The Haryana Veterinarian (June, 2024) **63(1),** 52-56 ISSN 0033-4359 (P) **Research Article**

EFFECT OF CHICKEN ANAEMIA VIRUS, INFECTIOUS BURSAL DISEASE VIRUS AND AVIAN REOVIRUS INFECTION ON IMMUNE STATUS OF COMMERCIAL POULTRY FLOCKS UNDER FIELD CONDITIONS

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Received: 28.07.2023; Accepted: 27.11.2023

ABSTRACT

The present study was conducted to diagnose chicken anemia virus (CAV) infection alone or its co-infection with other immunosuppressive viruses *viz*. Infectious Bursal Disease (IBD) virus and avian reovirus (ARV) in commercial chicken flocks and to access their effect on immune cells under field conditions. Tissue samples were collected from field cases of 100 birds, showing lesions in lymphoid organs i.e. thymus, spleen, bursa of Fabricius and caecal tonsils. The formalin-fixed, paraffin embedded sections were processed for immunolocalization and a total of 33 cases were detected as positive on the basis of immunoreactivity of various viral antigens. Further, 25 confirmed cases of CAV and multiple infections of CAV, IBDV and ARV were randomly selected and B-cell and T-cell markers were used on thymus, spleen and bursa. The results of the present study showed that in combined infections with CAV, IBDV and ARV, the immunosuppressive viruses had more tropism for T cells of spleen and in combined infections of CAV and ARV significant effect was produced on B cells of bursa of Fabricius. This indicates that spleen is the most affected organ in mixed infections of CAV, IBDV and ARV under field conditions.

Keywords: Avian Reovirus, Chicken anaemia Virus, Commercial chicken flocks, Immunosuppression, Infectious Bursal Disease Virus

How to cite: Andrabi, S.A., Singh, A. and Gupta, K. (2024). Effect of chicken anaemia virus, infectious bursal disease virus and avian reovirus infection on immune status of commercial poultry flocks under field conditions. *The Haryana Veterinarian* **63(1)**: 52-56.

Virus induced immunosuppression in poultry birds threaten the poultry industry because affected flocks respond poorly to administered vaccines, leading to heavy mortality and huge economic losses. Some of the viral pathogens affecting poultry cause irreversible immuno-deficiency and thus immunosuppression making birds susceptible to multiple secondary infections. The viruses causing immunosuppression follow various strategies to escape host immune surveillance and thus down regulate the immune response by causing imbalance in the immune cells (Naniche and Oldstone, 2000). In addition, different viral infections cause depletion of different immune cells and produce lesions in the organs they invade. Some of the important immunosuppressive viral diseases of poultry which have widespread occurrence in commercial chicken and cause heavy economic losses include chicken infectious anemia (CIA), infectious bursal disease (IBD), Marek's disease (MD), inclusion body hepatitis (IBH), avian reovirus (ARV) and retrovirus infections (Cui *et al.*, 2014; Singh *et al.*, 2006).

Chicken infectious anaemia is a re-emerging disease of poultry which significantly affects the cellular branch of the specific immune system causing depletion of CD4+ and CD8+ cells from thymus cortex and generalized lymphoid atrophy with depletion of cortical thymocytes and erythroblastoid cells from bone marrow leading to severe immunosuppression and anaemia (Noteborn, 2004). CIA leads to production losses in terms of high mortality and carcass condemnations (Hagood *et al.*, 2000).

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Immunosuppression by IBD is attributed mainly to apoptosis and necrosis of B cells (Vasconcelos and Lam, 1994; Ojeda *et al.*, 1997; Tanimura and Sharma, 1998; Nieper *et al.*, 1999). Recently IBDV positive T-cell populations have been detected in the bursal follicles as well (Mahgoub *et al.*, 2012). Avian reovirus (ARV) associated diseases may be a result of co-infection with other infectious pathogens under field conditions (Andral *et al.*, 1985). ARV causes atrophy of lymphoid organs and has efficiency to infect and replicate in blood monocytes (Hoerr, 2010). Co-infection of ARV with CAV and IBDV causes increased pathological effects and economic losses (Moradian *et al.*, 1990; Rios *et al.*, 2012).

Keeping in view, the importance of immuno-suppression caused by viruses in commercial poultry flocks; it is of utmost importance to understand the effect of these viruses on various immune cells in multiple viral infections under field conditions. The present study aimed at detection of multiple viral infections in poultry, by localizing the immunosuppressive viral antigens in various lymphoid organs and to access their combined effect on immune status of birds by correlating the presence of viral antigen(s) with percentage of B- and T-cells in lymphoid organs.

MATERIAL AND METHODS

The study included 100 cases presented for post mortem examination in the Poultry Disease Diagnostic Laboratory of the Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University

(GADVASU), Ludhiana and from three commercial poultry farms in and around Punjab. The details of the same are given in the (Table 1).

Thorough necropsy of birds was conducted and gross lesions were recorded. Tissue pieces of lymphoid organs showing lesions (thymus, bursa of Fabricius, spleen and caecal tonsils) were collected in 10% neutral buffered formalin for immunohistochemical studies. Further, tissue sections were mounted on Super frost/Plus, positively charged microscopic slides (Fisher Scientific, USA) and stained using specific antisera raised in chicken against CAV, IBDV and ARV (Polyclonal, Charles River Laboratories USA) at the dilution of 1:10000. The avidin biotin peroxidase complex (ABC) method for immuno-peroxidase staining was carried out for localization of viral antigens using commercial Vectastain ABC reagents (Vector Laboratories, Burlingame, USA) as described previously (Andrabi *et al.*, 2018). Briefly, heat induced epitope retrieval (HIER) was employed using EZ antigen retrieval solutions by EZ-Retriever TM System as per manufacturer's instructions (BioGenex Laboratories Inc., San Ramon, California, USA). The endogenous peroxidase was quenched in 3% H_2O_2 in methanol for 40 min at room temperature. The sections were incubated with 1:10 dilution of a normal goat serum (Vector Laboratories, Burlingame, USA) mixed with power block (1 drop in 1 ml) (Biogenex Laboratories Inc., San Ramon, California) to block non-specific protein binding for 60 min at room temperature. The sections were incubated with specific antisera as primary antibody at 4°C for overnight and then secondary biotinylated anti-chicken IgG (Vector Laboratories, Burlingame, USA) at a concentration of 1:400 for 30 min at room temperature the next day. Further incubation in Vectastain ABC reagent (Vector laboratories, Burlingame, USA) was done for 30 min at room temperature. The antigen-antibody-peroxidase reaction was developed with a freshly prepared 3, 3 '-diaminobenzidine (DAB) solution by mixing 1 drop of DAB chromogen with 1 ml of DAB buffer (Vector Laboratories, Burlingame, USA) and counterstained with Gill's hematoxylin (Merck, Germany). For each antibody, a negative control was run by replacing primary antibody with PBS buffer. Scoring was done as per Oladele *et al.*, 2009 by counting the number of cells showing positive reactivity using a 40× objective lens of the light microscope. Score -0 depicts no infection (<5 stained cells), 1 for mild infection (5-50 stained cells), 2 for medium infection (50-150 stained cells), and 3 for heavy infection (over 150 stained cells). The average of five fields was taken per slide.

In 25 confirmed cases of multiple viral infections, B-cells and T-cells were stained using CD79a and CD3 markers (Sigma-Aldrich) at the dilution of 1:200 and 1:100, respectively. The cells showing positive reactivity for CD3 and CD79a in lymphoid organs (thymus, bursa and spleen) of each case were scored by counting number of cells per field under oil immersion objective lens and taking average of five fields per slide, then percentage of CD3+ T-cells and CD79a+ B-cells was calculated for each case as shown below:

$$\text{Percentage of CD3+ /CD79a +cells per slide} = \frac{\text{Average of positive cells counted per slide}}{\text{Total number of cells}} \times 100$$

The Pearson correlation co-efficient and its significance was determined using HMISC package of R programming environment (ver 4.1.0). The correlogram were generated using package corrplot of R.

RESULTS AND DISCUSSION

Diagnosis of viral immunosuppressive diseases

The viral diseases causing immunosuppression viz. CIA, IBD and ARV were tentatively diagnosed based on gross lesions, which included generalized lymphoid atrophy and paleness of carcass with or without involvement of bone marrow. In thymus, bursa of Fabricius, spleen and caecal tonsils noticeable lesions were appreciated which included atrophy of thymus and bursa along with hemorrhages in some cases. Enlargement of spleen and congestion of caecal tonsils (Andrabi *et al.*, 2018) as reported in earlier studies (Balamurugan and Kataria, 2006; Van den Berg, 2000). However, confirmatory diagnosis was made by immunohistochemical localization of viral antigens (CAV, IBDV and ARV) in the lymphoid tissues which revealed moderate to strong reactivity for IBDV, CAV and ARV in both cytoplasm and nucleus of

Table 1. Farm wise details of poultry birds from which tissue samples were collected

S. No.	Farm	Type of Bird	Age	Number of Samples	Total
1.	Farm-1 (Organised)	Layer	8-12 Weeks	20	20
2.	Farm-2 (Organised)	Broiler	6 Weeks	3	43
			6-8 Weeks	5	
			6-12 Weeks	19	
			12-18 Weeks	3	
			Adult	9	
		Layer	Adult	4	
3.	Farm-3 (organised)	Broiler	6-8 Weeks	4	17
			12-18 Weeks	1	
			Adult	12	
4.	Farm-4 (unorganised)	Broiler	Adult	20	20
	Total				100

Table 2. Number of cases diagnosed based on immuno-histochemical localization of viral agents in lymphoid organs

S. No.	Disease	No. of cases
1.	*CAV	8
2.	CAV+**ARV	6
3.	CAV+***IBDV	7
4.	CAV+ARV+IBDV	9
5.	IBDV	2
6.	ARV+IBDV	1
	Total	33

*Chicken anaemia virus, **Avian Reovirus, ***Infectious Bursal Disease Virus

various lymphoid organs (Andrabi *et al.*, 2018) as reported previouslyfor CAV by Haridy *etal.* (2012), IBDVby Jackwood and Sommer (2010) and ARV by Engstrom (1988).

Based on immunoreactivity of viral antigens (Table 2) thirthy cases were positive for CAV out of hundred samples under study. These included eight positive cases of CAV alone (8%) six cases of mixed infection with both CAV and ARV (6%), seven cases of CAV and IBDV (7%) and nine cases of CAV, IBDV and ARV (9%). Only one case was found positive for IBDV and ARV (1%) and two cases for IBDV alone (2%) (Andrabi *et al.*, 2018).

Disease wise correlation of immunohistochemical score

Table 3. Disease wise correlation of Immunohistochemical score with percentage of immunolabelled B-cells and T-cells in lymphoid organs

Case	Thymus			Spleen			Bursa		
	IHC score	% B cells	% T cells	IHC score	% B cells	%T cells	IHC score	% B cells	% T cells
Correlation of B cells and T cells with Ihc in Cav									
A24	2.0	2.9	3.5	2.0	3.1	3.9	2.0	2.9	1.2
A38	2.4	3.4	4.2	2.2	3.1	3.8	2.4	3.1	1.4
A48	2.6	3.1	4.1	2.4	3.1	4.0	2.2	3.0	1.3
A53	1.6	3.3	3.8	1.8	3.0	4.1	1.6	2.7	1.2
A65	2.8	2.8	3.5	2.4	2.7	3.8	2.6	2.9	1.2
PCC		-0.36	0.07		-0.18	-0.46		0.69	0.18
Correlation of B cells and T cells with ihc in cav+ibd									
A5	1.8	2.9	4.0	2.4	3.1	3.9	2.4	3.2	1.6
A9	2.8	2.8	4.3	2.0	3.1	4.0	2	3.2	1.2
A10	1.8	2.8	4.6	1.6	3.2	4.1	2.8	2.9	1.3
PCC		-0.50	-0.06		-0.86	-0.96		0.77	-0.32
Correlation of B cells and T cells with ihc in cav+ibd+ARV									
A3	2.6	2.5	4.0	2.4	3.2	4.0	2.6	3.3	1.2
A4	2.4	2.8	4.0	1.8	3.3	4.0	2.4	3.1	1.3
A12	2.4	3.0	4.2	1.8	3.1	4.0	2.0	2.9	1.4
A19	2.8	3.0	4.6	2.4	3.2	4.2	2.8	2.9	1.2
A25	1.8	3.8	3.6	1.8	3.1	3.7	1.8	3.2	1.3
A37	2.8	2.6	4.0	2.6	3.2	4.1	2.6	3.1	1.3
A43	2.4	2.8	4.3	2.6	2.9	4.0	2.2	2.8	1.3
A44	2.6	3.4	4.5	2.4	2.8	4.1	2.4	3.1	1.2
A67	2.4	3.3	4.3	2.6	2.9	4.1	2.4	3.1	1.1
PCC		-0.69*	0.61		-0.38	0.67*		0.065**	-0.50
Correlation of B cells and T cells with ihc in cav+ARV									
A1	2.4	2.8	4.3	2.6	2.8	4.3	2.6	3.3	1.33
A2	2.4	2.8	4.4	2.4	3.3	4.1	2.4	3.2	1.2
A6	2.4	2.6	4.1	2.4	3.2	4.0	2.4	3.1	1.4
A7	2.8	2.6	4.2	1.6	3.1	3.8	2.0	2.8	1.3
A8	2.6	2.0	4.9	2.6	2.8	4.1	2.8	3.1	1.2
A11	2.8	3.5	4.4	2.6	2.9	4.0	2.8	3.1	1.3
A13	2.6	2.6	3.9	1.4	3.0	4.1	1.8	2.9	1.6
A14	2.4	2.8	3.8	2.6	2.9	4.2	2.2	3.1	1.4
PCC		0.21	0.24		-0.33	0.53		0.64	-0.67

*significant at P<0.05,**significant at P<0.008; PCC- Pearson Correlation Cofficient; A- name of sample
CAV-Chicken Anemia Virus, IBD-Infectious Bursal Disease, ARV- Avian Reovirus

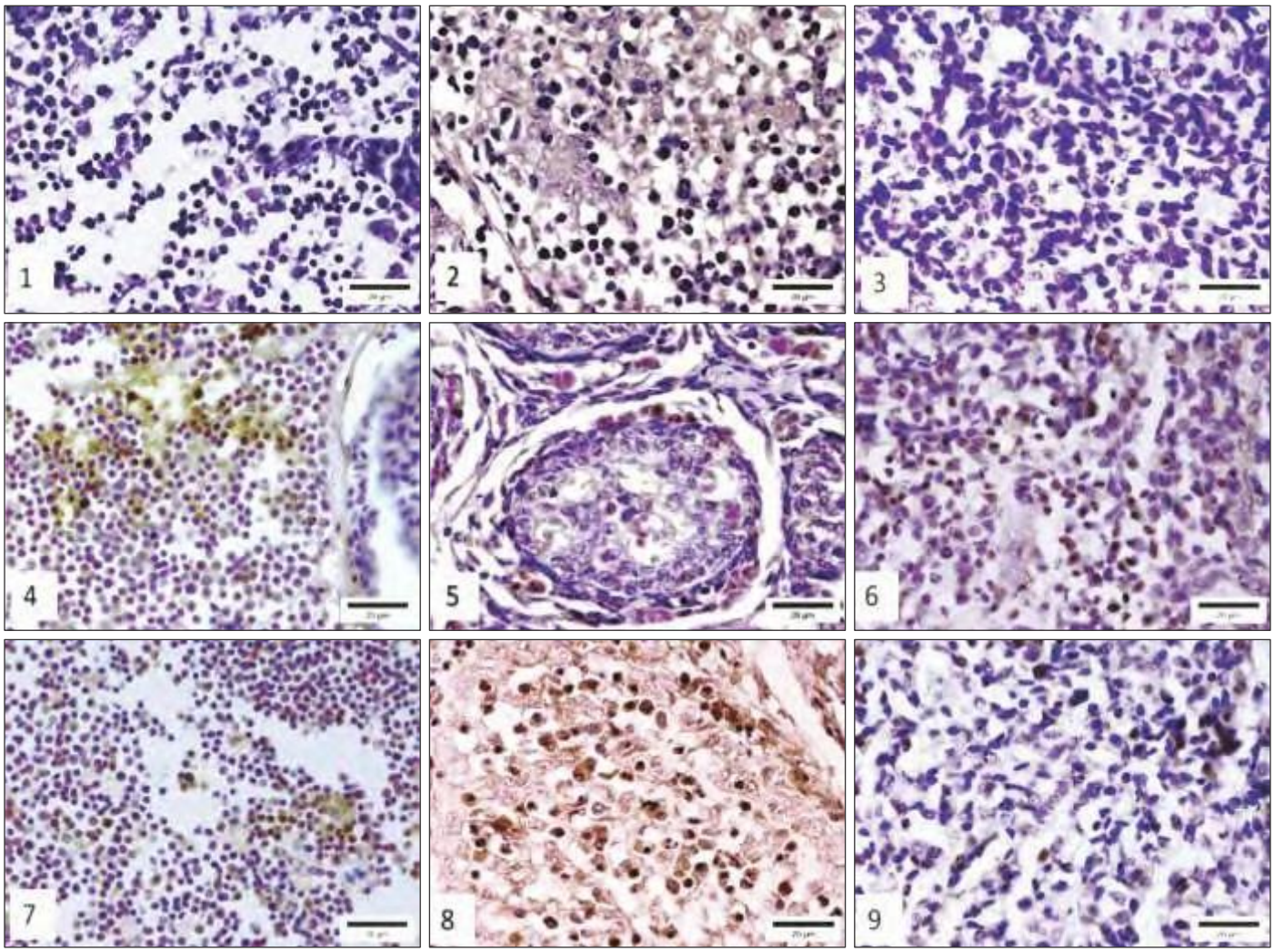

Fig. 1-9. Photomicrograph of Thymus **(1)**, Bursa **(2)** and Spleen **(3)** as control with no brown stain; **(4-6)** Photomicrograph of immunohistochemistry for CD3 in Thymus (4), Bursa (5) and Spleen (6); **(7-9)** Photomicrograph of immunohistochemistry for CD79 in Thymus (7), Bursa (8) and Spleen (9), IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 µm
Note that brown stain in nucleus, cytoplasm and membrane of cells depictes positive cells.

with percentage ofimmunolabelled B-cells and T-cells in lymphoid organs

In the present study, immunolocalization of CD3 and CD79a in lymphoid tissue of positive cases showed that most of the cells were CD3+ stained and were predominantly found in cortical region of thymus although CD79a+ cells were also present but staining was weak compared to CD3+ cells. In bursa of Fabricius CD79a+ cells were distributed mainly in the cortical region whereas CD3+ cells were present in both cortex and medulla in lesser number. In spleen, both CD3+ T-cells and CD79a+ B-cells were scattered in the parenchyma but the staining of CD3+ cells were more as compared to CD79a+ cells (Figs. 1-9). Since, higher density of CD3-cells in thymus and spleen, and CD79a-cells in bursa of Fabricius were observed in diseased birds as reported in earlier studies (Adair, 2000, Adair *et al.*, 1993 and Vaziry *et al.*, 2011) this indicated that viruses had tropism for T cells in thymus and spleen and B cells in bursa of Fabricius in mixed viral infections under field conditions.

Average score and percentage of B-cells and T-cells was calculated in thymus, bursa of Fabricius and spleen, and Pearson correlation coefficient (r) was evaluated to statistically ascertain the disease wise relationship between immunolocalization of viral antigens and percentage of immunolabelled B-cells and T-cells in lymphoid organs (thymus, spleen and bursa) as given in the Table 3. In combined infections of CAV, IBDV and ARV significant negative correlation was obtained between immunohistochemical score and percentage of immunolabelled B-cells in thymus ($r = -0.69$ $P<0.05$) and significant positive correlation was obtained between immunohistochemical score and percentage of immunolabelled T-cells of spleen ($r= 0.67$; $P<0.05$). In mixed infections of CAV and ARV a significant positive correlation was obtained between immunohistochemical score and percentage of B cells in bursa ($r=0.65$; $P<0.008$). However, effect was not significant on B and T cells in cases

solely diagnosed for CAV. Thus, the results of our study indicated that viral antigen load significantly increases in spleen in comparison to the thymus and bursa of Fabricius in combined infections of CAV, IBDV and ARV under field conditions. Moreover, CAV significantly produces depletion of lymphoid cells in combined infections of birds under field conditions. Although, immunosuppressive effect of CAV has been demonstrated experimentally where the mean lymphocyte proliferation stimulation index (SI) was found to be significantly lower in CAV inoculated group and percentages of CD3+, CD4+, CD8+ and NK cells were significantly decreased (Bounous *et al.*, 2000).

CONCLUSION

Although, it is an established fact that IBDV affects B cells of bursa of Fabricius and CAV affects T cells of thymus, but in the present study combined infections by immunosuppressive viruses *viz*. CAV, IBDV and ARV in poultry under field conditions produced significant effect on T cells in spleen. It was concluded that spleen is the most affected lymphoid organ in multiple viral infections of CAV, IBDV and ARV under field conditions owing to severe immunosuppression in commercial poultry flocks. However, CAV alone in chickens under field conditions did not produce much significant effect, suggesting that the virus produces substantial immunosuppression in presence of other immunosuppressive viruses under field conditions. Although, CAV is considered as an important immunosuppressive pathogen of poultry, it produces synergistic deteriorative effect and decreased immunological activity in presence of concurrent infection with other viruses. Therefore, there is a need to conduct epidemiological studies for accurate diagnosis and control of immuno-suppressive viral diseases, which pose great threat to the poultry industry worldwide and cause severe mortality and economic losses.

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