

## The Evaluation of Enzyme Histochemical and Histopathological Findings in the Diagnosis of Marek's Disease

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### Abstract

Marek's disease (MD) and other lymphoproliferative diseases such as lymphoid leucosis show similar clinical symptoms. Age of the bird, neural lesions, histopathologic and cytologic changes are important criteria in the differentiation of these diseases. T lymphocytes were reported as predominant in MD and Alpha Naphthyl Acetate Esterase (ANAE) demonstration has been used in the description of T cells in chickens. In this study, results of the lymphocyte enzyme histochemistry were compared with macroscopic and histopathologic findings, and also, value of enzyme histochemical findings were evaluated in the diagnosis of MD. For this reason, ANAE was demonstrated in peripheral blood and tissue samples in animals showing clinical signs of MD and compared with histopathological results. The proportion of ANAE positive lymphocytes was found significantly higher in both peripheral blood and lymphocytic infiltration in affected tissues of animals displaying clinical symptoms of MD and these results were supported by gross and microscopic findings. It is concluded that ANAE demonstration in peripheral blood and tissue sections is useful method with clinical and histopathological findings in the diagnosis of MD.

**Keywords:** Marek's disease, Alpha Naphthyl Acetate Esterase, Histochemistry, Histopathology

### Marek Hastalığı Teşhisinde Histokimyasal ve Histopatolojik Bulguların Değerlendirilmesi

#### Özet

Tavukların lenfoproliferatif bir hastalığı olan Marek hastalığı (MD), lenfoid lökozis gibi diğer lenfoproliferatif hastalıklar ile benzer klinik belirtiler gösterirler. Hastalığın ayırıcı tanısı için yaş, sinirsel belirtiler, histopatolojik ve sitolojik bulgular önemli kriterler olmakla birlikte yetersizdir. T lenfositlerin MD'de baskın hücre olduğu bildirilmektedir. Alfa Naftil Asetat Esteraz (ANAE) tespiti ise tavuklarda T lenfosit tanımlamasında kullanılan önemli bir yöntemdir. Bu çalışmada, hastalık şüpheli tavuklardan alınan kan ve doku örneklerinde ANAE demonstrasyonu yapıldı ve elde edilen sonuçlar makroskobik ve mikroskobik bulgularla karşılaştırıldı. Marek hastalığının teşhisinde lenfosit enzim histokimya sonuçları ile makroskobik ve histopatolojik sonuçlar karşılaştırılarak, Marek hastalığı teşhisinde enzim histokimyasal bulgular değerlendirildi. ANAE Marek hastalıklı tavukların hem perifer kanlarında hem de tümörlü dokularında önemli derecede yüksek olarak tesbit edildi. Sonuç olarak, klinik ve histopatolojik bulguların yanısıra perifer kan ve doku lenfositlerinde ANAE tesbitinin Marek hastalığının teşhisinde yararlanılabilecek bir metod olduğu kanısına varıldı.

**Anahtar sözcükler:** Marek hastalığı, Alfa Naftil Asetat Esteraz, Histokimya, Histopatoloji

## Introduction

Marek's disease (MD) is the most common lymphoproliferative diseases of chickens and it is characterised by a mononuclear infiltration of one or more of the peripheral nerves, gonad, iris, various viscera, muscle and skin. MD is caused by a herpesvirus and it is transmissible. MD can be distinguished etiologically from other lymphoid neoplasms of birds. Clinical symptoms are generally associated with asymmetric progressive paresis and later, complete paralysis of one or more of the extremities. Leucotic lesions in various visceral organs and muscles were referred to simply as visceral lymphomatosis. MD and Lymphoid leucosis show similar clinical symptoms. To differential diagnose of these diseases; however, following criteria have been suggested including age of the bird, occurrence of neural lesions, histopathologic and cytologic changes (1-5). Hudson and Payne (6) had reported that T lymphocytes were predominant in MD. Calnek and Witter (5) also reported that T lymphocytes were 60-90% in MD tumors. T lymphocytes show ANAE (78%) and ACP-ase (90.8%) positivity. Since ACP-ase also shows high positivity in unmaturation fetal lymphocytes (96%), ANAE positivity is broadly used on the determination of T lymphocytes (7-9). A correlation was reported between T lymphocyte in peripheral blood lymphocytes (PBL) and ANAE positivity rates in cattles (10), mouse (11), chickens (12, 13), dogs (14), Angora rabbits (15), and domestic ducks (16). In addition, the localizations patterns of ANAE positive T lymphocyte are ratherly harmonious with T lymphocyte localizations in tissue sections (7, 14).

The aim of this study is to compare the results of lymphocyte enzyme histochemistry by detecting ANAE positivity of blood and tissue lymphocytes with macroscopic and histopathological findings of MD and also to evaluate the value of ANAE enzyme histochemistry in the diagnosis of MD.

## Material and Methods

Twenty healthy (for control) and twenty diseased and suspected birds showing clinical signs of MD were obtained from different poultry farms and used as animal material of the study. Cardiac blood samples from animals were collected in to heparinised (Heparin 10 IU/ml blood, Liquemine Flc., Roche) tubes and were used in lymphocyte enzyme histochemistry. Then, all hens were tested for post-mortem examination, and gross findings were

recorded. Tissue samples were taken during the necropsy for histopathological and enzyme-histochemical studies.

### Preparing of ANAE Incubation Solutions

An incubation solution was prepared by mixing 20 mg of substrate, *alpha*-naphthyl-acetate (N-8505, Sigma, Steinheim, Germany) dissolved in 0.8 mL of acetone (Merck, Darmstadt, Germany), 4.8 mL of hexazotized pararosaniline [hexazotization was performed by mixing equal volumes (2.4 mL each) of 4% sodium nitrite (Merck) and 2% pararosaniline (Merck)], and 80 mL of phosphate buffer solution (pH 5). Final pH of the incubation solution was adjusted to 5.8 with 1 N NaOH, and the solution was filtered. Blood smears and tissue sections were frequently controlled under a light microscope during incubation approximately 2 hours and 30 minutes respectively at 37°C. After the incubation, the smears and sections were rinsed 3 times in distilled water, and nuclei were stained for 20 min in 1% methyl green prepared in acetate buffer (pH 4.2) (18).

### ANAE Demonstration in Peripheral Blood and Tissue Sections

Tissue samples from visceral organs, brain, peripheral nerves and skin were taken during necropsy and fixed in formol-sucrose (pH 6.8) at +4°C for 22 hours and kept in Holt solution for 22 hours. Frozen sections (Slee, London) were taken and ANAE activity was demonstrated according to Çelik et al. (18) whereas two slides were prepared from all blood samples. These blood samples were air dried and fixed in gluteraldehyde-acetone solution at -10°C for 3 minutes, rinsed in distilled water for 3 times and ANAE were demonstrated according to Çelik et al. (18).

All slides were stained with nuclear stain (Methyl green in acetate buffered, pH 5.2), and covered with Entellane (Entellane, Merck) and examined under the light microscopy. Those lymphocyte which contains 3-5 red-brown granules in the cytoplasm were evaluated as ANAE positive T lymphocyte (Figure 1), but the others which haven't this spesific granules were evaluated as ANAE negative B lymphocytes. Two hundred lymphocytes were counted on all smears.

### Histopathological procedures

Tissue samples from visceral organs, brain, peripheral nerves and skin were taken during necropsy and fixed in neutral buffered formalin solution (10%). After the routine histopathologic process, 5 µm paraffin sections were prepared and stained with Pappenheim’s panoptic stain and Hematoxylin and Eosine.

### Results

#### Clinical Findings

Clinical signs were associated with asymmetric progressive paresis and complete paralysis of one or more of the extremities. Depending on the affected nerves, drooping of limb or torticollis was detected. Weakness and emaciation were usually observed in all diseased birds.

#### Gross Lesions

Nervous lesions were prominent finding in all affected birds. In 14 chicks, affected peripheral nerves were characterised with edematous appearance, gray to yellow discoloration and loss of cross-striations. Localised or diffuse, unilateral enlargement of peripheral nerves were detected in these birds.

**Table 1.** ANAE (+) T lymphocyte rates and statistical results in peripheral blood of MD suspected and normal birds.

No	I (%)	II (%)	No	I (%)	II (%)
1	51.08	66.89	11	54.63	87.17
2	56.78	68.25	12	55.60	58.50
3	52.50	77.81	13	51.28	58.00
4	51.28	79.66	14	52.42	65.15
5	53.38	62.29	15	53.12	59.66
6	52.11	55.36	16	54.28	72.24
7	54.37	67.98	17	52.64	70.27
8	53.65	73.00	18	54.23	73.88
9	54.32	62.06	19	53.18	61.45
10	52.08	54.04	20	55.88	66.98
			X± SE	53.44 ± 2.58	64.26 ± 2.35*

I: Control group, II: Marek’s diseased group

(P< 0.05) It is significantly higher than control group.

Lymphoid tumors were localised in liver, spleen, ovary, kidney, proventriculus and heart. In the examination of the visceral organs, white to gray nodules in different sizes and shaped were observed on the livers and spleens. There were atrophy and paleness in skeletal muscles, especially in pectoral. Edema and wall thickness were observed in proventriculus.

#### ANAE Positivity in Peripheral Blood Lymphocytes

ANAE positive lymphocytes were demonstrated on periferic blood smear (Figure 1) and the rate of blood ANAE + T lymphocyte and statistical analyses were given in Table 1.

## Histopathology and Histochemistry

**Peripheral Nerves:** Both inflammatory and neoplastic changes were seen in peripheral nerves. There were masses of proliferating lymphoblastic cells in neoplastic cases. On the other hand, inflammatory changes were characterised by diffuse, mild to moderate infiltration of lymphocytes (Figure 2) and plasma cells with edema. Demyelination and Schwann cell proliferation were sometimes observed in both types. It was seen that the most of the infiltrated lymphocytes were ANAE positive in histochemistry (Figure 3).

**Cerebrum and cerebellum:** Perivascular lymphoid infiltrations in 7 cases and, also gliosis in another case were observed. ANAE positive lymphocyte infiltration has been diffusely detected in cerebral cortex (Figure 4). The number of ANAE positive monocyte/macrophage rate increased in stratum granulosum of the cerebellum. In addition to these changes, large intracytoplasmic vacuols have been detected in purkinje cells in stratum gangliosum (Figure 5).

**Bursa of Fabricius:** Atrophy and follicular destruction were seen in Bursa of Fabricius. Necrosis of lymphoid cells and cystic cavitations were shown in medulla. In addition, ANAE positive macrophages were detected in follicular medulla (Figure 6).

**Liver:** Periasinar, portal or diffuse lymphocytic cell infiltrations and granulomas were seen in the liver in

different degree. Some livers were entirely covered with pleomorphic lymphocytic cells. Strong ANAE positivity was observed in hepatocytes in these sections (Figure 7).

**Proventriculus:** This organ was infiltrated by pleomorphic lymphocytic infiltration in lamina propria, lamina muscularis and interglandular area in all diseased chicks.

**Heart:** lymphocytic infiltration was seen in the interstitial tissue of the myocard in 7 birds of 20.

**Kidney:** Perivascular lymphocytic infiltration and interstitial nephritis were observed in kidneys of 15 chicks.

**Spleen:** In 15 spleens of 20 chicks, it was seen enlargement of PALS (periarteriolar lymphoid sheaths). Because of severe lymphoid infiltration, normal histologic appearance of spleen completely changed in 1 animal.

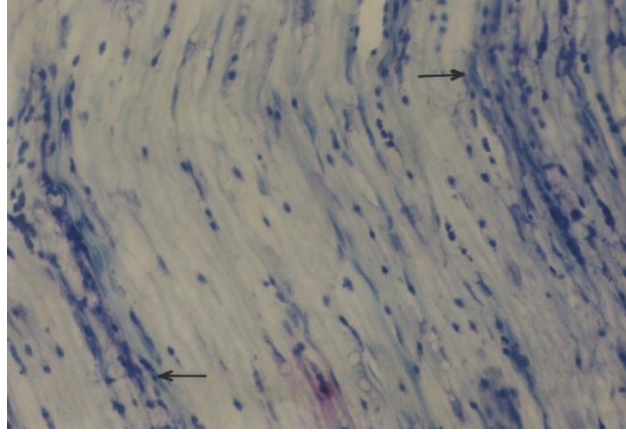
**Intestines:** There were prominent thicknesses of small intestinal wall, because of severe lymphocytic infiltrations in lamina propria, submucosa and tunica muscularis.

**Pancreas:** Lymphocytic infiltrations were detected in different degrees in 5 chicks.



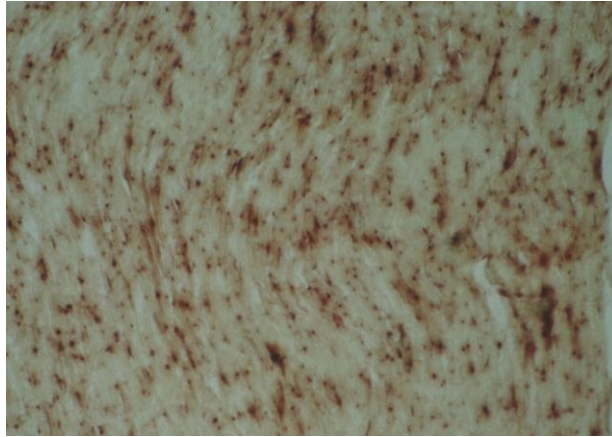
**Figure 1.** ANAE positive lymphocytes (arrows) in Peripheral blood. ANAE demonstration. X800.

**Şekil 1.** Periferik kanda ANAE pozitif lenfositler (oklar). ANAE boyama X800.



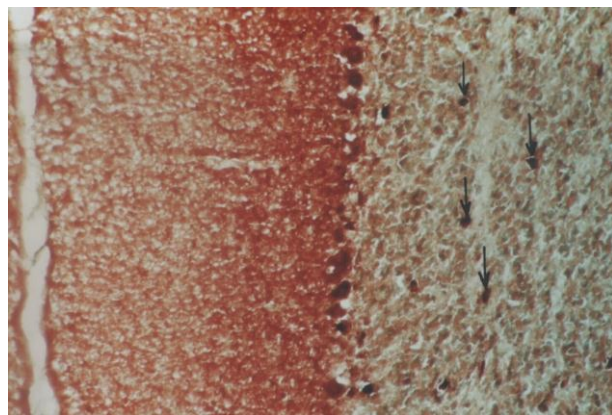
**Figure 2.** Nervus ischiadicus. Edema and lymphocytic infiltration (arrow). Pappenheim's panoptic stain. X382.

**Şekil 2.** Nervus ischiadicus. Ödem ve lenfosit infiltrasyonu (oklar). Pappenheim'in panoptik boyası. X382.



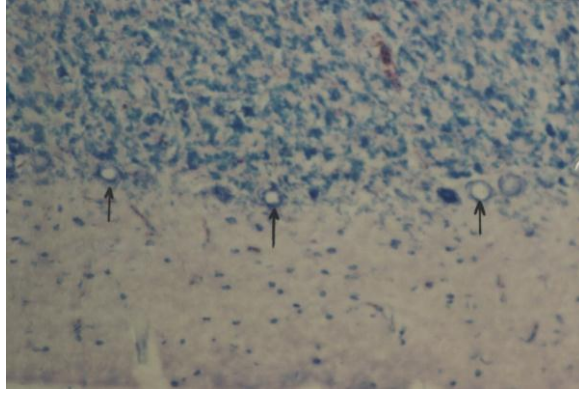
**Figure 3.** Nervus ischiadicus. ANAE positive lymphocytes. ANAE demonstration. X350

**Şekil 3.** Nervus ischiadicus. ANAE pozitif lenfositler. ANAE boyama. X350



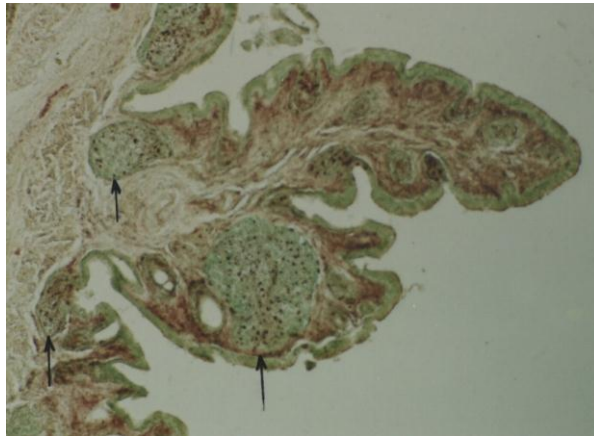
**Figure 4.** ANAE positive cells in the cerebellar cortex (arrows). ANAE demonstration. X256.

**Şekil 4.** Serebellar kortekste ANAE pozitif hücreler (oklar). ANAE boyama X256.



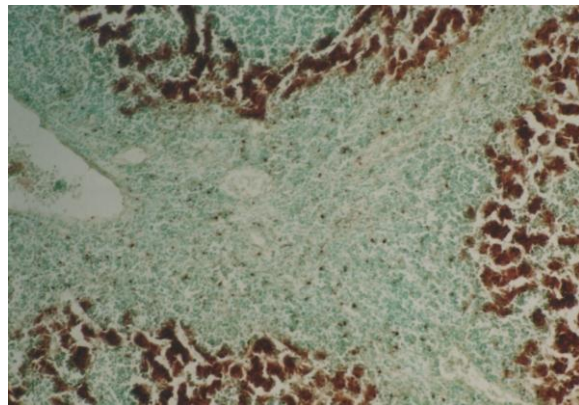
**Figure 5.** Large intracytoplasmic vacuols in stratum gangliosum of Cerebellum. Papanicolaou's panoptic stain. X280.

**Şekil 5.** Serebellum'un Stratum gangliosum tabakasında intrastoplazmik vakuoller, Papanicolaou'nun panoptic boyası. X280.



**Figure 6.** Bursa of Fabricius. Disorganisation of lymphoid follicles (arrows), cystic formations in medulla and macrophage type ANAE positivity. ANAE demonstration. X100.

**Şekil 6.** Bursa Fabricii. Follikülerde düzensizlik (oklar), medullada kist oluşumları ve ANAE pozitif makrofajlar. ANAE boyama, X100.



**Figure 7.** Liver. Perivascular lymphocytic infiltration in interlobular area. Strong ANAE positivity in hepatocytes. ANAE demonstration. X350.

**Şekil 7.** Karaciğer. İnterlobüler alanda lenfosit infiltrasyonu, Hepatositlerde güçlü ANAE pozitif boyanma, ANAE boyama. X350.

## Discussion

Some embryonic disorders or many factors affecting the lymphoid system cause different variations in both PBL (Peripheral Blood Lymphocytes) rates and lymphoid population in tissues. Enzyme histochemical methods have been used to detection of these disorders (10, 17, 18). This study was carried out to determine ANAE positive T lymphocyte rate and to correct that the numbers of T lymphocyte increase in Marek's disease. Basso et al. (19) indicated that T lymphocytes showed ANAE (78%) and ACP-ase (90.8%) positivity. Because ACP-ase also shows high positivity in unmaturation fetal lymphocytes (96%), ANAE positivity is broadly used on determination of T lymphocytes (7-9). ANAE shows diffuse and thin granular positivity in monocytes and macrophages. So, this peculiarity differs from T lymphocytes in both tissue and peripheral blood (7, 14). Kajikawa et al. (10), Mueller et al. (11), Pruthi et al. (12), Maiti et al. (13) and Wulff et al. (14) reported that the rates of ANAE positivity were harmonious with T lymphocyte in peripheral blood of cattle, mice, chickens and dogs, respectively.

In this study, it was determined that peripheral blood ANAE positive lymphocyte rate was 53.44% in control chicks. This result is higher than previous reports (12, 13). This difference may depend on the age, breeding and feeding properties of animals. ANAE positivity was detected much more higher ( $P < 0.05$ ) in Marek's diseased birds. This data supports Calnek and Witter (5) who reported that T lymphocyte rate could increase in Marek's disease. ANAE was also found positive in many of lymphocytes infiltrations of Nervus ischiadicus and visceral organs.

Emaciation, walking disorders, wing and extremity paralysis of chicks suffered from MD were clinically similar to previous reports (1, 2, 4, 5). In addition, complete atrophy of Bursa Fabricius, partial atrophy of thymus and hyperplasia of PALS indicated the loss of B lymphocyte reserves. Histopathological changes such as oedema, swelling and interglandular lymphoid infiltration of proventriculus were similar to the other reports (5). It was detected focal or diffuse pleomorphic lymphocytic infiltrations and ANAE positivity in many sections of proventriculus, liver and spleen in this study and it makes these organs are important for the diagnosis of Marek's disease.

According to Neuman and Witter (2); Neuman and Witter (3) and Calnek and Witter (5), lymphocytic leukemia tumors consist of B cell, But, MD tumors mainly consist of T cells. In our study, this important difference was exhibited by the demonstration of ANAE positivity of T cells in MD diseased animals.

Based on the findings, it is concluded that ANAE demonstration could be a useful aid in the differential diagnosis of Marek's disease in addition to conventional methods.

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