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Clinico-pathological and Immunocytochemical studies on Marek's Disease in poultry

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Abstract

The present investigation was carried out on layer flock belong to Livestock farm complex, College of Veterinary Science, Tirupati. Among 500 birds 41 birds revealed lesions suspected for Marek's disease and morphologically diagnosed on the basis of gross lesions, cytological smears and immunocytochemistry of different organs. Haematological and biochemical examination revealed significant increase in the lymphocytes count, AST, triglyceride levels and significant decrease in cholesterol levels in the Marek's disease affected birds when compared to normal birds. Clinically, birds affected with Marek's disease revealed emaciation, depression with drooping of the wings, anorexia, gasping, reduced weight gain, diarrhoea, pale combs, wattles with presence of nodules on combs, marked reduction in egg production and reduction in size of eggs. Few birds exhibited paralysis with inward curvature of the toes and death of the birds in few days after showing the clinical signs. Grossly, visceral organs (liver, heart, lungs, kidney, ovary, spleen, thymus, pancreas, proventriculus) were enlarged with presence of focal to discrete nodules. Cytological examination of the impression smears collected from the growths of the affected organs during necropsy revealed infiltration of pleomorphic lymphoid cells with thin rim of basophilic cytoplasm. Immunocytochemical staining of the smears revealed moderate to severe immunopositive reactivity for MDV antigens in the cytoplasm of the lymphoid cells.

Keywords: Chickens, cytology, haematobiochemical study, immunocytochemistry, Marek's disease

Introduction

Poultry industry in India is one of the fastest growing segments of the agricultural sector. A tremendous increase in number and size of the poultry flocks in India might be due to rapid increased demand for poultry products and at the same time the poultry farmers are facing lot of constraints due to disease out breaks especially the neoplastic disease conditions, even though vaccination was done at one day old chicken at hatchery itself leading to huge production losses. Marek's disease virus (MDV), an oncogenic avian herpesvirus is the causative agent and is responsible for great economic loss to the poultry industry worldwide. The birds presented for postmortem examination revealed the lesions suspected for Marek's disease and lymphoid leukosis. Hence an attempt is made to study the clinicopathology and immunocytochemistry of Marek's disease from the existing birds of Livestock Farm Complex, Tirupathi.

Materials and Methods

Collection of the samples

The biological materials for hematological studies were collected from the Livestock Farm Complex of the college and for immunocytochemistry the birds presented for necropsy conducted at department of Veterinary Pathology, for a period of 4 months.

Blood samples were collected from the birds showing the clinical signs as well as the normal birds and stored in 10% EDTA solution. The same were used for estimation of the Haemoglobin by Sahli's method and packed cell volume by microhaematocrit method, total erythrocytes count, differential leucocytes count and total leucocytes count (Jain 1986) [9].

Blood was collected from the infected birds directly in to the sterile test tube and allowed to clot. The serum was collected and stored at 4 °C until use and was used for estimation of AST, ALT, total protein, serum albumin, triglycerides, cholesterol, calcium, phosphorus for biochemical estimation with semiautomatic biochemical analyzer (Prietest Robonik) by using biochemical kits.

The impression smears were collected from the organs showing the lesions of the Marek's disease and were fixed in the methanol and preserved and stained with Geimsa stain for cytological examination (Viraraghavan and Nair, 1965) [22].

Immunocytochemistry: Impression smears of various organs like liver, heart, spleen, lungs, kidneys, ovaries, intestines, proventriculus having nodular growths from Marek's disease infected birds. The smears made on the APES (Amino Propyl Ethoxy Saline) precoated slides were dried, fixed with methanol and subjected to immunocytochemistry protocol. The polyclonal primary antibodies of chicken origin were procured from Charles River Laboratories (USA) and reconstituted with 1 ml of sterile deionized water for immunocytochemical staining. In the present study primary antibodies (Marek's disease antiserum) were diluted to 1:5000 and used. The immunological staining protocol used in the present study was similar to previous authors with slight modifications (Yavuz and Erer, 2017 and Andrabi *et al.*, 2018) [23, 2].

Procedure: Methanol poured on the impression smears and kept for 5 minutes and then the slides were washed in distilled water for 10 minutes in Tris buffer for 5 min and were kept in dark humid chambers and pour peroxidase block solution (3% H₂O₂ - freshly prepared) on the slides for 30 minutes to block endogenous peroxidase. Care was taken not to expose towards light. Washed in Tris buffer saline for 3 times 5 minutes each. The power block solution using 1.5% BSA was poured on the smeared portion and incubated for 30 minutes. Primary antibodies were added on the sections and slides were kept at room temperature for 2 hrs or at 4 °C overnight. Washed in Tris buffer saline for 5 min each in 3 changes. Secondary antibody Horse Radish Peroxidase (HRP) was added and kept for 1 hour and then washed in Tris buffer saline for 5 min, each for 3 changes. DAB (2,2 Dichloro Diamino benzidine), coloring reagent was prepared by adding one drop of DAB to 1 ml of substrate. The sections were kept in the coloring reagent for 30 min to 1 hr. Washed in Tris buffer saline for 2 times and in tap water for 10 min and stained with Harris haematoxylin for 1 min. Washed in tap water for 5 min. Air dried the slides and mounted with DPX. The development of brown color in the cells indicate positive reaction where as blue colour development is due to staining with hematoxylin.

Results

Clinical signs: The clinical signs were noted from the history

collected from the records available with the farm in charge and signs observed during the necropsy examination revealed reduced appetite, weight gain, depression, diarrhoea, pale combs, wattles with presence of nodules on combs, marked reduction in egg production and reduction in size of eggs and in few birds atrophied breast and thigh muscles, weakness of legs sometimes paralysis with inward curving of the toes were observed.

Gross lesions

The birds presented for necropsy revealed enlarged liver, heart, lungs, kidneys, ovary, spleen, thymus, pancreas, proventriculus, Intestine with presence of focal to discrete greyish-white nodules. Ovary appeared as cauliflower like structure. Proventriculus was thickened and haemorrhages were observed in the mucosa.

Cytological examination

Cytological examination of the impression smears taken from the tumorous growths of the affected tissues of liver, kidney, spleen, proventriculus, ovary and heart revealed pleomorphic lymphocytes, lymphoblasts with large, round vesicular and hyperchromatic nucleus with few nucleoli having thin rim of basophilic cytoplasm.

Haematological and biochemical profiles

A total of 12 blood and serum samples were collected randomly from the birds affected with Marek's disease showing the clinical signs as mentioned above. 12 more samples were collected from the healthy birds as control and analyzed.

Haematological parameters

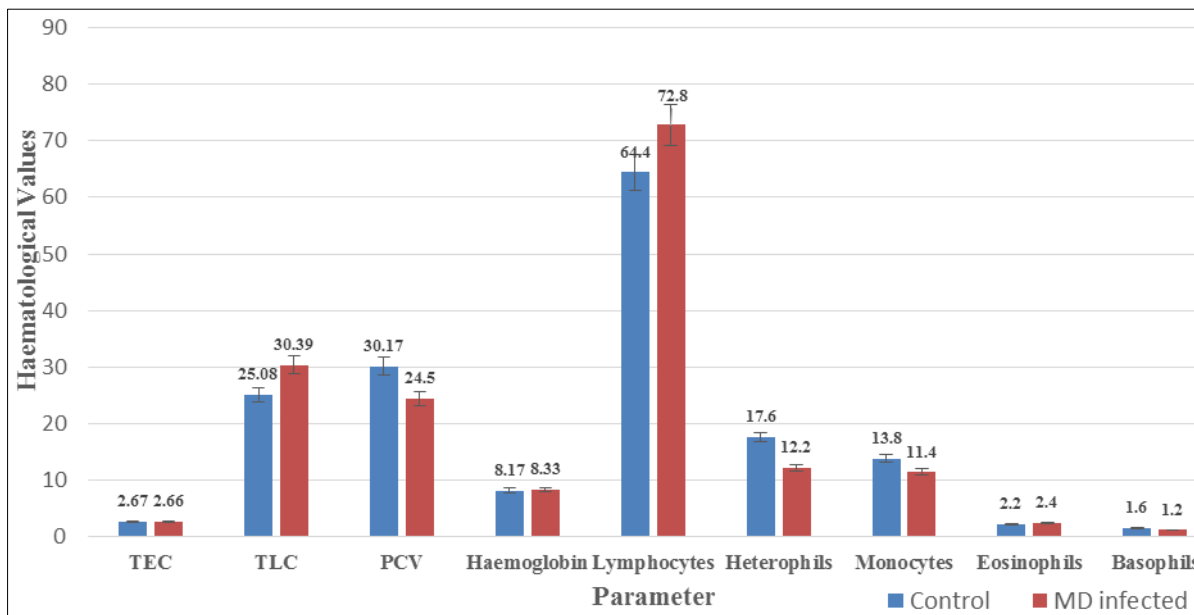
On haematological examination, the mean±SE values of TEC, TLC, PCV, haemoglobin, lymphocytes, heterophils, monocytes, eosinophils, basophils in normal birds and infected birds were indicated in the table 1, graph 1.

The lymphocytes count in the Marek's disease affected birds was increased significantly when compared to the normal birds but the mean values of TEC, TLC, PCV, haemoglobin, number of heterophils, monocytes, eosinophils, basophils count among the Marek's disease affected birds and normal birds were varied non significantly. More particularly non significant increase in the TLC, decrease in the heterophils and monocytes in the Marek's disease affected birds was noted with respect to the normal birds.

Table 1: Mean values of various hematological parameters of the birds suspected for Marek's disease

S. No	Parameter	Normal birds (Mean±SE)	MD infected birds (Mean±SE)
1	TEC (x10 ⁶ /mm ³)	2.67±0.18	2.66±0.22
2	TLC (x10 ³ /mm ³)	25.08±0.50	30.39±5.37
3	PCV (%)	30.17±1.80	24.50±1.52
4	Haemoglobin (g%)	8.33±0.45	8.17±0.30
5	Lymphocytes (%)	64.4±1.36*	72.8±1.66*
6	Heterophils (%)	17.6±2.50	12.2±0.86
7	Monocytes (%)	13.8±1.24	11.4±1.08
8	Eosinophils (%)	2.2±0.66	2.4±0.24
9	Basophils (%)	1.6±0.51	1.2±0.37

* Significant ($P < 0.05$) analysed by two-sample t-test



Graph 1: Graph showing mean values of various haematological parameters of the birds suspected for Marek’s disease

Biochemical parameters

The mean±SE values of AST, ALT, Total protein, Albumin, Globulin, Triglycerides, Cholesterol, Calcium, Phosphorus in the normal birds and in Marek’s disease infected birds were indicated in table 2, graph 2.

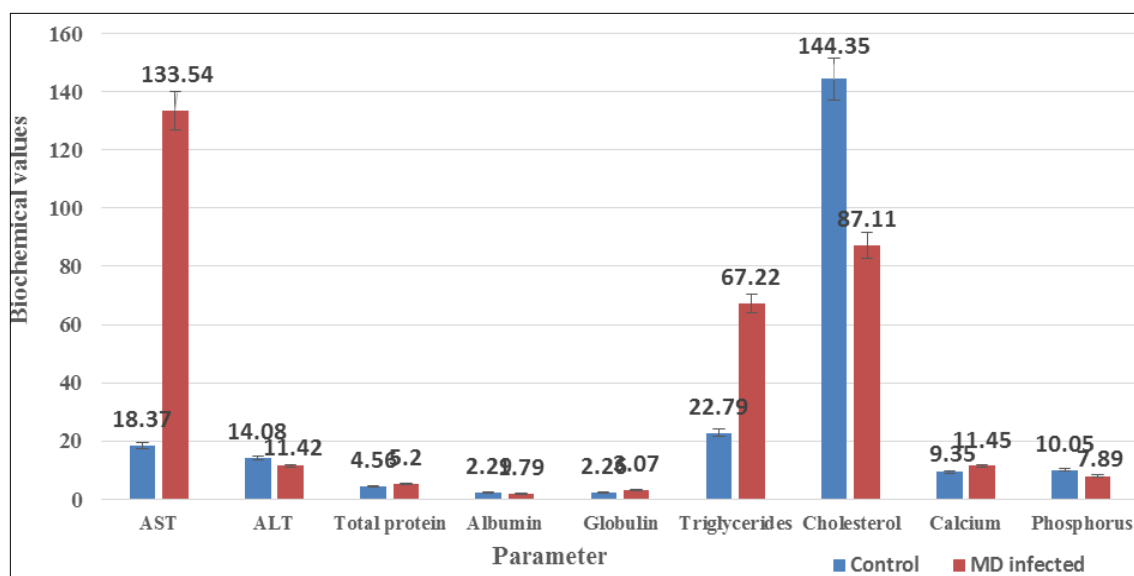
Significant increase in the AST and triglyceride levels and significant decrease in cholesterol levels in the Marek’s disease affected birds were observed when compared to

normal birds. The mean values of ALT, total protein, albumin, globulin, calcium and phosphorous levels between Marek’s disease affected birds and normal birds were varied non significantly. More particularly non significant decrease in albumin, ALT and phosphorus levels and non significant increase in total protein, globulin, calcium levels were noted in Marek’s disease affected birds with respect to normal birds.

Table 2: Mean values of various biochemical parameters of the birds suspected for Marek’s disease

S. No	Parameter	Normal birds (Mean±SE)	MD infected birds (Mean±SE)
1	AST (IU/L)	18.37±14.90*	133.54±29.21*
2	ALT (IU/L)	14.08±2.44	11.42±2.72
3	Total protein (g/dl)	4.56±0.17	5.2±0.49
4	Albumin (g/dl)	2.29±0.21	1.79±0.11
5	Globulin (g/dl)	2.26±0.15	3.07±0.30
6	Triglycerides (mg/dl)	22.79±4.01*	215.74±79.76*
7	Cholesterol (mg/dl)	144.35±8.33*	87.11±11.39*
8	Calcium (mg/dl)	9.35±0.96	11.45±0.91
9	Phosphorus (mg/dl)	10.05±0.62	7.89±1.32

* Significant ($P < 0.05$) analysed by two-sample t-test



Graph 2: Graph showing mean values of various biochemical parameters of the birds suspected for Marek’s disease

Immunocytochemistry

Immunocytochemical staining of impression smears collected from various organs like liver, heart, spleen, lungs, kidneys, ovaries, proventriculus having nodular growths from Marek's disease infected birds revealed moderate to severe immunopositive reactivity for MDV antigens in the cytoplasm of the lymphoid cells.

Discussion

Marek's disease is one of the economically important disease in the poultry industry and recurrence of this disease is often being noted though the birds are vaccinated. Keeping this in view the investigation is carried out by analyzing the hematobiochemical, cytological and immunocytochemical studies.

Clinically birds affected with Marek's disease were emaciated, depressed with drooping of the wings, anorexia, gasping, reduced weight gain, diarrhoea, pale, anemic combs and wattles with nodular growths were observed on combs. These observations were in agreement with earlier reports by Fujimoto *et al.* (1971)^[6], Okwor and Eze (2011)^[16], Ali *et al.* (2014)^[1], Sawale *et al.* (2014)^[20], Balasubramaniam *et al.* (2017)^[3], Makwana *et al.* (2018)^[14] and Das *et al.* (2018)^[5]. Paleness of combs might be due to viral induced anaemia (Heidari *et al.*, 2016)^[7]. In addition there was marked reduction in egg production, reduction in size of eggs was observed in the present study was supported by Okonkwo (2015)^[15] and Das *et al.* (2018)^[5]. In the present study few birds also revealed atrophied breast and thigh muscles, weakness of legs, sometimes paralysis with inward curvature of the toes and death of the birds in few days after showing the clinical signs was noticed. These observations of paralysis of legs was similar to the previous reports by Fujimoto *et al.* (1971)^[6], Santin *et al.* (2006)^[19], Keles *et al.* (2010)^[11], Sawale *et al.* (2014)^[20], Biswas *et al.* (2018)^[4] and Das *et al.* (2018)^[5] in the chickens affected with Marek's disease.

Significant increase in the lymphocytes count and non significant increase in the TLC, decrease in the heterophils and monocytes in the Marek's disease affected birds was noted in comparison to the normal birds. Our results are in line with the findings of earlier study by Payne and Rennie (1976)^[17] in which they observed increase in total lymphocytes, eosinophils count and decrease in monocytes, heterophils and basophils count in the lymphoproliferative phase of the chickens affected with Marek's disease. Similarly Das *et al.* (2018)^[5] reported, significant increase in the TLC values and no significant difference between the mean values of haemoglobin concentration, total erythrocytes count, PCV, DLC among Marek's disease affected birds and the normal birds.

Serum biochemical examination revealed significant increase in the triglycerides, AST levels and decrease in cholesterol levels and non significant decrease in albumin, ALT and phosphorus levels and increase in total protein, globulin, calcium levels in Marek's disease affected birds when compared to the control birds. The increase in AST values and decrease in albumin levels might be due to the liver damage observed grossly. Similarly, the significant increase in cholesterol, globulins and significant decrease in albumin, AST levels with no change in glucose, total protein, ALT, calcium, phosphorus and chloride levels were reported in the Marek's disease affected birds when compared to normal birds by Shanker (1974)^[21].

Grossly, visceral organs (liver, heart, lungs, kidneys, ovary,

spleen, thymus, pancreas, proventriculus, Intestine) were diffusely enlarged with presence of focal to discrete nodules. Ovary was diffusely enlarged with nodular growths which appeared as cauliflower like structure. Proventriculus was thickened and haemorrhages were observed in the mucosa. Similar observations were reported by Lobago and Woldemeskel (2004)^[13], Jayalakshmi *et al.* (2016)^[10] and Prathibha *et al.* (2018)^[18]. Prathibha *et al.* (2018)^[18] on Marek's disease.

Cytological examination of the impression smears taken from the nodular growths of the affected tissues of liver, kidney, spleen, proventriculus, ovary and heart revealed pleomorphic lymphocytes and tumorous lymphoblast cells with large, round vesicular and heterochromatic nucleus with few nucleoli and having thin rim of basophilic cytoplasm in the birds affected with Marek's disease. Similar observations were reported earlier by Hughes *et al.* (2016)^[8], Das *et al.* (2018)^[5] and Kumar *et al.* (2018)^[12].

Immunocytochemical studies of impression smears taken from various organs like liver, heart, spleen, lungs, kidneys, ovaries, proventriculus having nodular growths from Marek's disease infected birds revealed immunopositive reaction in the cytoplasm of the lymphoid cells. These observations were in agreement with Yavuz and Erer (2017)^[23] where they reported intra-cytoplasmic immuno-positive staining in lymphoid cells in liver and in lymphoblastoid cells and plasma cell in spleen.



Fig 1: Photograph showing misshaped heart and enlarged liver with presence of focal greyish-white nodules



Fig 2: Photograph showing pale, focal greyish-white nodule on the right anterior lobe of kidneys

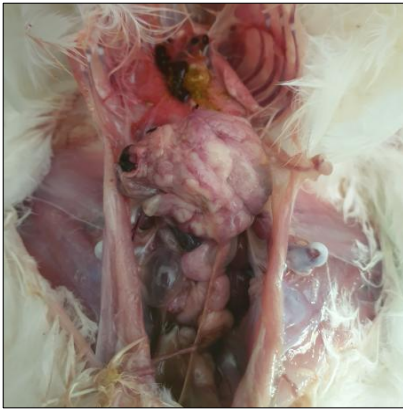


Fig 3: Photograph showing extremely enlarged ovary with irregular greyish-white firm cauliflower like growths



Fig 4: Photograph showing thickened glands with presence of haemorrhages in the mucosa of proventriculus

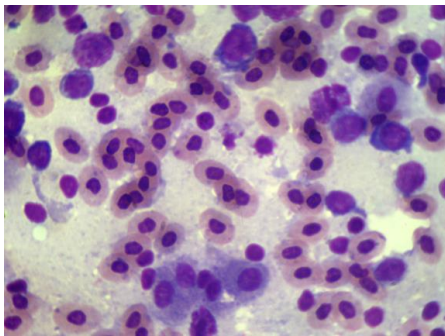


Fig 5: Cytological smear of spleen showing pleomorphic lymphoid cells with heterochromatic nucleus and basophilic cytoplasm. Giemsa stain x 1000

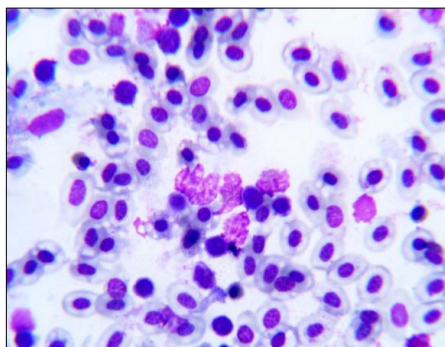


Fig 6: Cytological smear of liver showing pleomorphic lymphoid cells with heterochromatic nucleus and basophilic cytoplasm. Giemsa stain x 1000

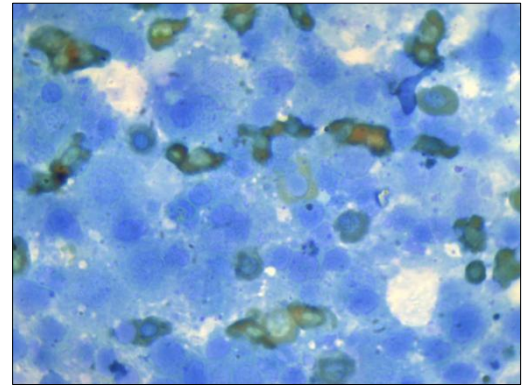


Fig 7: Immunocytochemical staining of liver showing positive reactivity in the lymphoid cells. ICC x 1000

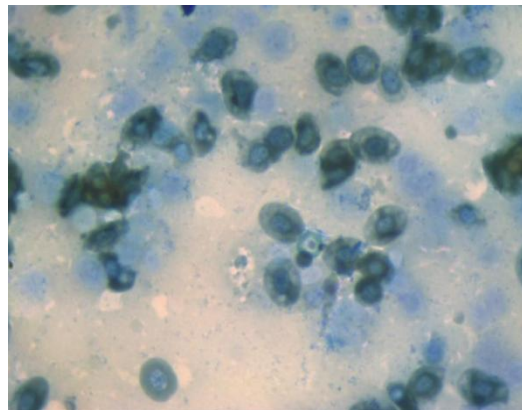


Fig 8: Immunocytochemical staining of spleen showing positive reactivity in the lymphoid cells. ICC x 1000

Conclusion

The above study concluded that the incidence of Marek’s disease among the vaccinated chicken flock suspected clinically and diagnosed as Marek’s disease through haematobiochemical studies, gross pathology at necropsy, cytological and immunocytochemical examination.

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