Kafkas Univ Vet Fak Derg
15 (5): 725-732, 2009
DOI:10.9775/kvfd.2009.095-A

RESEARCH ARTICLE

Pathologic Findings and Immunohistochemical Distribution of Viral Antigen in Experimental Avian Encephalomyelitis of the Chick and Quail [1]

Nihat TOPLU * Günay ALÇIĞIR **

[1] *This study was summarized from PhD thesis entitled "The Use of Light Microscopy and Fluorescent Antibody technique in the Diagnosis of Experimental Avian Encephalomyelitis in Chicks and Quails"*

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

Makale Kodu (Article Code): 2009/095-A

Summary

In this study, the differences and diagnostic importances of the pathological and immunohistochemical findings in avian encephalomyelitis were investigated comparatively in chicks and quails by inoculating van Roekel strain (reference strain) via intracerebral and intramuscular routes. Histopathologically, nonpurulent encephalomyelitis characterized with infiltration of perivascular mononuclear cells and gliosis was described in both species. Neuronal lesion in the chicks consisted of central chromatolysis and neuronal shrinkage (neuronal pyknosis), whereas in quails it was composed of only neuronal shrinkage. Lymphoid aggregates and/or infiltration of mononuclear cells were observed in the proventriculus, gizzard, intestines, liver, pancreas, kidneys, myocardium, lungs, spleen and eyes. However, these lesions in quails were restricted only in the proventriculus, intestines, myocardium and eyes. In the immunofluorescein examination, viral antigen was found especially in neurons of the midbrain, cerebral hemispheres, cerebellum, and occasionally in medulla oblongata and medulla spinalis in both species. Viral antigen in the brain and spinal cord samples of the chicks decreased gradually by increasing inflammatory reaction. Viral antigen in both species was continously detected in the proventriculus, gizzard, intestines, myocardium, pancreas, spleen, liver, kidneys, lungs and eyes.

Keywords: *Avian encephalomyelitis, Immunohistochemistry, Chick, Quail*

Civciv ve Bıldırcınların Deneysel Avian Ensefalomyelitis'inde Patolojik Bulgular ve Viral Antijenin İmmunohistokimyasal Dağılımı

Özet

Bu çalışmada, avian ensefalomyelitis virusunun van Roekel suşunun (referans suş) intraserebral ve intramuskuler yollarla inokule edildiği civciv ve bıldırcınlarda; patolojik ve immunohistokimyasal bulgulardaki farklılıklar ve tanıdaki önemleri karşılaştırmalı olarak incelendi. Histopatolojik incelemede, her iki türde perivasküler mononüklear hücre infiltrasyonları ve gliozisi içeren nonpurulent ensefalomyelitis tanımlandı. Civcivlerde nöronal lezyonlar, sentral kromatolizis ve nöronal büzüşme (nöronal piknoz) ile karakterize olurken, bıldırcınlarda ise yalnızca nöronal büzüşme görüldü. Viseral organlardan bezli mide, kaslı mide, bağırsaklar, dalak, karaciğer, pankreas, böbrekler, kalp, akciğerler ve gözlerde; lenfoid kümeler ve/ya da mononüklear hücre infiltrasyonları şeklinde yangısal yanıt gözlendi. Ancak, bıldırcınlarda benzeri yangısal reaksiyonlar bezli mide, bağırsaklar, kalp ve gözlerde sınırlı kalmıştı. Immunofloresan incelemede, viral antijen özellikle orta beyin, serebral hemisferler ve serebellum nöronlarında dikkati çekerken, medulla spinalis ve medulla oblongatada ise daha zayıftı. Immunreaksiyonun civcivlerin beyin ve medulla spinalislerinde, yangısal reaksiyonların şiddetiyle doğru orantılı olarak azaldığı dikkati çekti. Her iki türde viral antijen bezli mide, kaslı mide, bağırsaklar, kalp kası, pankreas, dalak, böbrekler, akciğerler ve gözde belirlendi.

Anahtar sözcükler: *Avian ensefalomyelitis, İmmunohistokimya, Civciv, Bıldırcın*

İletişim (Correspondence)
☎ +90 256 2470700/183
✉ nihat.toplu@gmail.com / nihattoplu@hotmail.com

INTRODUCTION

Avian encephalomyelitis (AE), also known as epidemic tremor, is an infectious viral disease of birds naturally affecting young birds including chicks, pheasents, turkey, quail and pigeon [1-5]. The etiologic agent, AE virus (AEV), is a Hepatovirus in the family Picornaviridae [6]. AE described comprehensively in chicks causes neurological signs such as ataxia, paresis or paralysis and rapid tremors of the head and neck [7-10], whereas in older birds infection is subclinical, resulting in declines in egg production and hatchability [11-12]. AE in turkey, pheasents and pigeons have been investigated in field cases [1,3,5], but not enough in quails [2].

Histopathological findings in the central nervous system (CNS) have a great value in the diagnosis of AE. Histologically, AE lesions are of two general types. Changes in the CNS have been characterized as non-purulent encephalomyelitis accompanied by neuronal lesions [10,13-15]. Lesions in visceral organs consist of lymphoid aggregates which are either increased in size or frequency or are found in unusual places [7,16-18]. Lymphoid aggregates in proventriculus are considered to be pathognomic, especially when coupled with neuronal change (central chromatolysis) from lesions of the CNS [19].

The aim of this study is to describe the pathologic and immunohistochemical findings comparatively in experimentally infected chicks and quails.

MATERIAL and METHODS

Animals

Three days old broiler chicks and quails (Coturnix Coturnix Japonica) having no neutralizan antibodies against AEV were chosen for the study. A total of 100 animals were used: 80 of them were allocated to the experimental groups and the others comprised the control group. Chicken embryo brain homogenate including van Roekel strain of AEV (2x10-7 EID50/ml) was used for infecting the animals. Chicks and quails were divided four experimental groups and the others were used for control *(Table 1)*. Animals in each group were separeted different room throughout experiment, and were fed ad libitum. All animals received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published the National Institute of Health.

Table 1. *Experimental Design.*
Tablo 1. *Deney düzeni*

Group	Old	Animals	Application	Application rough
A (n=20)	Three days	Chicken	0.05 ml virus	Intracerebral
B (n=5), (Control)	Three days	Chicken	0.05 ml PBS	Intracerebral
C (n=20)	Three days	Chicken	0.15 ml virus	Intramuscular
D (n=5), (Control)	Three days	Chicken	0.15 ml PBS	Intramuscular
E (n=20)	Three days	Quail	0.03 ml virus	Intracerebral
F (n=5), (Control)	Three days	Quail	0.03 ml PBS	Intracerebral
G (n=20)	Three days	Quail	0.10 ml virus	Intramuscular
H (n=5), (Control)	Three days	Quail	0.10 ml PBS	Intramuscular

The terminally ill and death animals from infected groups following incubation period of the disease were euthanized and necropsied without planed time. In infected groups, animals showing no symptoms were euthanized and necropsied at 60th days of inoculation. The animals in control groups were euthanized on 5th, 10th, 15th, 20th ve 30th days of inoculation. Tissue samples were collected from the brain and medulla spinalis, pancreas, proventriculus, gizzard, intestine, liver, spleen, kidneys, lungs, heart and eyes. The tissues were then fixed in 10% neutral-buffer formalin solution, embedded in parafin, sectioned at 4 μm and stained haematoyxylin and eosin (HE). The selected brain and medulla spinalis sections were stained by cresyl violet for Nissl granules of neurons [20]. The replicate sections were used for immunohisto-chemical staining.

The scores of histopathological findings in CNS were evaluated under light microscope with a 10X ocular with grids and a 20X objective according the following categories: 0: No lesion; 1: Only neuronal changes (mild lesion); (2): Neuronal changes, slight perivascular infiltrtions and focal gliosis (moderate lesion); (3): Neuronal changes, severe perivascular infiltrations and diffuse gliosis (severe lesion).

Preparation of hyperimmun serum and conjugate

The preparation of chicken hyperimmune serum against AE and fluorescein isothiocyanate (FITC)-conjugate was carried out as described by Gialetti et al.[21] and Johnson et al.[22]. Specific pathogen free chickens (Veterinary Control and Research Enstitute, Manisa), 6 weeks of age, were inoculated IM with a mixture of 1 ml van Roekel virus strain and 1 ml of Freund's complete adjuvant three times at 7-days intervals. The chicks were bled by cardiac puncture 15 days after the last injection and serum was pooled.

The gamma-globulins from the pooled antiserum

were fractionated and conjugated with FITC. Gamma-globulins were initially separated using ammonium sulphate precipitation. IgG was fractioned using saphadex G-25 (Pharmacia Fine Chemicals) and conjugated with FITC (Fluka AG). This complex was purified.

Immunohistochemistry

For the direct immunofluorescence technique (IF), the selected sections were placed on glass microscope slides coated with poly-L-lysine. After 2 h incubation at 37ºC, sections were deparaffinized in xylene and washed briefly in Phosphate-Buffered-Saline (PBS) solution (pH 7.3). Tissues were digested with 0.1% protease K for 10 min at 37ºC. Slides were washed for 15 min in PBS for fluorescein antibody staining. Sections were incubated with chicken anti-AEV serum conjugated with FITC in a 1:16 dilution for 45 min at 37ºC, 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 negative control, chicken anti-Newcastle virus FITC-conjugate was used instead of AEV antibody-FITC conjugate. The results of IF were determined using a fluorescent microscope (Leitz- Laborlux D, binocular microscope equipped with a dry darkfield condensor).

The percentage of the total area of the fluorescent labelling positive cells were assessed semi-quantitatively under fluorescent microscope 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: slight staining (1-2% positive cells); 2: moderate staining (3-5% positive cells); 3: marked staining (>6% positive cells).

RESULTS

Clinical findings

In group A, the first clinical findings were observed at the 4th day of inoculation, and all chicks had clinical signs by the 22th day of the inoculation. In group C, 11 chicks showed clinical findings between on 7th and 23th days of inoculation. In group E, the first symptoms were observed at 3th day of inoculation, and 11 quails had clinical findings by 15th days of inoculation. Nine quails in group G showed clinical signs between 4th and 14th days of the inoculation.

At the beginning, affected chicks showed stagnation, unwillingness against feed and water. When developed ataxia related to incoordination of the muscles, animals showed an inclination to sit their hocks. With progression of the disease, they came to rest or fall on their side related to paralysis of legs and wings. Clinical appearence of the sick animals in the infected groups of both quail groups was similar to chicks. However, the disease in quails was more rapidly progressing in comparing with chicks. After stagnation and ataxia, four and three quails from group E and G, respectively died without paresis or paralysis developed.

Tremors of the head and neck or body were seen in three chicks from group A and two quails from E. Torticollis was described only in one chick from group A. Animals with no symptoms in infected groups of both species had growth retardation when compared to control animals.

Necropsy finding

Gross lesion was not observed in the chicks. Pericardium of two quails from group E showed grayish-white in colour. In five chicks from group A and four chicks from group C, generalized atrophia of the skeletal muscles were observed due to inactivity caused by long time paralysis.

Histopathological findings

The intensity of histopathological findings was summarized in *Fig 1*. Histopathological examination exhibited partly different findings between animal species. The most conspicuous lesion in CNS was nonpurulent encephalomyelitis including perivascular infiltrations, gliosis, neuronal degeneration and necrosis. In infected chick groups, the intensity of vascular lesions, gliosis and neuronal lesions was generally stronger in chick infected groups than those observed in quail infected groups. Moreover, the chicks and quails inoculated by intracerebral via had more severe CNS lesions when compared to those of the animals infected intramuscularly. In CNS of the chicks and quails with no symptoms in infected groups, there were also slight perivascular mononuclear infiltrations and occasional glial foci.

Neuronal lesions composed of mainly central chromatolysis and occasionally of neuronal shrinkage and neuronal lysis in the chick inoculation groups. Central chromatolysis and neuranal lysis were recognized particularly in motor neurons of the medulla oblongata and medulla spinalis, and in neurons of nuclear groups of the midbrain and, occasionally in Purkinje

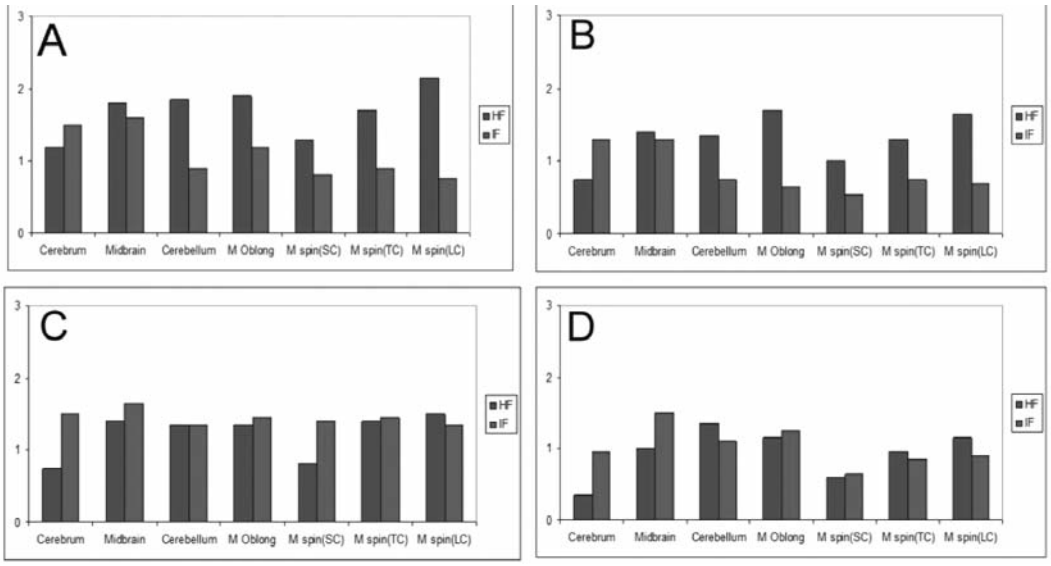

Fig 1. Mutual relation between histo-pathological and immunohistochemical scores in CNS tissues in infected groups. A: Intracerebrally inoculated chicks (group A). B: Intramuscularly inoculated chicks (group C). C: Intracerebrally inoculated quails (group E). D: Intramuscularly inoculated quails (group G). M oblong: Medulla oblongata, M spin: Medulla spinalis, SC: Spinal cord, TC: Thoracal cord, LC: Lumbosacral cord, HF:Histopathological finding, IF:Immunofluorescence.

Şekil 1. Enfekte grublarda, merkezi sinir sistemi dokularında histopatolojik ve immunohistokimyasal bulgular arasındaki ilişki. A: İnraserebral yolla enfekte civcivler (grup A). B: İntramuskuler yolla enfekte civcivler (grup C). C: İntraserebral yolla enfekte bıldırcınlar (grup E). D: İntramuskuler yolla enfekte bıldırcınlar (grup G). M oblong: Medulla oblongata, M spin: Medulla spinalis, SC: Spinal kort, TC: Thoracal kort, LC: Lumbosacral kort, HF:Histopathological finding, IF:Immunofluorescence.

cells of the cerebellum. Additionally, neuronal shrinkage was also seen occasionally in these sites of the CNS. In neurons with central chromatolysis, Nissl's substances disappeared in centrum of cell and cytoplasm stained homogeneously pink, and enlarged nucleus was eccentric *(Fig 2A)*. In advanced stages, such neurons were necrotized, whose nucleus were completely disappeared, and Nissl's substances only remained in periphery of the cell. Such archetypical appearenced neurons were stained poorly with HE and cresyl violet, and resembled ghost cell *(Fig 2B)*.

Neuronal lesions in the infected quail groups were characterized with only neuranal shrinkage (neuronal pyknosis) especially in Purkinje cells of the cerebellum, and motor neurons of the medulla spinalis and medulla oblongata, and neurons of nuclear groups in midbrain. Such neurons with deep red in colour showed a marked decrease in size and irregular shaped and their nucleuses were generally pyknotic. Necrotic neurons were surrounded by microglia and then replaced by focal gliosis *(Fig 2C)*.

Vasculitis was one of the most conspicious findings in CNS of the animals, with proliferation of endothelial and adventitial cells and perivascular mononuclear infiltrations (mainly composed of lymphocytes and lymphoblasts, and occasionally of plasmocytes and macrophages). Chicks in infected groups generally showed intense vasculitis (3 to 12 cells layer), whereas in the quails it was relatively thinner (2 to 5 cells layer). Lumens of some vessels especially in the chicks were narrowed associated with proliferation of endothelial and adventitial cells.

Gliosis in CNS was one of the most conspicuous findings particularly in chicks, and occasionally in the quails. Gliosis gradually increased with progression of the vessel and neuronal lesion especially in the midbrain, medulla oblongata and medulla spinalis in the chicks *(Fig 2D)*. Focal or diffuse gliosis was pronounced lesion in molecular layer of the cerebellum in the chicks, but was not found or was characterized with scattered proliferation of glial cells in the quails. Another different lesion was found in four and one quail from group A and C, respectively was polio-encephalomalacia in the midbrain, cerebral hemi-spheres and medulla oblongata.

Changes in the visceral organs of the infected quails and chicks were of lymphoid aggregates and/or infiltrations of mononuclear cells. Similar lesions were also observed in visceral organs of animals with no symptoms in infected groups. Infiltration of mono-nuclear cells in the animals appeared in the lamina propria and muscular layer of the proventriculus and

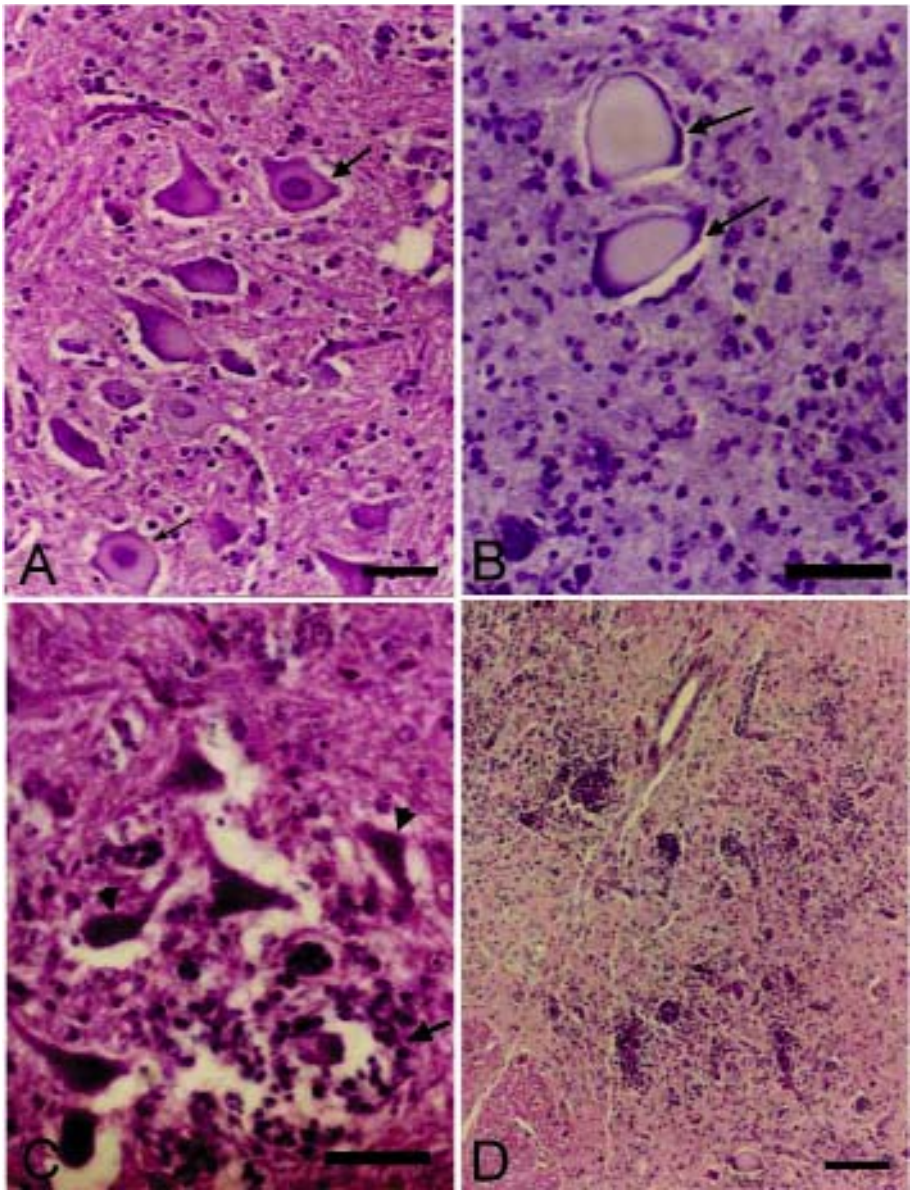

Fig 2. (A) Degenerated motor neurons with central chromatolysis (arrows). Lumbosacral cord of medulla spinalis, chick. HE. Bar: 50 µm. (B) Necrotic motor neurons (arrows) with poorly stained and disappeared nucleus. Medulla oblongata, chick. Cresyl violet. Bar: 50 µm. (C) Neuronal pyknosis with deep red in colour and a marked decrease in size (arrow heads), and neuronaphagie (arrow). Lumbosacral cord of medulla spinalis, quail. HE. Bar: 50 µm. (D) Infiltration of perivascular mononuclear cells, gliosis and disappeared motor neurons. Thoracal cord of medulla spinalis, chick. HE. Bar: 50 µm.

Şekil 2. (A) Sentral kromatolizisli dejenere motor nöronlar (oklar). Medulla spinalis, lumbosakral bölüm, civciv. HE. Bar: 50 µm. (B) Silik boyalı ve çekirdeklerini kaybetmiş nekrotik motor nöronlar (oklar). Medulla oblongata, civciv. Cresyl violet. Bar: 50 µm. (C) Nöronlarda, koyu kırmızı renkte ve büzüşmeyle karakterize nöronal piknozis(ok başları) ve nöronofaji (ok). Medulla spinalis, lumbosakral bölüm, bıldırcın. HE. Bar: 50 µm. (D) Perivasküler mononüklear hücre infiltrasyonları, gliozis ve gözden silinmiş motor nöronlar. Medulla spinalis torakal bölüm, civciv. HE. Bar: 50 µm

gizzard, and in portal and perivascular areas in the liver, and in ventriculus of myocardium, and in lamina propria and muscular layer of the small intestines, and in corpus ciliare and iris of eyes. In addition, two chicks from group A showed the infiltrations of perivascular mononuclear cells with haemorrhages in iris of the eyes. No lesion was detected in control animals.

Immunohistochemical findings

Immunolabelling of AE viral antigen in CNS of both species was recognized particularly in the cerebral hemispheres, midbrain and cerebellum. Medulla oblongata and medulla spinalis had less immuno-reactivity compared with other portions of CNS. Viral labelling was abundant in both large neuron and small neurons of CNS in the chicks with weak inflammatory reactions. With increasing inflammatory reaction, viral antigen was then only limited in glial cells and few small neurons.

Immunostaining of AE viral antigen was in intense granular and/or diffuse patterns particularly in the cytoplasm of neurons especially in cerebral hemispheres and nuclear groups of midbrain in both species (*Fig 3A* and *3B*). In the cerebellum, immunoreactivity was localized mainly in neurons of the granular and molecular layers. No immunostaining was observed in Purkinje cells of the cerebellum. In the medulla oblongata and medulla spinalis, viral antigen was detected in small neurons and motor neurons *(Fig 3C)*. Moderate to strong immunoreactivity was generally in endotheliums and glial cells in all portions of CNS. Additionally, the

viral labelling was also detected in neurons of dorsal root ganglia. In CNS of both species with no symptoms, there were slight to moderate immunopositive reactions. Control animals did not show immunoreactivity in CNS.

Immunolabelling of AE viral antigen in visceral organs of both species was present. The scores of immunolabellings were generally superior in chicks compared with quails. AE viral antigen was detected in cytoplasm of infiltrating mononuclear cells and intraepithelial lymphocytes in the proventriculus, gizzard, small intestines and caecum in both species. In the pancreas, immunoreactivity was stronger in the chicks than the quails, and localized particularly in islets of Langerhans and occasionally in the exocrin glands and infiltrating macrophages. In the liver, immunostaining appeared in infiltrating macrophages, endothelial cells and Kupffer's cells. Immunolabelling in the kidneys was observed in epitheliums of the proximal tubulus and infiltrating mononuclear cells. The labelling was strongly detected especially in alveolar macrophages of the lungs in all infected groups. AE viral antigen was densened in lymphocytes and reticular cells in surrounding areas of the lymphoid follicles of the spleen. In the myocardium, moderate to strong immunoreactivity was detected in infiltrating macrophages and myocytes in both species *(Fig 3D)*. In the eyes, AE viral antigen was recognized in infiltrating macrophages of the iris and corpus ciliare. In addition, immunoreactivity was also seen in ganglion cells of the retina in two chicks of group A. In bursa of Fabricius, immunopositive reactions in the chicks were detected in intraepithelial lymphocytes, and lymphocytes of lymphoid follicles, but were limited only in intraepithelial lymphocytes in the quails. In control animals, visceral organs did not show antigenic reactions.

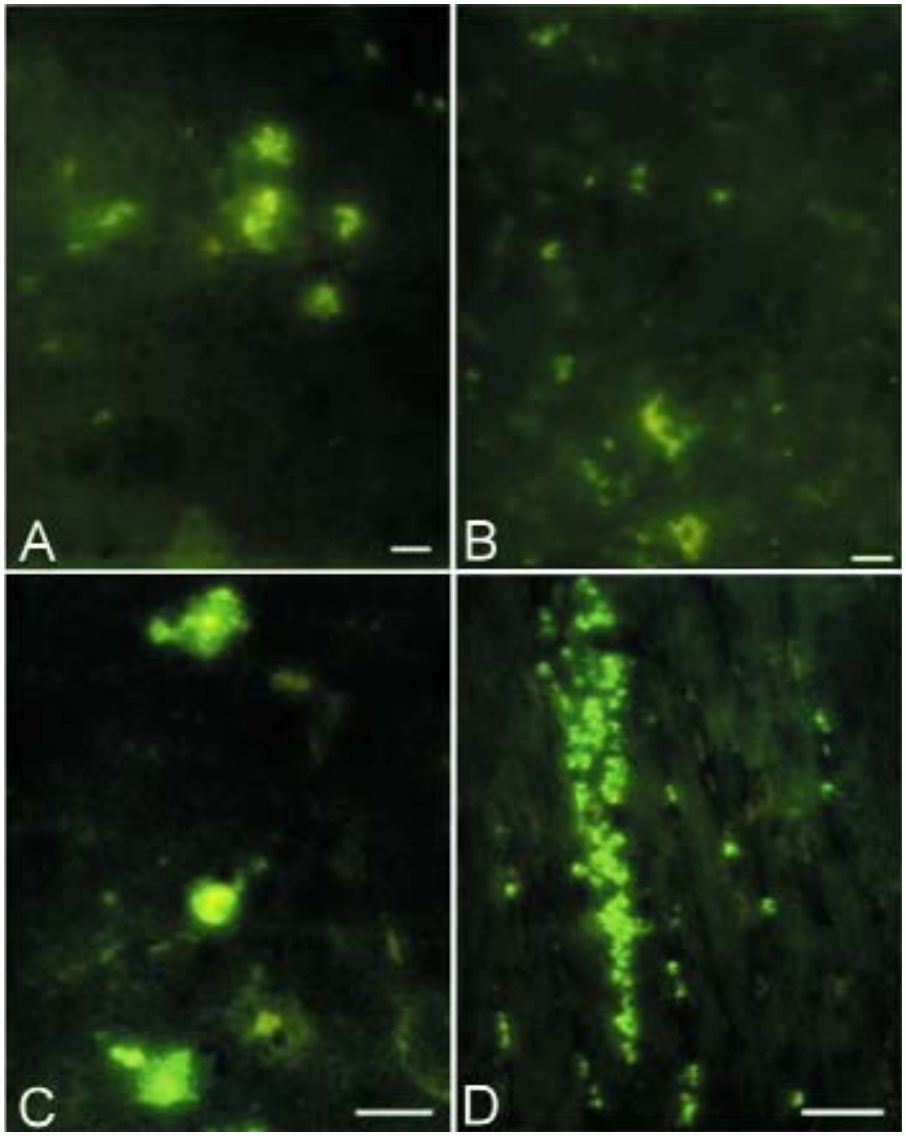

Fig 3. (A) Fluorescent labelling of AE viral antigen in cytoplasm of neurons of the midbrain. Chick. Bar: 50 µm. (B) Granular immunolabelling in neurons of the cerebral hemispher. Chick. Bar: 50 µm. (C) Strong immunopositive reaction in motor neurons of the medulla oblongata. Chick. Bar: 50 µm. (D) Immunolabelling of infiltrated macrophages in the myocardium. Chick. Bar: 50 µm

Şekil 3. (A) Orta beyinde nöronların sitoplazmalarında floresan pozitif reaksiyonlar. Civciv. Bar: 50 µm. (B) Serebral hemisferde nöronlarda granüler pozitif reaksiyonlar. Civciv. Bar: 50 µm. (C) Medulla oblongatanın motor nöronlarında immunpozitif reaksiyonlar. Civciv. Bar:50 µm. (D) Kalp kasında infiltre makrofajlarda immunpozitif reaksiyonlar. Civciv. Bar: 50 µm

DISCUSSION

Clinical and pathologic findings of AE may easily be confused particularly with Newcastle disease (ND) and Marek's disease (MD) [19,23]. AE should be differentiated from these diseases and definite diagnosis needs immunohistochemical methods or virus isolation [24]. For this purpose, IF method has been used in detection of AE viral antigen in tissue sections [21,25]. At the same time, this method has also been applied in pathogenesis studies of AE [24,26,27].

In CNS, histopathological findings characterized by nonpurulent encephalomyelitis as well as neuronal lesions are distinctive for AE. Moreover, it is pointed out that neurotropic van Roekel strain of AEV cause more severe CNS and neuronal lesions according to those of natural infections caused by enteretropic strains [15,17,28]. However, central chromatolysis of the neurons, a pathognomic lesion for AE, may absent or limited only in the midbrain of naturally infected chicks [10]. On the other hand, quails of the present study showed neuronal shrinkage rather than central chromatolysis. In experimental studies, it is speculated that central chromatolysis is associated with intensity of the vascular lesion and gliosis, and has been regressed with decreasing of vascular lesions in recovering chicks [18,29,30]. Recent a study indicate that neuronal lesions and tissue necrosis caused by a pestivirus could be triggered by inflammatory mediators elaborated by the infiltrating macrophages and glial cells in inflamed areas, including NO, type I IFN and other excitotoxic signals [31]. On the contrary, central chromatolysis in neurons was widespread in the chicks including slight vascular and glial changes, as well. Absence of central chromatolysis in CNS of quail could be insufficient for AE. Thus, it is suggested that pathogenesis of neuronal lesions in AE should be investigated for detailed approach in future experiments.

IF method showed that AE viral antigen in CNS was strongly localized in nuclear groups of the midbrain, cerebral hemispheres and, to a lesser extend, in the cerebellum, medulla oblongata and medulla spinalis, in aggrement with other studies [13,21,26,32,33]. On the other hand, AE viral antigen in CNS of the chicks gradually decreased with the progression of intense inflammatory changes, and even was not detectable in some segments of CNS. However, such mutual relation was not recorded in CNS of the quails. It is possible that pronounced AE viral antigen weakness in such cases of AE is due to circulating antibodies or restricted protein syntesis and/or rapid degradation.

Previous studies show that visceral organs have strongly AE viral antigen especially in organs of digestive system in natural outbreaks and experimental infections by field strains [21,27,32]. However, viral antigen in the present study was weak in the visceral organs, and generally limited in the infiltrating macrophages. This discrepancy according to field strains was most probably caused by poorly enterotropic van Roekel strain.

Consequently, it has been concluded that clinical and pathological findings in diagnosis of AE was useful in the chicks, but not in the quails. However, seeing that the positive reaction is weak in the cases with severe histopathological changes, it will be better to evaluate histopathological findings together with immunohistochemistry in the diagnosis of the disease. We suggest that further studies with in-situ hybridization technique and in-situ polymerase change reaction would be required in detection of AEV genome, and then their results should be compared with immunohistochemical methods.

Acknowledgements

Authors wish to thank Research Fund of Turkish Technical Research Council, project number VHAG-1373 ADP; Dr. Yavuz Selim Sağlam supplying virus homogenate.

REFERENCES

1. Mathey WJ: Avian encephalomyelitis in pheasents. *Cornell Vet,* 45, 89-93, 1955.

2. Hill RW, Raymond G: Apparent of natural infection of coturnix quail hens with the virus of avian encephalomyelitis - case report. *Avian Dis,* 6, 226-227, 1962.

3. Hohlstein WM, Deshmukh DR, Larsen CT, Sautter JH, Pomoroy RS, Mcdowell JR: An epiornithic of avian encephalomyelitis in turkey in minnosota. *Am J Vet Res,* 31, 2233-2242, 1970.

4. Deshmukh DR, Hohlstein WM, Mcdowell JR, Pomeroy BS: Prevalence of avian encephalomyelitis in turkey breeder flock. *Am J Vet Res,* 32, 1263-1267, 1971.

5. Toplu N, Alcigir G: Avian encephalomyelitis in naturally infected pigeons in Turkey. *Avian Pathol,* 33, 381-386, 2004.

6. 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.

7. Markson LM, Blaxland JD: Infectious avian encephalomyelitis. *Vet Rec,* 20, 1208-1213, 1958.

8. Maas HJ, Helmboldt CF: Aviare Encephalomyelitis. Opmerkingen over de klinische en de patho-histologishe

diagnotiek. *Tijdschr Diergeneesk,* 87, 371-397, 1962.

9. Hoekstra J: Experiments with avian encephalomyelitis. *Brit Vet J,* 120, 322-334, 1964.

10. Itakura C, Goto M: Avian encephalomyelitis in embryos and abnormal chick on day of hatching-neurohistopathological obseravtions. *Jap J Vet Res Sci,* 37, 21-28, 1975.

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. Calnek BW, Taylor PJ, Sevoian M: Studies on avian encephalomyelitis. IV. Epizootiology. *Avian Dis,* 4, 325-347, 1960.

13. Mohanty GC, West JL: Pathogenesis and pathologic features of avian encephalomyelitis in chicks. *Am J Vet Res,* 29, 2387-2400, 1968.

14. Yamagiwa S, Itakura C, Shimizu Y: Poliomyelitis of newborn chicks (epidemic tremor of chickens, avian encephalomyelitis). *Jap J Vet Sci,* 31, 105-118, 1969.

15. Hishida N, Odagiri Y, Kotani T, Horiuchi T: Morphological changes of neurons in experimental avian encephalomyelitis. *Jpn J Vet Sci,* 48, 169-172, 1986.

16. Springer WT, Schimittle SC: Avian encephalomyelitis: A chronological study of the histopathogenesis in selected tissues. *Avian Dis,* 12, 229-239, 1968.

17. Butterfield WK, Helmbold CF, Luginbuhl RE: Studies on avian encephalomyelitis IV. Early incidence and longevity of histopathology lesions in chickens. *Avian Dis,* 13, 53-57, 1969.

18. Malik G: Histopathological lesions of avian encephalomyelitis in Hungary. *Act Vet Acad Sci Hung,* 19, 279-298, 1969.

19. Calnek BW, Luginbuhl RE, Helmboldt CF: Avian encephalomyelitis. **In,** Calnek BW, Barnes HJ, McDougald LL, Saif, YM (Eds): Disease of Poultry 10th edn. Ames, Iowa State University Press, pp. 571-581, 1997.

20. Luna LG: Manual of histologic staining methods of the armed forces institute of pathology. New York, McGrow-Hill Book Co, pp, 212-213, 1968.

21. Gialetti L, Tittoli F, Asdruballi G: La diagnosi dell'encefelomielite aviare mediante immunoflourescenza. *Fol Vet Lat,* 2, 292-309, 1972.

22. Johnson GD, Holborow EJ, Dorling J: Immunofluorescence and Immunoenzyme Techniques. **In,** Neir DM (Ed): Handbook of experimental immunology. Vol. 1. Blackwell Scientific Publication, pp, 15.2-15.18, 1978.

23. Helmboldt CF: Histopathologic differantiation of diseases of the nervous system of the domestic fowl *(Gallus gallus). Avian Dis,* 16, 229- 240, 1972.

24. Heide L Van Der: De diagnostic van aviare encephalomyelitis met behalp van de directe immunofluorescente. *Techniek Tijdschr Diergeneesk,* 95, 639-641, 1970.

25. Braune MO, Gentry RF: Avian encephalomyelitis virus. II. Pathogenesis in chickens. *Avian Dis,* 15, 648-653, 1971.

26. Braune MO, Gentry RF: Avian encephalomyelitis virus. 1. Pathogenesis in chicken embryos. *Avian Dis,* 15, 638-647, 1971.

27. 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.

28. Mohanty GC, West JL: Research note; some observation on experimental avian encephalomyelitis. *Avian Dis,* 12, 689-693, 1968.

29. Mohanty GC, West JL: Avian encephalomyelitis; pathogenesis and histologic features of dorsal root ganglia lesions in chicks. *Avian Dis,* 17, 31-42, 1973.

30. Sağlam YS, Erer H: Civcivlerde deneysel avian encephalomyelitis hastalığında klinik ve histopatolojik bulguların incelenmesi. *Etlik Vet Mikrobiol Derg,* 9, 31-46, 1997.

31. Bielefeldt-Ohmann H, Tolnay AE, Reisenhauer CE, Hansen TR, Smirnova N, Van Campen H: Transplacental infection with non-cytopathic bovine viral diarrhoea virus types 1 and 2: Viral spread and molecular neuropathology. *J Comp Pathol,* 138, 72-85. (2008)

32. Miyamae T: Ecological survey by the immunoflourescent method of virus in enzootics of avian encephalomyelitis. *Avian Dis,* 18, 369-377, 1974.

33. Miyamae T: Immunofluorescent study on egg-adapted avian encephalomyelitis virus infection in chickens. *Am J Vet Res,* 38, 2009-2012, 1977.