission of chicken anaemia agent in experimentally infected laying hens. Avian Pathology, 21(3), pp.493-501.
58. Mostafa, D.I.A., Hamed, R.I., Salem, S.M., Abdallah, F. and Tolba, H.M.N., (2021). Pathological and immunopathological studies on broiler chicks infected with chicken anemia virus. Adv. Anim. Vet. Sci, 9(4), pp.508-518.
59. Chansiripornchai, N., (2016). Field study of seroconversion of three different commercial vaccines of chicken infectious anemia virus in Thailand. The Thai Journal of Veterinary Medicine, 46(4), p.699.
60. Miller, M.M. and Schat, K.A., (2004). Chicken infectious anemia virus: an example of the ultimate host–parasite relationship. Avian diseases, 48(4), pp.734-745.
61. Castaño, P., Benavides, J., Lee, M.S., Fernández, M., Fuertes, M., Royo, M., Fernández, J.M., Pérez, V. and Ferreras, M.C., (2019). Tissue tropism of chicken anaemia virus in naturally infected broiler chickens. Journal of comparative pathology, 167, pp.32-40.
62. Hosokawa, K., Imai, K., VAN HIEU, D., Ogawa, H., Suzutou, M., Linn, S.H., Kurokawa, A. and Yamamoto, Y., (2020). Pathological and virological analysis of concurrent disease of chicken anemia virus infection and infectious bronchitis in Japanese native chicks. Journal of Veterinary Medical Science, pp.20-0006.
63. Sawant, P.M., Dhama, K., Rawool, D.B., Wani, M.Y., Tiwari, R., Singh, S.D. and Singh, R.K., (2015). Development of a DNA vaccine for chicken infectious anemia and its immunogenicity studies using high mobility group box 1 protein as a novel immunoadjuvant indicated induction of promising protective immune responses. Vaccine, 33(2), pp.333-340.
64. Wani, M.Y., Dhama, K., Latheef, S.K., Barathidassan, R., Tiwari, R., Chakraborty, S., Chawak, M.M. and Singh, S.D., (2014). Experimental pathological studies of an Indian chicken anaemia virus isolate and its detection by PCR and FAT. Pakistan journal of biological sciences: PJBS, 17(6), pp.802-811.
65. Tan, J. and Tannock, G.A., (2005). Role of viral load in the pathogenesis of chicken anemia virus. Journal of General Virology, 86(5), pp.1327-1333.
66. Kuscu, B. and Gürel, A., (2008). Lesions in the thymus and bone marrow

Online ISSN: 2537-1045
Print ISSN: 1110-1423

in chicks with experimentally induced chicken infectious anemia disease. Journal of Veterinary Science, 9(1), pp.15-23.
67. Toro, H., Ramirez, A.M. and Larenas, J., (1997). Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. Avian Pathology, 26(3), pp.485-499.
68. Smyth, J.A., Moffett, D.A., McNulty, M.S., Todd, D. and Mackie, D.P., (1993). A sequential histopathologic and immunocytochemical study of chicken anemia virus infection at one day of age. Avian diseases, pp.324-338.
69. Taniguchi, T., Yuasa, N., Maeda, M. and Horiuchi, T., (1983). Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. National Institute of Animal Health Quarterly, 23(1), pp.1-12.
70. Nadeem, S.M., Aslam, A., Sheikh, A.A., Ahmad, A., Latif, A.A. and Anjum, A., (2021). Clinico-Pathological and Hemato-Biochemical Assessment of Field Originated Chicken Anemia Virus in Experimentally Challenged Broiler Chicken. Indian Journal of Animal Research, 55(8).
71. Engström, B.E. and Luthman, M., (1984). Blue wing disease of chickens: signs, pathology and natural transmission. Avian Pathology, 13(1), pp.1-12.
72. Islam, A.F., Alsharari, M., Renz, K.G., Burgess, S.K. and Walkden-Brown, S.W., (2016), November. Lateral Transmission of Chicken Anaemia Virus: Is It Faecal-Oral or Dust Inhalation?. In 9th International Poultry Conference, Hurghada (pp. 07-10).
73. Alsharari, M., Islam, A.F., Renz, K.G., Burgess, S.K. and Walkden-Brown, S.W., (2016). Distribution of Chicken Anaemia Virus in Tissues, Faeces and Environmental Samples up to Day 56 Following Artificial Infection in Specific Pathogen Free Chickens. In Proceedings of the 9th International Poultry Conference. WPSA: World's Poultry Science Association.

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Table 1: Oligonucleotide sequences for the detection of chicken anaemia virus DNA using real-time TaqMan assays as reported in Markowski-Grimsrud *et al.* [50].

Primer/Probe	**Sequence**
Forward primer (CAV Q5')	5'-GCCCCGGTACGTATAGTGTGAG-3'
CAV probe	5' - (6FAM)-CTGCCGAACCCCCAATCTACTATGACTATCC-(TAMRA)-3'
Reverse primer (Cux-1* specific Q3')	5'-CCGTGAGAAAGATGACCCCTT-3'

* GenBank accession number M55918.

Table 2: Histopathological lesions score induced by CIAV in bone marrow at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	**Bone marrow**						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	0.00±0.00b	3.00±0.00a	4.00±0.00a	3.00±0.577a	4.00±0.00a	3.00±0.00a	3.00±0.577a
2	2.00±0.577a	3.00±0.577a	4.00±0.00a	3.00±0.00a	3.00±0.00a,b	2.00±0.577a	4.00±0.00a
3	0.00±0.00b	4.00±0.00a	4.00±0.00a	3.00±0.577a	3.00±0.577a,b	2.00±0.577a	4.00±0.00a
4	0.00±0.00b	3.00±0.00a	3.00±0.577a	2.00±0.577a	2.00±0.00b	0.00±0.00b	3.00±0.577a
5	0.00±0.00b	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00c	0.00±0.00b	0.00±0.00a

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

Table 3: Histopathological lesions score induced by CIAV in thymus at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	**Thymus**						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	2.00 ± 0.00^{a}	2.00 ± 0.00^{a}	$2.00\pm0.00^{a,b}$	1.00 ± 0.00^{a}	2.00 ± 0.00^{a}
2	1.00 ± 0.577^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	2.00 ± 0.00^{a}	$1.00\pm0.00^{b,c}$	1.00 ± 0.577^{a}	1.00 ± 0.577^{a}
3	1.00 ± 0.00^{a}	1.00 ± 0.577^{a}	2.00 ± 0.577^{a}	2.00 ± 0.00^{a}	$1.00\pm0.577^{b,c}$	2.00 ± 0.577^{a}	1.00 ± 0.00^{a}
4	2.00 ± 0.577^{a}	2.00 ± 0.00^{a}	1.00 ± 0.577^{a}	1.00 ± 0.577^{a}	3.00 ± 0.00^{a}	3.00 ± 0.577^{a}	1.00 ± 0.577^{a}
5	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

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Table 4: Histopathological lesions score induced by CIAV in spleen at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	Spleen						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	1.00±0.577^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a}	1.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}
2	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a}	0.00±0.00^{b}	0.00±0.00^{a}
3	2.00±0.577^{a}	2.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a,b}	1.00±0.00^{a}
4	1.00±0.00^{a}	2.00±0.577^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a}	2.00±0.00^{a}	1.00±0.577^{a}
5	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

Table 5: Histopathological lesions score induced by CIAV in bursa of Fabracious at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	Bursa of Fabracious						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	2.00±0.00^{a}	2.00±0.577^{a}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a,b}	0.00±0.00^{b}
2	2.00±0.577^{a}	2.00±0.00^{a}	2.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}
3	1.00±0.577^{a}	2.00±0.577^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.577^{a}	2.00±0.577^{a}	0.00±0.00^{b}
4	1.00±0.00^{a}	1.00±0.577^{a}	1.00±0.577^{a}	1.00±0.00^{a}	1.00±0.00^{a}	2.00±0.00^{a}	1.00±0.00^{a}
5	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

Table 6: Histopathological lesions score induced by CIAV in cecal tonsils at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	Cecal tonsils						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	1.00±0.577^{a}	0.00±0.00^{b}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}
2	1.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}	1.00±0.577^{a}	1.00±0.577^{a}	1.00±0.577^{a}	1.00±0.00^{a}
3	1.00±0.577^{a}	0.00±0.00^{b}	0.00±0.00^{b}	1.00±0.00^{a}	0.00±0.00^{a}	2.00±0.577^{a}	0.00±0.00^{b}
4	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	2.00±0.577^{a}	1.00±0.577^{a}	2.00±0.00^{a}	0.00±0.00^{b}
5	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

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Table 7: Histopathological lesions score induced by CIAV in liver at 3, 7, 15, 22, 29, 36 and 41 dpv

Group	Liver						
	3 dpv	**7 dpv**	**15 dpv**	**22 dpv**	**29 dpv**	**36 dpv**	**41 dpv**
1	2.00±0.00[a]	2.00±0.577[a]	1.00±0.577[a]	2.00±0.577[a]	1.00±0.00[a]	2.00±0.00[a]	1.00±0.00[a]
2	1.00±0.00[a]	2.00±0.00[a]	1.00±0.00[a]	1.00±0.00[a]	1.00±0.577[a]	1.00±0.00[a,b]	1.00±0.577[a]
3	2.00±0.00[a]	1.00±0.00[a]	2.00±0.577[a]	2.00±0.00[a]	2.00±0.00[a]	1.00±0.577[a,b]	2.00±0.00[a]
4	2.00±0.577[a]	2.00±0.577[a]	2.00±0.00[a]	2.00±0.577[a]	2.00±0.577[a]	2.00±0.00[a]	1.00±0.577[a]
5	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[b]	0.00±0.00[a]

Values expressed as mean values ± SEM: Standard Error of Mean, within the same row, different superscripts a, b and c letters indicates a statistically significant difference (at $p \leq 0.05$) between values at the same dpv time.

Table 8: CIAV load in livers, the mean Log_{10} $TCID_{50}$ ± SD (standard deviation), of group 1, 2, 3 and 4 at 7, 15 and 21 days post vaccination (dpv).

	Group 1	**Group 2**	**Group 3**	**Group 4**
7 dpv	3.052259333 ± 0.999283256	2.814462 ± 0.975482	3.185484 ± 1.113226	2.934621 ± 1.099598
15 dpv	3.720646667 ± 0.940898425	3.862736 ± 1.127491341	3.834405 ± 1.023284	3.218817333 ± 1.163545156
21 dpv	3.419312667 ± 0.99639109	3.001286667 ± 1.05428304	3.803017 ± 1.094634	2.93462 ± 1.16405

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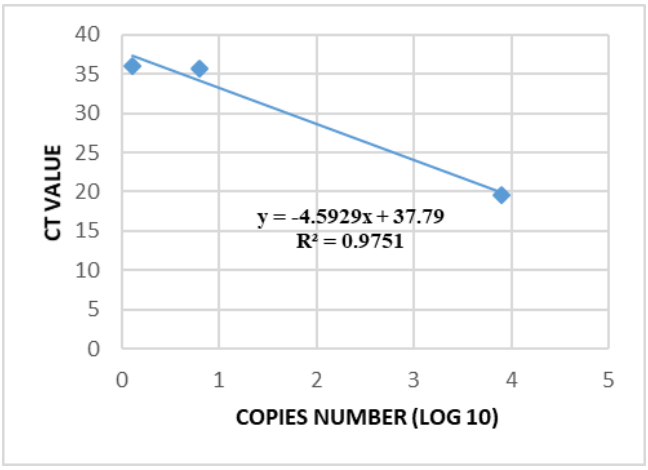

Fig .1: The standard curve for qPCR, showing the linear equation y = -4.5929x + 37.79 with R^2 = 0.9751.

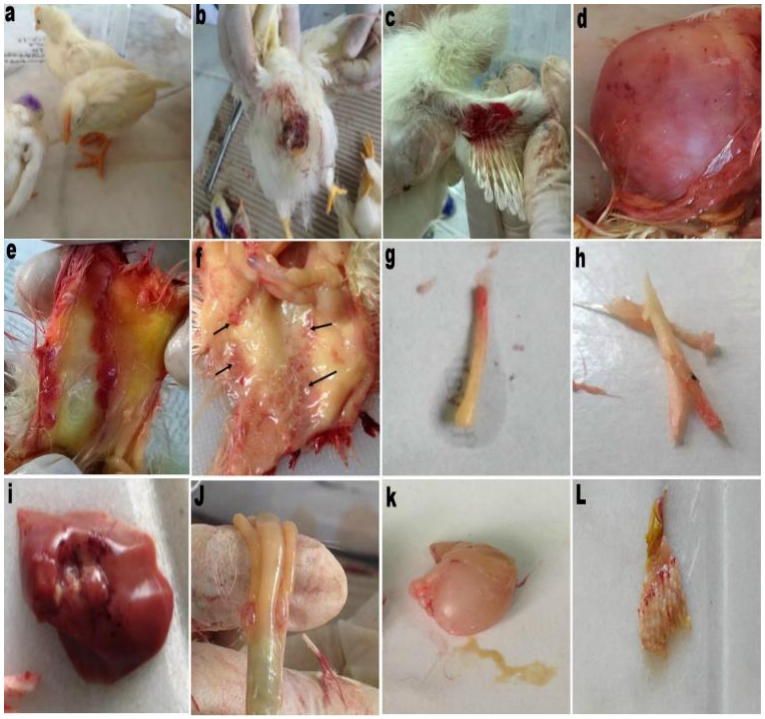

Fig .2: Clinical and necropsy findings, **(a)** Chicks at 29 dpv with pale comb and prostration of groups 1, 3 and 4. **(b)** Chick of group 1 with vent lesion due to cannibalism at 22 dpv. **(c)** Chick of group 2 with wing lesion due to cannibalism at 4 dpv. **(d)** Hemorrhagic streaks on thigh muscle at 41 dpv in group 4. **(e)** Thymus with dark reddish colour at 3 dpv in group 3. **(f)** Atrophied thymus at 15 dpv in group 1. **(g)** Yellowish bone marrow at 29 dpv in group 3. **(h)** Whitish fatty bone marrow at 15 dpv in group 3. **(i)** Liver with petechial hemorrhage at 41 dpv in group 3. **(j)** Cecal tonsils with petechial hemorrhage at 22 dpv in group 4 **(k)** Swollen Bursa of Fabricius at 15 dpv in group 4. **(L)** Hemorrhagic petechiae on the tips of proventricular glands at 36 dpv in group 4.

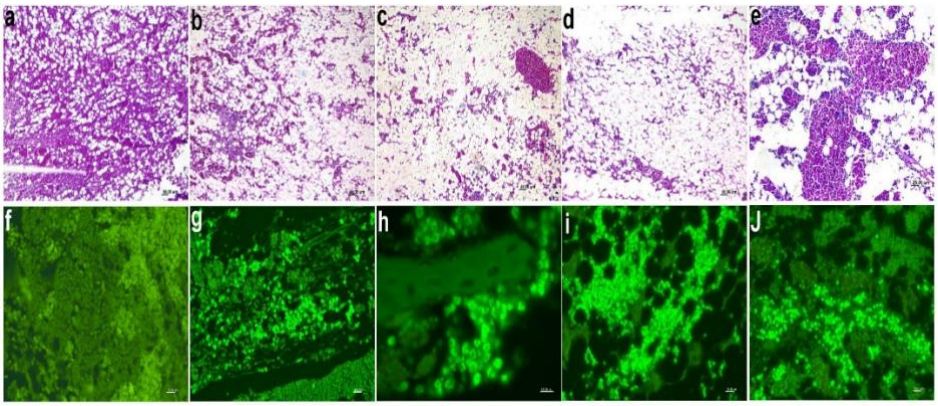

Fig .3: Bone marrow histological sections and immunofluorescence expression of CIAV antigen, showing green-apple-coloured characteristic intranuclear granular fluorescence. **(a)** apparently normal bone marrow architecture of group 5 Bar, 50 µm. **(b)** group 1 at 36 dpv showing severe depletion of haemopoietic cells Bar, 50 µm. **(c)** group 2 at 41 dpv showing very severe depletion of haemopoietic cells Bar, 50 µm. **(d)** group 3 at 7 dpv showing very severe depletion of haemopoietic cells replaced with fat cells Bar, 50 µm. **(e)** group 4 at 41 dpv showing congested sinuses with depletion of haemopoietic cells and proliferation of adipocytes Bar, 25 µm. **(f)** group 5 showing negative immunofluorescence expression for CIAV Bar, 15 µm. **(g)** group 1 at 36 dpv. Bar, 15 µm. **(h)** group 2 at 36 dpv. Bar, 10 µm. **(i)** group 3 at 7 dpv. Bar, 15 µm. **(J)** group 4 at 7 dpv. Bar, 15 µm.

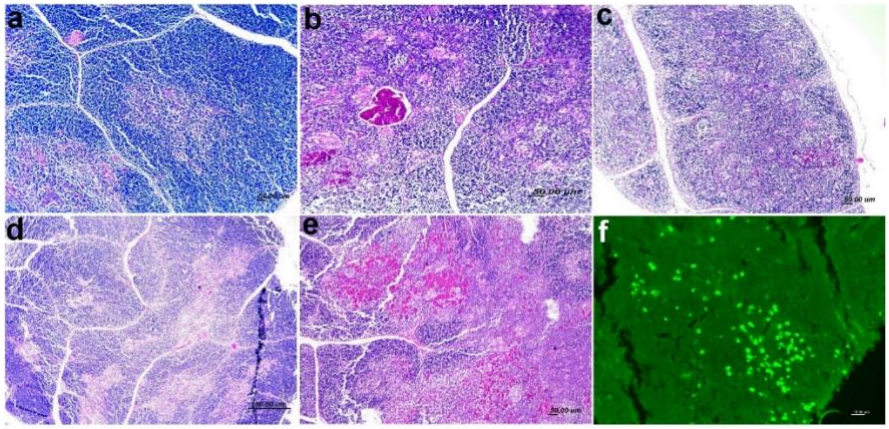

Fig .4: Thymus histological sections and immunofluorescence expression of CIAV antigen **(a)** apparently normal thymus architecture of group 5 Bar, 50 µm. **(b)** group 1 at 22 dpv showing pronounced congested BVs with mild haemorrhage and diffuse degeneration of Hassal's corpuscles cells Bar, 50 µm. **(c)** group 2 at 22 dpv showing loss of distinction between cortex and medulla with moderate lymphocytes depletion Bar, 50 µm. **(d)** group 3 at 22 dpv showing moderate lymphocytes depletion Bar, 100 µm. **(e)** group 4 at 7 dpv showing moderate depletion of lymphocytes within cortex and medulla with pronounced haemorrhage Bar, 50 µm. **(f)** group 2 at 7 dpv showing green-apple-coloured characteristic intranuclear granular fluorescence of CIAV antigen Bar, 15 µm.

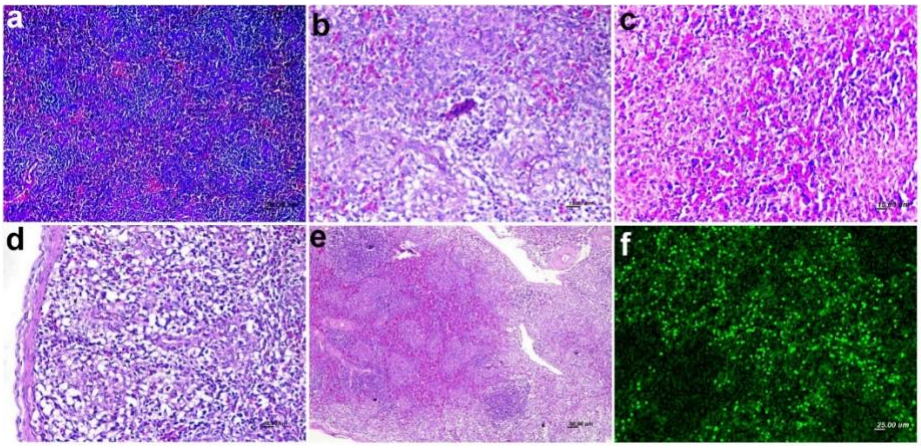

Fig .5: Histological sections of spleen and immunofluorescence expression of CIAV antigen **(a)** group 5 apparently normal spleen architecture Bar, 50 µm. **(b)** group 1 at 3 dpv showing mild depletion of lymphocytes with focal few extravasated RBCs Bar, 15 µm. **(c)** group 2 at 3 dpv showing mild depletion of lymphocytes with mild haemorrhage Bar, 15 µm **(d)** group 3 at 3 dpv showing moderate depletion of lymphocytes Bar, 15 µm. **(e)** group 4 at 7 dpv showing moderate haemorrhage and depletion of lymphocytes Bar, 50 µm. **(f)** group 3 at 36 dpv showing green-apple-coloured characteristic intranuclear granular fluorescence of CIAV antigen Bar, 25 µm.

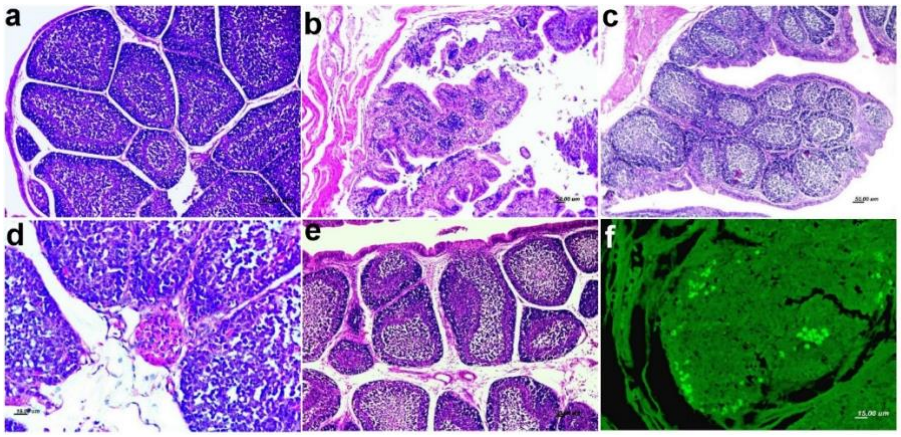

Fig .6: Histological sections of bursa of fabracious and immunofluorescence expression of CIAV antigen **(a)** group 5 apparently normal architecture of bursa of Fabracious Bar, 50 µm. **(b)** group 1 at 3 dpv showing atrophy of plicae, depletion of lymphocytes, corrugated hyperplasia of lining epithelium and epithelization Bar, 50 µm. **(c)** group 2 at 15 dpv showing moderate depletion of lymphocytes mainly in medulla with hyperplasia of plical lining epithelium Bar, 50 µm. **(d)** group 3 at 7 dpv showing heterophils aggregation Bar, 15 µm. **(e)** group 4 at 29 dpv showing depletion of lymphocytes, proliferation of corticomedullary lining epithelium and interfollicular oedema Bar, 25 µm. **(f)** group 2 at 7 dpv showing green-apple-coloured characteristic intranuclear granular fluorescence of CIAV antigen Bar, 15 µm.

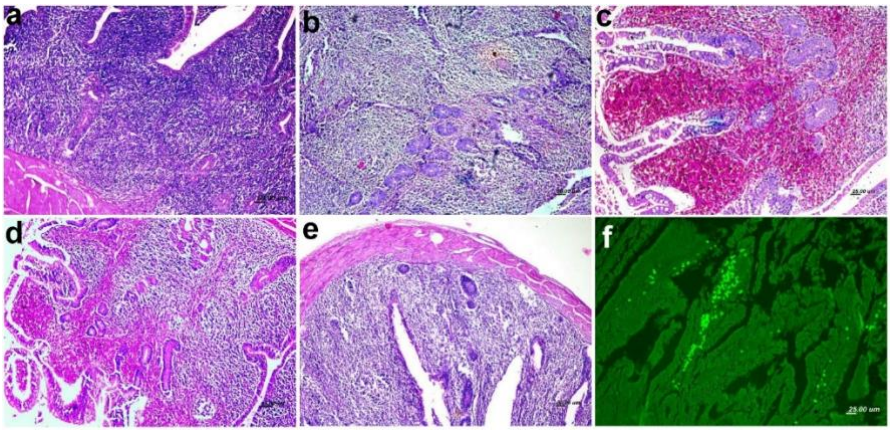

Fig .7: Histological sections of cecal tonsils and immunofluorescence expression of CIAV antigen **(a)** group 5 apparently normal architecture of cecal tonsils Bar, 50 µm. **(b)** group 1 at 41 dpv showing mild lymphocytic depletion Bar, 50 µm. **(c)** group 2 at 41 dpv showing severe haemorrhage in lamina propria Bar, 25 µm. **(d)** group 3 at 36 dpv showing hyperplasia with vacuolar degeneration of lining epithelium and moderate hemorrhage in lamina propria Bar, 15 µm. **(e)** group 4 at 22 dpv showing depletion of lymphocytes and mucous glands focal atrophy Bar, 50 µm. **(f)** group 2 at 22 dpv showing green-apple-coloured characteristic intranuclear granular fluorescence of CIAV antigen Bar, 25 µm.

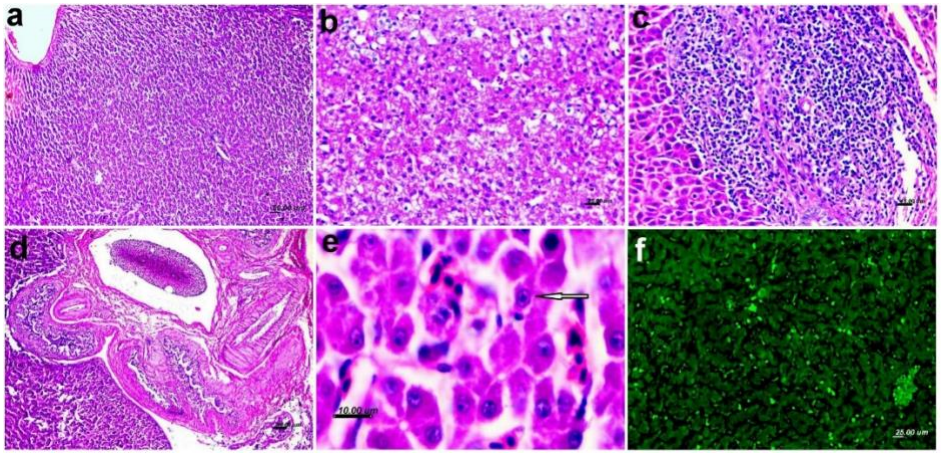

Fig .8: Histological sections of liver and immunofluorescence expression of CIAV antigen **(a)** group 5 apparently normal architecture of liver Bar, 50 µm. **(b)** group 1 at 3 dpv showing perivascular vacuolar degeneration with hepatocellurar necrosis Bar, 15 µm. **(c)** group 2 at 22 dpv showing aggregation of mononuclear cells Bar, 15 µm. **(d)** group 3 at 41 dpv showing pronounced congested blood vessel and hyperplasia of bile ductules Bar, 50 µm. **(e)** group 4 at 22 dpv showing intranuclear inclusion body with marginalized chromatin (arrow), 10 µm. **(f)** group 4 at 18 dpv of a dead chick showing green-apple-coloured characteristic intranuclear granular fluorescence of CIAV antigen Bar, 25 µm.

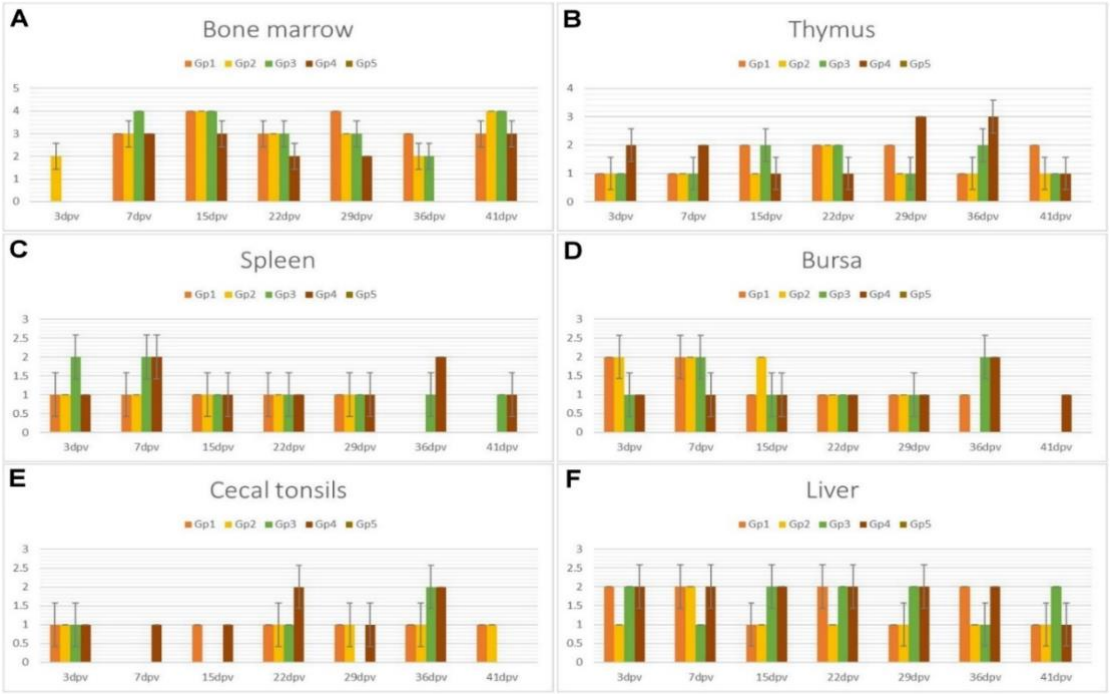

Fig .9: Histopathological lesions score at 3, 7, 15, 22, 29, 36 and 41 day-post-vaccination (dpv) of (A) Bone marrow. (B) Thymus. (C) Spleen. (D) Bursa of Fabracious. (E) Cecal tonsils. (F) Liver.

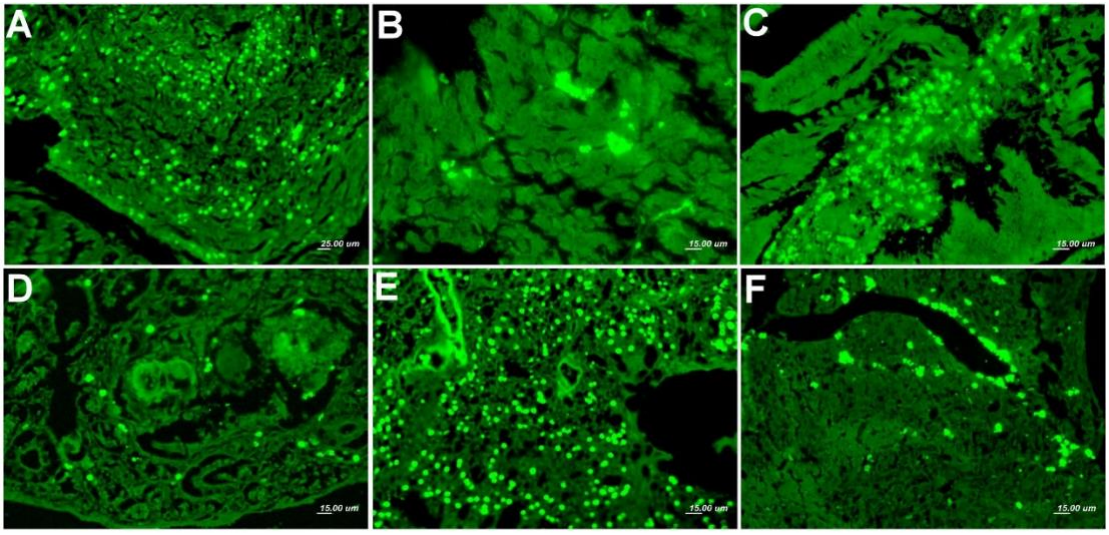

Fig .10: Immunofluorescence expression of CIAV antigen in non-lymphoid organs, showing green-apple-coloured characteristic intranuclear granular fluorescence **(A)** Proventriculous at 18 dpv of a dead chick of group 4 Bar, 25 µm. **(B)** Pancreas at 22 dpv of group 4 Bar, 15 µm. **(C)** Duodenum at 36 dpv of group 1 Bar, 15 µm. **(D)** Kidney at 18 dpv of a dead chick of group 4 Bar, 15 µm. **(E)** Lung at 6 dpv of a dead chick of group 2 Bar, 15 µm. **(F)** Heart at 6 dpv of group 2 Bar, 15 µm.

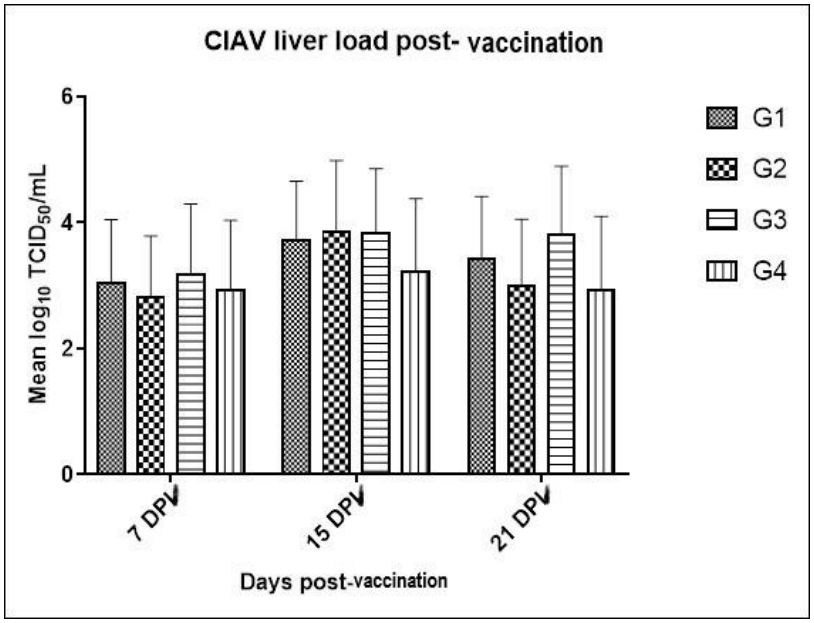

Fig .11: CIA virus load**,** mean Log_{10} $TCID_{50}$ at 7, 15, and 21 days-post-vaccination (DPV) of group (G) 1, 2, 3 and 4.