

Pathologic Changes and Use of Immunohistochemical Methods in Naturally Occuring Avian Encephalomyelitis of the Chicks

Nihat TOPLU *  Günay ALÇIĞIR ** Recai TUNCA *

* Department of Pathology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın - TURKEY

** Department of Pathology, Faculty of Veterinary Medicine, Ankara University, Ankara - TURKEY

Makale Kodu (Article Code): 2009/094-A

Summary

The present study describes the pathological and immunohistochemical findings of avian encephalomyelitis (AE) in naturally infected 31 chicks obtained from three broiler rearing farms. Clinically, ataxia, paresis, paralysis, tremor and torticollis were observed in the chicks. Nonpurulent encephalomyelitis accompanied by neuronal degeneration and necrosis was the most pronounced histopathological findings of the central nervous system (CNS). Lymphoid aggregations and/or mononuclear cell infiltrations were observed in the visceral organs. The avidin-biotin peroxidase complex (ABC) and immunofluorescence (IF) methods were compared for AE viral antigen detection in formalin-fixed, paraffin embedded tissues. With the both tests, condensed viral immunolabelling was detected particularly in the CNS, intestines, proventriculus, kidneys, lungs and heart and, to a lesser extend, in the gizzard, liver, pancreas, spleen, eyes and fabricius of Bursa. Compared of both methods according to labelling intensity and distribution of the viral antigen, ABC method was found to be more sensitive than IF method.

Keywords: Avian encephalomyelitis, Avidin-biotin peroxidase complex method, Chick, Immunofluorescence method

Civcivlerin Doğal Avian Ensefalomyelitis'inde Patolojik Değişiklikler ve İmmunohistokimyasal Metotların Uygulanması

Özet

Çalışmada, üç kümeleden sağlanan doğal enfekte civcivlerdeki avian ensefalomyelitis (AE)'in patolojik ve immunohistokimyasal bulguları tanımlanır. Civcivlerde, ataksi, parezis, paralizis, tremor ve tortikollis göze çarpan klinik bulgulardır. Santral sinir sistemin (CNS)'de nöron dejenerasyonu ve nekrozlarını da içeren nonpurulent ensefalomyelitis en dikkat çekici histopatolojik bulgulardır. Visceral organlardaki değişiklikler; lenfoid kümeler ve/ya da mononükleer hücre infiltrasyonlarından oluşmaktaydı. Viral antijeni belirlemede; formalin tespitli parafin kesitlere avidin-biotin peroksidaz kompleks (ABC) ve immunofloresan (IF) metotlar uygulandı ve sonuçları karşılaştırmalı olarak değerlendirildi. Her iki testte de, AE viral antijen pozitifliği CNS, bağırsaklar, bezli mide, böbrekler, akciğerlerde ve kalpte şiddetli iken; kaslı mide, karaciğer, pankreas, dalak, gözler ve Bursa fabricius'ta ise zayıftı. Her iki metot, immunreaksiyonların şiddeti ve dokulardaki dağılımı yönünden karşılaştırıldığında, ABC metot IF'ye göre daha duyarlı idi.

Anahtar sözcükler: Avian ensefalomyelitis, Avidin-biotin peroksidaz kompleks metot, Civciv, İmmunofloresan metot

INTRODUCTION

Avian encephalomyelitis (AE) is an infectious viral disease affecting young chickens, pheasants, quail, turkey and pigeon ¹⁻⁶. The virus is classified in the family Picornaviridae, the sequences of its inclusions in the genus Hepatovirus ⁷.

Clinically, marked symptoms in young birds are

characterized with neurological signs such as ataxia, paresis or paralysis and rapid tremors, especially of the head and neck ^{8,9}, whereas in older birds infection is subclinical, resulting in declines in egg production and hatchability ¹⁰⁻¹². Histologically, lesion in CNS has been characterized with nonpurulent encephalomyelitis ^{8,13-15}. Changes in visceral organs consist of lymphocyte



İletişim (Correspondence)



+90 256 2470700/183



nihat.toplu@gmail.com / nihattoplu@hotmail.com

aggregates, which are either increased in size or frequency or are found in unusual places^{9,16-18}. Lymphoid aggregates in the proventriculus are considered to be pathognomic, especially when coupled with central chromatolysis from lesions of CNS¹. For immunohistochemical diagnosis, the immunofluorescence method (IF) has been successfully used in definitive diagnosis and pathogenesis studies of AE to date^{14,19-21}. It is reported that AE viral antigen gradually increases in the brain tissue with the progression of neuronal degeneration and necrosis. However, labelling intensity of viral antigen may be decreased due to intense inflammation such as perivascular infiltrations, gliosis and mononuclear infiltration, even it may not be detectable by IF^{14,18,21,22}. For this reason, IF method may not be adequate for certain diagnosis of AE in CNS sections including strong inflammatory reactions. On the other hand, the immunoperoxidase method, another sensitive diagnostic test, have not been applied for detection of AE.

The subjects of this study are to describe the pathological findings and to compare the avidin-biotin peroxidase complex (ABC) and IF methods for detection and tissue distribution of AE viral antigens in natural AE infected chicks.

MATERIAL and METHODS

Animals

Farm I. Six 17-day of age chicks, only complaining of standing and ataxia, were presented for laboratory investigation by the practitioner. After that seven 35 days of age chicks with nervous clinical signs including paresis, paralysis, tremor and torticollis were obtained from the same farm.

Farm II. Twelve 20-day of age broiler chicks had clinical findings of ataxia, tremor, torticollis and paralysis.

Farm III. Six one-day of age broiler chicks were presented with ataxia, tremor and paralysis. Practitioner of this farm also pointed out that similar symptoms were seen in broiler chicks of other farms delivered from the same breeding hen flock with the chicks in farm III.

Each farm was belonging to different commercial firms. The animals were subjected to necropsy. The tissue samples were collected from intestines, proventriculus, gizzard, pancreas, fabricius of Bursa, liver, spleen, kidneys, lungs, cardiac muscles, eyes and brain,

then fixed in 10% neutral-buffer formalin solution, embedded in paraffin wax, sectioned at 5 μ m and stained hematoxylin and eosin (HE). Selected sections were used for immunohistochemical stainings.

Immunohistochemistry

For ABC method (Kwik-kit; Shandon, Pittsburg, USA) was performed, essentially as described by Toplu⁶. Briefly, sections (5 μ m) were mounted placed on poly-L-lysine coated glass slides. After incubation for 2h at 40°C, sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase was then blocked with H₂O₂ 3% in 70% methanol. The tissues were digested with 0.1% protease K for 10 min at 37°C and the slides washed for 10 min in phosphate-buffered-saline (PBS; pH 7.3). Nonspecific staining was blocked by treatment with 2% normal goat serum for 10 min. The blocking serum was then replaced by rabbit anti-AE virus serum diluted 1 in 64, followed by overnight incubation at 4°C. After washing for 10 min, sections were flooded with biotinylated goat anti-rabbit immunoglobulin for 10 min. After another wash, sections were covered with streptavidin-peroxidase and incubated for 10 minutes. Finally, they were treated for 7 min with diaminobenzidine (DAB) containing H₂O₂ 3%. The sections were then counterstained with Mayer's haematoxylin, washed in tap water, dehydrated in graded alcohols, and mounted. For control slides, replicate sections of selected infected tissues were processed, substituting chicken anti-Newcastle disease virus antibody (against La Sota strain) for anti-AE serum. All incubations were performed at room temperature in a humidified chamber.

For IF method, the sections were deparaffinized in xylene and washed briefly in PBS solution. Tissues were digested with 0.1% protease K for 10 min at 37°C. Slides were washed for 15 min in PBS for IFA. Sections were incubated with chicken anti-AE serum conjugated with FITC in a 1:16 dilution for 45 min at 37°C, and then washed for 15 min in PBS. They were then washed in PBS for 15 min and mounted in phosphate-buffered glycerin (pH 9.0). For control slides, replicate sections of selected infected tissues were processed, substituting chicken anti-Newcastle disease virus antibody (against La Sota strain) conjugated with FITC for anti-AE serum conjugate. The results of the fluorescent antibody reaction were determined using a fluorescent microscope (Leica DMLB).

Score of Immunohistochemical Data

The percentage of the total area of the fluorescent and peroxidase labelling positive cells were assessed semi-quantitatively under light and fluorescent microscopes with a 10X ocular with grids and a 20X objective. The labelling intensity in a given cellular compartment was assessed according the following categories; 0+: no positively staining cells; 1+: weak staining (1-2% positive cells); 2+: moderate staining (3-5% positive cells); 3+: marked staining (>6% positive cells).

RESULTS

At necropsy, 3 animals from farm I showed fibrinoid pericarditis. On bacteriologic examination, *E. coli* was isolated from heart tissue specimens. However, the other birds had not gross lesions, and no bacteria were isolated from tissue specimens.

The most remarkable histopathological findings were in the brain and segments of the spinal cord. The lesion in all portions of CNS were characterized with nonpurulent encephalomyelitis: perivascular infiltrations consisted of lymphocytes, few macrophages and plasma cells; gliosis were in focal and/or diffuse patterns. Perivascular infiltration and gliosis were not intense in cases of farm III and 6 cases of farm I. In the cerebellum, there were intense gliosis from granular to molecular layer and perivascular infiltrations in molecular layer and substantia alba (Fig 1A). Neuronal degeneration and necrosis characterized by central chromatolysis and neuronal shrinkage were marked

especially in Purkinje cells and motor neurons. Central chromatolysis was observed especially in motor neurons of the medulla oblongata and spinal cord, and brain stem of seven cases in farm I and II (Fig 1B), but not a common finding for all cases. With exception from farm I and farm II, the birds of farm III showed polio-leucomalacic changes of the cerebellum in three cases. In these cases, infiltration of eosinophil leucocytes as well as mononuclear cells was recognized from the meninges to brain tissue, and rarely in choroid plexus. No central chromatolysis was observed in all cases of farm III. Additionally, lytic alterative changes as well as neuronal shrinkage were observed in the locations of Purkinje cells and motor neurons in all cases of farm III.

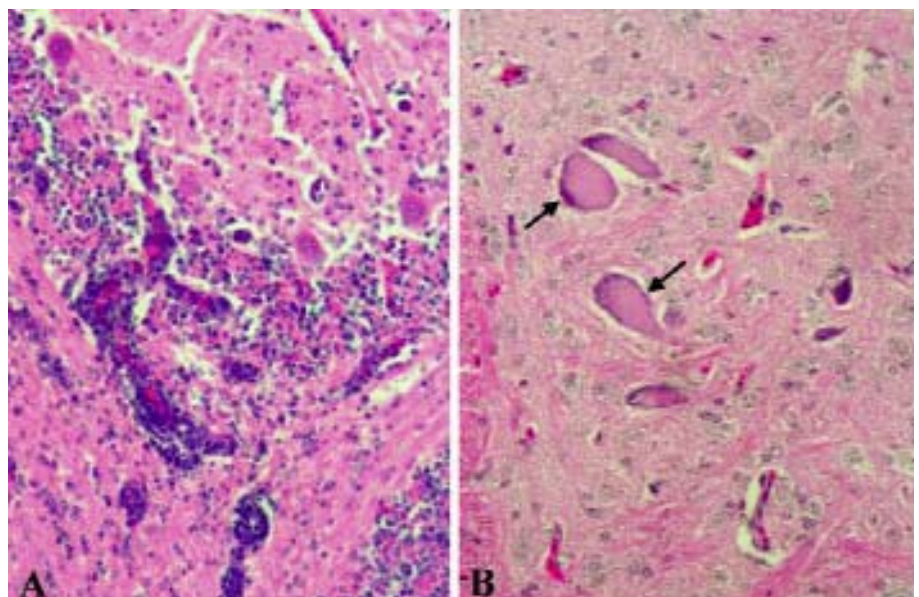
In visceral organs, lymphoid aggregations and/or mononuclear cells infiltration were most conspicuous findings especially in cases of farm I and farm II. Infiltration of focal or diffuse lymphocytes was present in the lamina propria and rarely in the muscular layer of the proventriculus, and in the muscular layer and crypts of the gizzard. Small intestines, especially duodenal cryptes, showed mononuclear cells infiltration and occasional lymphoid aggregation in the muscular layer. Lymphoid aggregations were also recognized in the liver, pancreas, spleen, lungs, and cardiac muscle. Diffuse or focal nonpurulent interstitial nephritis along with partly destroyed and disappeared tubules was marked lesions in the kidneys. However, lymphoid aggregations were restricted only in the liver of 4 cases in farm III.

Immunohistochemical findings

The results are summarized in Fig 2, Tables 1 and 2.

Fig 1. (A) Intense gliosis from granular to molecular layer and infiltration of perivascular mononuclear cells in the cerebellum. HE. x180. (B) Central chromatolysis of motor neurons (arrows) in lumbosacral cord of medulla spinalis. HE. x180

Şekil 1. (A) Serebellumda granular tabakadan moleküler katmana uzanan şiddetli gliozis ve perivasküler mononükleer hücre infiltrasyonları. HE. x180. (B) Medulla spinalisin lumbosakral bölümündeki motor nöronlarda sentral kromatolizis (oklar). HE. x180



There were significant differences in the intensity scores and location of AE viral antigen especially in CNS and visceral organs between ABC and IF. On the 31 CNS samples analysed, positive staining (31/31, 100%) with both ABC and the IF methods was identical in cerebral hemispheres, midbrain and spinal cord, but it was weaker in the cerebellum with ABC (30/31, 96.7%) and IF (26/31, 83.8%) (Table 2). ABC and IF showed positive staining in visceral organs as follow: proventriculus (25/31, 80.6% ; 22/31, 70.9%), intestines (28/31, 90% ; 26/31, 83.8%), liver (20/31, 64.5% ; 16/31, 51.6%), kidneys (24/31, 77.4% ; 19/31, 61.2%), lungs (26/31, 83.8% ; 29/31, 93.5%), heart (18/31, 58.% ; 15/31, 48.3%), spleen (21/31, 67.7; 22/31, %70.9) and pancreas (22/31, 70.9% ; 12/31, 38.7%), respectively (Table 2). Additionally, the staining intensity observed with the 2 methods varied according

to the tissues (Fig 2 and Table 1). Although this parameter was identical in 171/338 (50.5%) tissue samples, intensity of ABC labelling was stronger in 204 (61.9%) samples (23 cerebral hemispheres, 22 cerebellum, 18 midbrain, 20 spinal cord, 23 medulla oblongata, 16 proventriculus, 20 intestines, 11 liver, 23 kidney, 1 lungs, 8 heart, 19 pancreas samples) and was weaker in lungs (13 samples) and spleen (15 samples) than the fluorescent labelling (Table 2).

In CNS, AE viral antigen was markedly expressed by both methods, on the contrary viral antigen in IF was only limited in a few small neurons, glial cells and endotheliums in CNS samples including strong inflammatory reaction. However, ABC method showed moderate to strong positive reaction in small and large neurons as well as glial cells, mononuclear

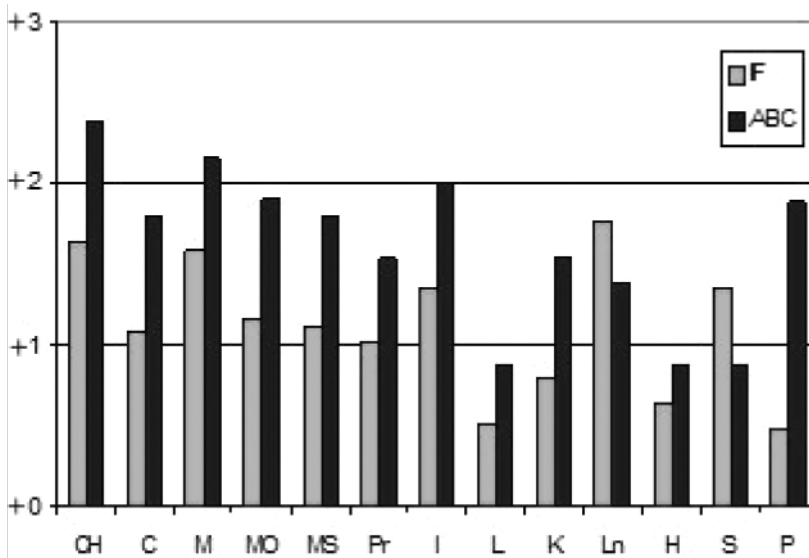


Fig 2. Mean immunohistochemical scores of AE viral antigen levels by ABC and IF methods in the tissues. CH: Cerebral hemispheres; C: Cerebellum; M: Midbrain; MO: Medulla oblongata; MS: Medulla spinalis; Pr: Proventriculus; I: Intestines; L: Liver; K: Kidney; Ln: Lungs; H: Heart; S: Spleen; P: Pancreas

Şekil 2. ABC ve IF metotlarla, dokularda AE viral antijenin ortalama immunohistokimyasal skorları. CH: Serebral hemisferler, C: Serebellum, M: Orta beyin, MO: Medulla oblongata; MS: Medulla spinalis; Pr: Bezli mide; I: Bağırsaklar, L: Karaciğer, K: Böbrek, Ln: Akciğer, H: Kalp, S: Dalak, P: Pankreas

Table 1. Scores of AE viral antigen immunolabelling by ABC and IF methods in the tissues of naturally AE infected chicks (n = 31)
Tablo 1. Doğal enfekte civcivlerde, ABC ve IF metotlarla dokularda AE viral antijen immunpozitif skorları (n=31)

	Tissues	Intensity of AE viral antigen labelling	
		ABC method	IF method
CNS	Cerebral hemispheres	0+:0 / 1+:2 / 2+:15 / 3+:14	0+:0 / 1+:12 / 2+:18 / 3+:1
	Cerebellum	0+:1 / 1+:10 / 2+:14 / 3+:6	0+:5 / 1+:19 / 2+:6 / 3+:1
	Midbrain	0+:0 / 1+:3 / 2+:20 / 3+:8	0+:0 / 1+:13 / 2+:18
	Medulla spinalis	0+:0 / 1+:10 / 2+:17 / 3+:4	0+:0 / 1+:27 / 2+:4
	Medulla oblongata	0+:0 / 1+:7 / 2+:20 / 3+:4	0+:0 / 1+:26 / 2+:5
	Visceral organs	Intestines	0+:6 / 1+:10 / 2+:11 / 3+:4
Proventriculus		0+:6 / 1+:8 / 2+:11 / 3+:6	0+:9 / 1+:12 / 2+:10
Liver		0+:11 / 1+:13 / 2+:7	0+:15 / 1+:16
Kidneys		0+:7 / 1+:6 / 2+:12 / 3+:6	0+:12 / 1+:13 / 2+:6
Lungs		0+:5 / 1+:11 / 2+:13 / 3+:2	0+:2 / 1+:8 / 2+:16 / 3+:5
Heart		0+:13 / 1+:11 / 2+:5 / 3+:2	0+:16 / 1+:11 / 2+:3 / 3+:1
Spleen		0+:10 / 1+:15 / 2+:6	0+:9 / 1+:6 / 2+:12 / 3+:4
Pancreas		0+:9 / 1+:13 / 2+:6 / 3+:3	0+:19 / 1+:9 / 2+:3

Table 2. Comparison of ABC and IF methods for the detection of AE viral antigens in tissues (CNS, proventriculus, intestines, liver, kidney, lung, heart, spleen and pancreas) of naturally infected chicks (n = 31)

Table 2. Doğal enfekte civcivlerin dokularında (CNS, bezli mide, bağırsaklar, karaciğer, böbrek, akciğer, kalp, dalak ve pankreas), AE viral antijenin belirlenmesinde ABC ve IF metotlar arasında bir karşılaştırma (n=31)

ABC and IF scores		Overall	CH	C	M	MO	MS	PV	I	L	K	LN	H	S	P
Number of	ABC+	338	31	30	31	31	31	25	28	20	24	26	18	21	22
	IF+	311	31	26	31	31	31	22	26	16	19	29	15	22	12
	ABC-	65	0	1	0	0	0	6	3	11	7	5	13	10	9
	IF-	92	0	5	0	0	0	9	5	15	12	2	16	9	19
$\Delta_{ABC/IF}$	-(1+)	28	0	0	0	0	0	0	0	0	0	13	0	15	0
	0	171	8	9	13	8	11	15	11	20	8	17	23	16	12
	+(1+)	204	23	22	18	23	20	16	20	11	23	1	8	0	19

CH: Cerebral hemispheres; **C:** Cerebellum; **M:** Midbrain; **MO:** Medulla oblongata; **MS:** Medulla spinalis; **PV:** Proventriculus; **I:** Intestines; **L:** Liver; **K:** Kidney; **LN:** Lungs; **H:** Heart; **S:** Spleen; **P:** Pancreas

$\Delta_{ABC/IF}$: Difference between labelling scores with the 2 immunohistochemical methods (the ABC and the IF methods)

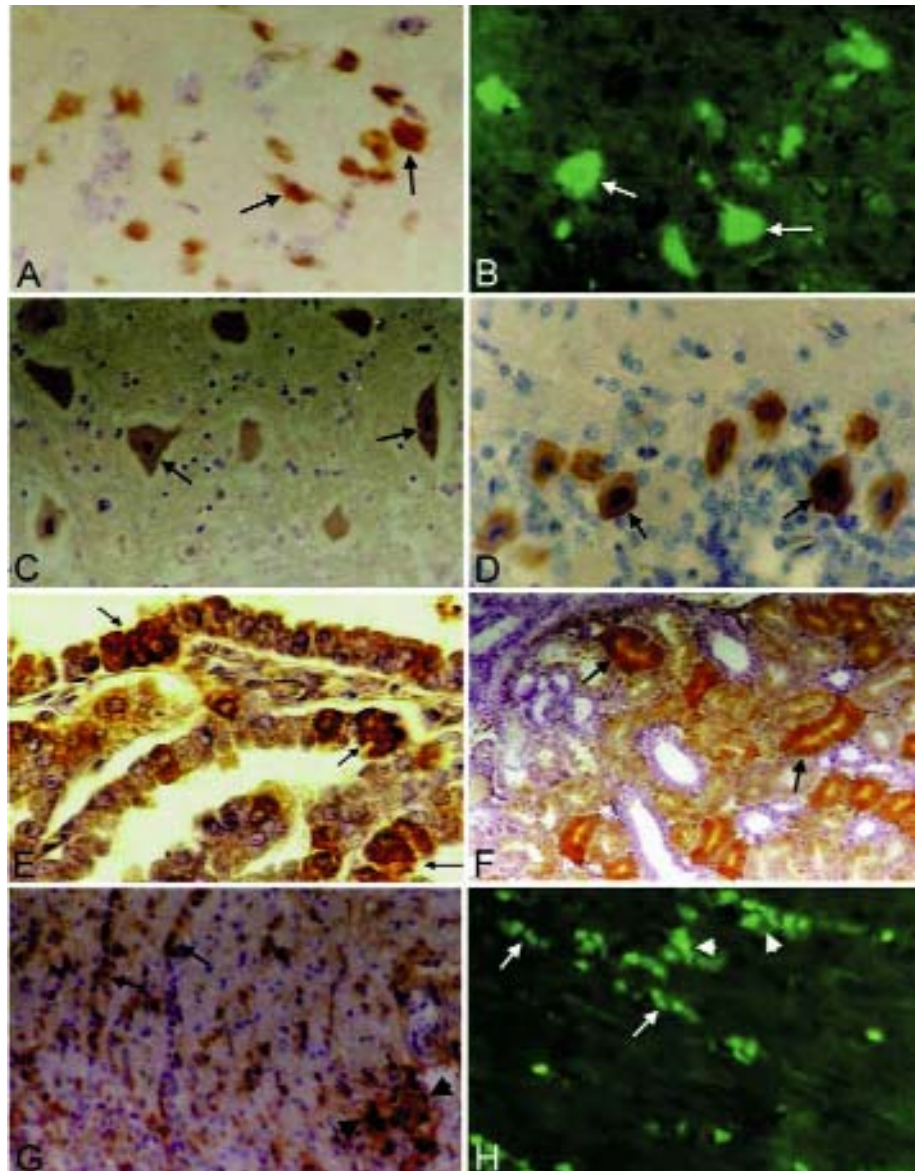
cells and endotheliums in such cases. The viral antigen in both methods was continuously strong in visceral organs of the cases from farm I and farm II. Whereas, viral immunolabelling in cases of farm III found to be in a lesser extend in the visceral organs in comparison with brain tissues.

In both methods, immunolabelling of AE viral antigen occurred particularly in neurons of the cerebral hemispheres, midbrain, medulla oblongata and medulla spinalis (Figs 3A and 3B). Viral antigen in IF was explicitly weak in neurons in comparison with to ABC. Additionally, the viral antigen in both methods was localized in endothelium of the vessels, and infiltrating mononuclear cells and glial cells especially in perivascular areas. In 5 cases, both peroxidase and fluorescent labellings formed a linear positive staining from the meninx to neurophil in brain sections. Positive reactions in both methods were generally weak in the medulla oblongata and medulla spinalis possessing strong inflammatory reaction, and were limited in some small neurons and glial cells. Beside this, as a difference, ABC also showed prominent positive reactions within motor neurons of medulla oblongata and medulla spinalis in six cases (Fig 3C). Intensity scores of immunolabelling were strong in the cerebellum in farm III in comparison to those observed from the cases of farm I and II. Both labellings in the cerebellum were found especially in neurons of the molecular and granular layer as well as neurons of the substantia alba. Additionally, ABC also showed immunolabelling of Purkinje cells in five cases (Fig 3D).

In the proventriculus, gizzard, small and large intestines, peroxidase labelling was detected

especially in the epitheliums of tubular glands and crypts and infiltrating mononuclear cells in the propria mucosa (Fig 3E), but was restricted in only infiltrating mononuclear cells in these tissues by IF method. In the pancreas, remarkable immunolabelling was localized in the cytoplasm of the epitheliums of the exocrin glands and infiltrating cells. Slight positive reactions in both methods were recognized in the endotheliums and Kupffer's cells of the liver, and in mononuclear cells of aggregates as well as the infiltrating macrophages. Additionally, viral labelling of Kupffer's cells was remarkable in peroxidase method. Immunoreaction in ABC method was found particularly in the epitheliums of the proximal convoluted tubules and the infiltrating mononuclear cells of the kidneys (Fig 3F). However, positive staining was restricted in the infiltrating mononuclear cells in IF method. In the lungs, immunolabelling in IF method was detected in the infiltrating cells, pneumocytes and alveolar macrophages, and was stronger compared to reactions observed in ABC. In the myocardial tissues, immunostaining in both methods was localized in myofibrils, fibroblast and infiltrating mononuclear cells (Figs 3G and 3H). In the spleen and fabricius of Bursa, IF method was stronger for demonstration of AE viral antigen than ABC method (Fig 2) and viral antigen was found in reticular cells and lymphocytes especially in surrounding areas of the lymphoid follicles. With exception from IF, ABC also showed immunolabelling in epitheliums of the bursal crypts. In the eyes, viral antigen in both methods was observed in the infiltrating mononuclear cells in the iris and ciliary body of eight cases. Control slides for all tissues were negative.

Fig 3. Peroxidase (A, C, D, E, F, G) and fluorescent (B, H) immunopositive reactions in CNS and visceral organs. x180. AE viral antigens in neurons (arrows) of the cerebral hemisphere (A and B), and in motor neurons (arrows) of the medulla spinalis (C), and in Purkinje cells (arrows) in the cerebellum (D). Immunopositive reactions in tubular glands (arrows) of the pro-ventriculus (E), and in epitheliums of the proximal convoluted tubules (arrows) in kidney (F), and in myocytes (arrows) and infiltrating mononuclear cells (arrowhead) in the cardiac muscle (G and H)



Şekil 3. CNS ve viseral organlarda peroksidaz (A, C, D, E, F, G) ve floresan (B, H) immunpozitif reaksiyonlar. x180. Serebral hemisfer nöronlarında (oklar) (A ve B), medulla spinalis motor nöronlarında (oklar) (C) ve serebellum Purkinje hücrelerinde (oklar) (D) immunpozitif reaksiyonlar. Bezli midenin bez epitellerinde (oklar) (E), böbrek proksimal tubul epitellerinde (oklar) (F) ve kalp kasında infiltre mononuclear hücrelerde (okbaşları) ve myositlerde (oklar) immunpozitif reaksiyonlar (G ve H)

DISCUSSION

AE is essentially an enteric infection with virus shedding in the faeces. In enteric infection, horizontal spread of infection is easily effected by tracking or by fomites, because the virus is relatively resistant to usual environmental conditions and can remain in the litter for long periods ^{1,10}. In addition to horizontal spread, vertical transmission is a very important means of AE virus dissemination, based on both field evidence and experimental results. Breeder hens first exposed to virus after sexual maturity infect a variable proportion of their eggs during the second week after infection. Most of these hatch is infected subclinically and excrete virus, thus such animals infects their hatcmates or brooder mates ¹⁰⁻¹². In congenitally infected chicks, clinical disease ensues at an early age

(<7 days of age) ¹. In the present study, it is possible that vertical transmission could be interpreted so that the sick animals in farm III were newly hatched.

In spontaneous cases, clinical and histo-pathological findings of AE may easily be confused particularly with Newcastle disease (ND) and Marek's disease (MD) ²³. AE and MD are predominantly the diseases of 1- to 3-week, and 4- to 6-week of age, respectively. ND is a disease of any period of age ¹. These diseases have also closely associated with microscopical brain lesions characterized with nonpurulent encephalomyelitis. In AE, vascular changes consisting of intense mononuclear cell infiltrations in perivascular cuff type and gliosis are generally more severe according to ND and MD ^{1,7,9,17}. Moreover, central chromatolysis of the neurons also strongly supports AE, especially when

coupled with lymphoid aggregates especially in the proventriculus, gizzard and pancreas ^{1,17,24}. In the present study, brain lesions were unequivocal among the cases. In the chicks of farms I and II, infiltration of strong perivascular mononuclear cells and gliosis in the brain was generally marked lesions, but not central chromatolysis in each case. However, the brain lesions of the chicks in the farm III mainly consisted of alterative changes with malacic changes. Thus, findings in CNS of the chicks had not perfectly corroborated histopathologic findings of AE. Therefore, immunolabelling of AE viral antigens was essential to clarify certain diagnosis ^{6,20,22}.

Previous studies show that the intensity of AE viral antigen by IF method especially in brain tissue is changeable in various condition such as long incubation period, strong inflammatory reaction, age of the chicks (newly hatched, unhatched chicks and chicks) ^{19,21,22,25}. In the present study, immunohistochemical methods strongly revealed that viral antigen was localized especially in the small and large intestines, proventriculus, lungs, kidneys, cardiac muscles, pancreas and partly in gizzard, liver, fabricius of Bursa, spleen and eyes. However, the intensity and localization of the viral antigen were unequivocal particularly in the brain tissues in comparison with visceral organs for each case, except for farm III.

The previous reports show that AE viral antigen gradually increases with the progression of neuronal degeneration and necrosis, but with intense inflammation, such as perivascular infiltration, gliosis and mononuclear infiltration, viral antigen decreases until it is not detectable by immunohistochemistry ¹⁴. Likewise, it is reported that AE viral antigen could not be detected after 32 days of postinoculation in CNS of chickens infected orally at one day old ²¹. In an experimental infection with field strain of AE, the prolonged incubation period may cause a decrease in infectivity titer of the virus in the brain tissue ¹⁴. On the other hand, detection rates and/or intensity of viral antigen in CNS vary in newly hatched and chicken embryos infected by transovarial transmission according to chicks infected by oral transmission ^{19,22,25-27}. In trans-ovarial transmission, virus first localizes in dorsal root ganglia and then spread to medulla spinalis and brain tissues ^{22,25}. Similarly, immunolabelling in the brain of the hatched chicks in farm III (by transovarial transmission) was much stronger than those of farm I and II (by oral transmission). On the contrary, viral immunolabelling

was weak in visceral organs in the chicks of farm III. In the present study, several possible explanations for severity of viral antigen could be host-related environmental influences, viral mutations, circulating antibodies or restricted protein synthesis and/or rapid degradation under in field conditions. Alternatively, surface proteins may be expressed at a level below the sensitivity of the method used. Furthermore, the humoral immune response could result in reduced expression, antigenic modulation or masking and subsequent shedding of the peplomers.

In the present study, ABC method would be more sensitive than the IF test, since the enzyme fixed on antibodies can have a continuous action on the substrate leading to the massive production of the reaction product and to the consequent amplification of the signal linked to the formation of immune complexes with AE antigens. By contrast, in IF test, the observed fluorescence is directly proportional to the amount of AE antigens available in the specimen for binding labelled antibodies ^{6,20,22}. Thus, both immunohistochemical methods were equally sensitive and specific for detection of AE viral antigens in cerebral hemispheres, midbrain and medulla spinalis, but there was a marked difference to advantage of ABC method from the point of view of staining intensity score. On the other hand, sensitivity and staining intensity scores of the ABC method were also over than IF method in CNS and visceral organs. However, the sensitivity and staining intensity score only in the lungs, fabricius of Bursa and spleen samples was being to the fluorescent method's advantage, ABC method appeared to be more advantageous for AE diagnosis since this method required the utilisation of more simplified microscopes and allowed the storage of stained slides for a long time.

In conclusion, the present study indicates that the clinical and pathological findings may not sufficient for certain diagnosis of AE in field conditions. The present study suggest that detection of the viral antigen is certainly required for differential diagnosis. Compared to both methods, ABC method would be useful as a strong method in detection of AE viral antigen in natural infections. Furthermore, it is suggested that molecular biological methods such as in-situ hybridization and in-situ PCR should be applied for certifying the AE diagnosis in future experiments due to difficulties in detection of AE viral antigen especially in CNS sections with strong inflammatory reactions.

REFERENCES

1. Calnek BW, Luginbuhl RE, Helmboldt CF: Avian encephalomyelitis. In: Calnek BW, Barnes HJ, McDougald LR, Saif YM (Eds): Disease of Poultry. 10th ed. pp. 571-581, Iowa State University Press, Ames, 1997.
2. Deshmukh DR, Hohlstein WM, McDowell JR, Pomeroy BS: Prevalence of avian encephalomyelitis in turkey breeder flock. *Am J Vet Res*, 32, 1263-1267, 1971.
3. Hill RW, Raymond G: Apparent of natural infection of coturnix quail hens with the virus of avian encephalomyelitis. *Avian Dis*, 6, 226-227, 1962.
4. Hohlstein WM, Deshmukh DR, Larsen CT, Sautter JH, Pomoroy RS, McDowell JR: An epizootic of avian encephalomyelitis in turkey in Minnesota. *Am J Vet Res*, 31, 2233-2242, 1970.
5. Mathey WJ: Avian encephalomyelitis in pheasants. *Cornell Vet*, 45, 89-93, 1955.
6. Toplu N, Alcigir G: Avian encephalomyelitis in naturally infected pigeons in Turkey. *Avian Pathol*, 33, 381-386, 2004.
7. Marvil P, Knowles NJ, Mockett APA, Britton P, Brown TDK, Cavanagh D: Avian encephalomyelitis virus is a picornavirus and is most closely related to hepatitis virus. *J Gen Virol*, 80, 653-662, 1999.
8. Itakura C, Goto M: Avian encephalomyelitis in embryos and abnormal chick on day of hatching-neurohistopathological observations. *Japan J Vet Sci*, 37, 21-28, 1975.
9. Markson LM, Blaxland JD: Infectious avian encephalomyelitis. *Vet Rec*, 20, 1208-1213, 1958.
10. Calnek BW, Taylor PJ, Sevoian M: Studies on avian encephalomyelitis. IV. Epizootiology. *Avian Dis*, 4, 325-347, 1960.
11. Feibel MS, Helmboldt CF, Jungher EL, Carson JR: Avian encephalomyelitis. Prevalence, pathogenicity of the virus and breed susceptibility. *Am J Vet Res*, 13, 260-266, 1952.
12. Taylor LW, Dorothy CL, Raggi LG: Effects of an outbreak of avian encephalomyelitis (Epidemic Tremor) in a breeding flock. *Poult Sci*, 34, 1036-1045, 1955.
13. Hishida N, Odagiri Y, Kotani T, Horiuchi T: Morphological changes of neurons in experimental avian encephalomyelitis. *Japan J Vet Sci*, 48, 169-172, 1986.
14. Mohanty GC, West JL: Pathogenesis and pathologic features of avian encephalomyelitis in chicks. *Am J Vet Res*, 29, 2387-2400, 1968.
15. Yamagiwa S, Itakura C, Shimizu Y: Poliomyelitis of newborn chicks (epidemic tremor of chickens, avian encephalomyelitis). *Japan J Vet Sci*, 31, 105-118, 1969.
16. Malik G: Histopathological lesions of avian encephalomyelitis in Hungary. *Acta Vet Acad Sci Hung*, 19, 279-298, 1969.
17. Springer WT, Schmitt SC: Avian encephalomyelitis: A chronological study of the histopathogenesis in selected tissues. *Avian Dis*, 12, 229-239, 1968.
18. Toplu N: The use of light microscopy and fluorescent antibody technique in the diagnosis of experimental avian encephalomyelitis virus infection in chicks and quails. *PhD Thesis*, Institute of Health Science, University of Ankara, 2000.
19. Braune MO, Gentry RF: Avian encephalomyelitis virus. II. Pathogenesis in chickens. *Avian Dis*, 15, 648-653, 1971b.
20. Heide L Van Der: De diagnostiek van aviare encephalomyelitis met behulp van de directe immunofluorescente Techniek. *Tijdschr Geneeskde*, 95, 639-641, 1970.
21. Miyamae T: Invasion of encephalomyelitis virus from the gastrointestinal tract to the central nervous system in young chickens. *Am J Vet Res*, 44, 508-510, 1983.
22. Miyamae T: Ecological survey by the immunofluorescent method of virus in enzootics of avian encephalomyelitis. *Avian Dis*, 18, 369-377, 1974.
23. Helmboldt CF: Histopathologic differentiation of diseases of the nervous system of the domestic fowl (*Gallus gallus*). *Avian Dis*, 16, 229-240, 1972.
24. Hazirolu R, Alcigir G, Karademir N: Piliçlerde avian encephalomyelitis. *Ankara Üniv Vet Fak Derg*, 37, 207-213, 1990.
25. Braune MO, Gentry RF: Avian encephalomyelitis virus. I. Pathogenesis in chicken embryos. *Avian Dis*, 15, 638-647, 1971.
26. Sağlam YS, Erer H: Cıvcıvlerde deneysel avian encephalomyelitis hastalığında klinik ve histopatolojik bulguların incelenmesi. *Etlik Vet Mikrobiol Derg*, 9, 31-46, 1997.
27. Yılmaz F, Özer H: The morphological investigations of experimental avian encephalomyelitis in Turkey embryo. *Turk J Vet Anim Sci*, 23, 205-213, 1999.