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# Histopathological and Immunohistochemical Detection of Pigeon Paramyxovirus-1 (*pPMV-1*) in Pigeons

G. Yuzbasioglu-Ozturk, A. Gurel

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## ABSTRACT

**Background:** The study's main objective was to determine the occurrence, frequency and primary histopathological lesions of pigeon paramyxovirus-1 infection in Istanbul's pigeon population and demonstrate the viral antigen immunohistochemically to reach a definitive diagnosis and to help elucidate the pathogenesis of the disease.

**Methods:** Sera were collected for ELISA from thirty-seven pigeons suspected of *pPMV-1* infection, housed at different flocks in various Istanbul districts. The study was carried out with a total of ninety pigeons. Immunohistochemistry was applied to demonstrate tissue distribution of the virus with mAb 617/161 antibody.

**Result:** ELISA results for positive, suspected and negative cases were 89.18%, 5.4% and 5.4%, respectively. Tubulointerstitial nephritis, pancreatitis and necrotizing hepatitis were commonly encountered histopathological lesions. Viral antigen was demonstrated in 60.25% of all cases by immunohistochemistry. It was concluded that infection was present in pigeon flocks in various districts of Istanbul and immunohistochemistry was considered as a useful tool in reaching a definiteve diagnosis through the demonstration of antigenic particles in the lymphoid cells and macrophages localized in various organs, particularly the kidney, pancreas, spleen and liver.

**Key words:** ELISA, Immunohistochemistry, Newcastle disease, Pigeon, *pPMV-1*.

## INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal disease affecting various bird species. Avian Paramyxovirus type-1 (*a-PMV-1*) has been defined as the disease's causative agent. The ND virus (NDV) serotype isolated from sick pigeons during the panzootic in Europe in the 1980s was demonstrated to have differed from the well-known conventional (*a-PMV-1)* serotype and was afterward referred to as the pigeon paramyxovirus type 1 (*pPMV-1*). The infection is known to be enzootic in the pigeon population and is a threat for the poultry industry worldwide (Mishra *et al*., 2000a; Alexander and Senne, 2008).

There are a few references concerning the relationship between viral antigen distribution and the histopathological lesions in *pPMV-1* infection of pigeons in the literature (Barton *et al*., 1992; Johnston and Key 1992; Shaheen *et al*., 2005; Isidoro-Ayza *et al*., 2017). This study aims to specify the presence and prevalence of *pPMV-1* infection among the pigeon population in Istanbul through serology and immunohistochemistry (IHC), to present the clinical signs and to determine the microscopic lesions that develop during the disease.

## MATERIALS AND METHODS

This study was approved by The Ethics Committee of Istanbul University (decision no: 2005/130, dated 2nd of August, 2005).

Thirty different flocks from different districts of Istanbul were monitored for the study and samples were collected from 13 of these flocks with pigeons showing clinical signs and positivity with the ELISA method.

The study included a total of 90 pigeons. Eighty of them comprised the study group while 10 were kept as the control.

Department of Pathology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa, 34500 Buyukcekmece/Istanbul, Turkey.

**Corresponding Author:** G. Yuzbasioglu-Ozturk, Department of Pathology, Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, 34500 Buyukcekmece/Istanbul, Turkey.
Email: gulayoz@iuc.edu.tr

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Seventy-four out of 80 birds were members of the 13 different unvaccinated flocks with different breed and age distributions that died during the study. According to the information given by the breeders, 48 of the birds in the study group, were under one year old and 32 of them were over one year old. Four cases submitted to the pathology department with the suspicion of *pPMV-1* infection and 2 birds from the control group that came out to be positive with ELISA from the control group were also included in the study group. Serum samples could be collected from forty-seven birds (37 from the study and 10 from the control groups). ELISA was conducted following the instructions indicated in the data sheets (Svanova Biotech AB, Uppsala, Sweden. Catalogue No. 10-1500-02).

For histopathological and immunohistological examination, tissue samples were fixed in 10% neutral-buffered formalin and paraffin after routine tissue processing methods. 4 µm-thick serial sections were obtained and stained with hematoxylin and eosin (H&E). Unstained sections were deparaffinized and stained using the avidin-biotin

complex method described before (Hsu *et al.,* 1981). Negative controls included substituting the primary antibody with non-related sera. Tissue samples of ELISA positive cases with evidential clinical signs served as the positive control. Antigen retrieval was carried out by microwaving in sodium citrate buffer (0.01 M, pH 6.0). Endogenous peroxidase activity was blocked by immersing the slides for 20 min in a 3% solution of $H_2O_2$ made in methanol then incubated overnight at +4°C with the primary antibody (mAb 617/161, 1:500 dilution) specific for Hemaglutinin-Neuraminidase (HN) spike protein of *pPMV-1* virus (the antibody was kindly provided by Dr. Manvell Ruth, Veterinary Laboratories Agency-Weybridge, Great Britain). The slides were further incubated with rabbit/mouse reagent conjugated HRP Polymer for 15 min at room temperature after rinsing. The sections were treated with 3,3'-diaminobenzidine (DAB) (Lab Vision, Catalogue No. TA-125-HDX) and counterstained with Harris' hematoxylin. Immunohistochemically stained sections were assessed as positive or negative according to the presence or absence of intracytoplasmic immuno reactivity by scanning all microscopic fields on a slide using a light microscope.

Independent-samples t-test was applied to compare the ELISA results of the study and control groups. The chi-square test was used to compare various tissue samples in terms of IHC positive staining. Specificity, sensitivity, positive predictive value and negative predictive value of ELISA results were calculated according tothe method proposed by Ozdamar (1999).

## RESULTS AND DISCUSSION

In most cases, the infection spread among birds within 2-3 weeks of the onset of the clinical signs in a flock. The morbidity rate was relatively high, ranging from 40% to 100%, while mortality was low. Polyuria, followed by polydipsia, was the main finding in the infected flocks. Nervous system signs such as incoordination, head tremors and torticollis were encountered in 52 birds. Additionally,decreased activity, fluffy feathers and reluctance to fly were seen in most flocks. 43 birds with neurological symptoms were younger than one year old, while nine were over one year of age. At postmortem examination, even though most of the birds were in good body condition, some had mild to moderate dehydration and cachexia. There were no significant gross findings that might have been attributed to *pPMV-1* infection other than hyperemia in the kidney, liver, pancreas and brain.

ELISA results of the 37 serum samples revealed 33 (89.19%) were positive, two (5.4%) were negative and two (5.4%) were suspected animals. The most prominent histopathological findings were detected in the kidney, pancreas, bursa of Fabricius, spleen, liver and brain (Table 1). There were no histopathological lesions in 11 birds (14.77%), with clinical symptoms and ELISA positivity evidence of *pPMV-1* infection. Viral antigen immunolabelling, visualized as intracytoplasmic fine brown granules, was detected in various organs of 49 (61.25%) out of 80 pigeons (Fig 1-8). Positive immunohistochemical reaction was detected mainly in the kidney (n=42), bursa of Fabricius (n=15), pancreas (n=28), spleen (n=29) and the liver (n=27), which paralleled

**Table 1:** Distribution of the histopathological lesions in 80 *pPMV-1* positive pigeons.

| Histopathological lesion | Number of affected pigeons (n=80) |
|---|---|
| **Kidney** | |
| Disseminated lymphoplasmacytic inflammatory infiltration accompanied by heterophil infiltration | 62 |
| Slight degenerative alterations to necrosis localized mainly in the cortical renal tubules | 56 |
| Dense hyperemia | 64 |
| Areas of subcapsular and interstitial hemorrhage | 22 |
| **Pancreas** | |
| Degenerative alterations and vacuolisation in the acinar cells accompanied by multifocal lymphoid cell infiltrations | 46 |
| **Spleen** | |
| Diffuse moderate lymphoid depletion replaced by fibrosis | 33 |
| **Bursa of fabricius** | |
| Severe lymphoid depletion of the follicular medula and the cortex | 8/29* |
| **Liver** | |
| Sinusoidal dilatation and dense congestion | 54 |
| Disseminated parenchymatous degeneration and vacuolisation in the hepatocytes | 64 |
| **Brain** | |
| Demyelination in white matter | 55 |
| Neuronal degeneration and neuronophagia | 36 |
| Lymphocytic infiltration and focal gliosis | 14 |
| Leptomeningeal lymphohistiocytic infiltration | 10 |
| Proliferative alterations in the vascular endothelial cells | 9 |

* bursa of Fabricius obtained from 29 birds in total.

concurrent histopathological lesions. Positive immuno staining occurred mainly in the cytoplasm of the inflammatory mononuclear cells in all tissues. Based on the chi-square test results, the differences among the organs in terms of positive IHC staining were significant ($\chi^2$=357.26; P<0.001). Moreover, there was a highly significant difference (t=6.649; P<0.001) between the study and the control groups as per the ELISA results.

Specificity, positive predictive value, negative predictive value and sensitivity of ELISA test were 38.46, 75.75, 0 and 78.94%, respectively.

The *pPMV-1* isolates cause severe outbreaks in chickens as well as pigeons. *pPMV-1* was isolated and identified as the causative agent of various NDV outbreaks previously observed in poultry farms from different regions across Turkey (Oncel *et al.*,1997; Coven *et al.*, 1999).

Greenish watery droppings, polydipsia and neurological symptoms were reported to be the most commonly seen

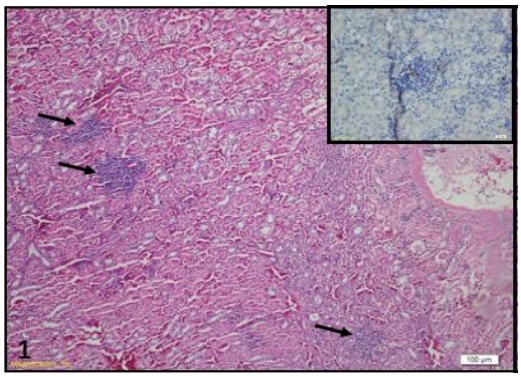

**Fig 1:** Tubulointerstitial nephritis. 4-month-old. Disseminated multi-nodular lymphoplasmacytic infiltration accompanied by heterophil infiltration in the interstitial areas and degenerative alterations in the cortical renal tubules of kidney (arrows). H&E. 100X Bar=100 µm *Inset.* Immunohistochemical labeling for *pPMV-1* antigen in inflammatory mononuclear cell cytoplasms in the kidney.

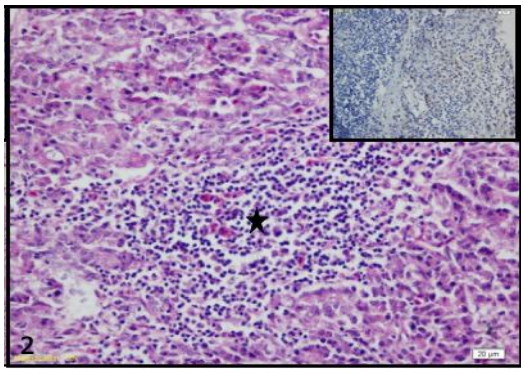

**Fig 2:** Pancreas. 3-month-old. Degenerative alterations and vacuolisation in the acinar cells accompanied by multifocal lymphoid cell infiltrations (star). H&E. 400X Bar=20 µm *Inset.* Immunolabeling of pPMV-1 antigen in the cytoplasms of inflammatory lenfoid cells.

*pPMV-1* infection-associated clinical signs in pigeons (Eisa and Omer, 1984; Tangredi, 1985; Chowdhary *et al.*, 2020). The birds manifesting the related clinical signs were included in the study group; however, some revealed neither histopathological lesions nor positivity with immuno histochemistry. Furthermore, 2 birds from the control group with no clinical signs were positive, both with the ELISA and IHC methods. Therefore, it is considered that a diagnosis based merely on clinical symptoms might be misleading and the asymptomatic birds likely serve as the reservoir of the infection. Furthermore, six pigeons with clinical symptoms and ELISA positivity revealed negative results with IHC. These birds were considered to have completed the acute phase of the infection and entered the chronic form, which was manifested by the neurological symptoms that might have been permanent as previously reported (Lumeij and Stam, 1985; Barton *et al.*, 1992). It was deduced that antigen density decreased once the infection had entered the chronic phase, which negatively affected the IHC results despite the positivity in these animal sera.

It has been reported that mortality rate is high in young birds with natural infections (Tangredi, 1985; Vindevogel and Duchatel, 1988). Eventhough the owner's inclination to euthanize the sick birds hamper the detection of the mortality rates, young animals' mortality rates were found higher in our study.

There was no evidence of gross lesions in the pigeons infected with *pPMV-1* in the majority of the studies (Isidoro-Ayza *et al.*, 2017), while a few demonstrated hemorrhage in the digestive system, pancreas and the brain and dehydration in acute cases (Eisa and Omer, 1984; Johnston and Key 1992). No prominent gross findings atributable to the disease other than hyperemia in the kidney, liver, pancreas and brain were detected in the study.

In the study group, all kidneys with positive immuno histochemical labeling revealed tubulointerstitial lymphoplasmacytic infiltration. Similar lesions were previously demonstrated in the kidney by several researchers (Zanetti *et al.*, 2001; Shaheen *et al.*, 2005; Isidoro-Ayza *et al.*, 2017). Based on findings in our study, it can be deduced that the virus had an affinity to the kidney tissue. Ojok and Brown (1996), in an experimentally induced chicken model with the viscerotropic velogenic NDV strain, detected the viral antigens only in the inflammation areas. Even though degeneration and necrosis were widespread in the tubular epithelial cells, no positive immuno histochemical staining in the epithelial cells was observed in the study.

Vacuolisation and necrosis in the acinar epithelial cells, focal mononuclear inflammatory cell infiltration and necrosis and reticular cell proliferation in the pancreatic islets of affected birds have been reported (Zanetti *et al.*, 2001; Isidoro-Ayza *et al.*, 2017). No necrosis was noted in the pancreatic islets in the study, yet the other findings were consistent with the literature. Slight degenerative alterations accompanying interstitial mononuclear cell infiltration of the

liver were compatible with the literature (Shaheen *et al*., 2005).

In this study, histopathological lesions in the central nervous system were observed consisting mainly of hyperemia, demyelination, neuronal degeneration and to a lesser extent gliosis, lymphocytic perivascular infiltration and proliferative alterations of the vascular endothelial cells were compatible with the literature (Maeda *et al*., 1987; Shaheen *et al*., 2005). In the brain and cerebellum tissues with positive immunoreaction, viral antigen was observed subsequently with gliosis, neuronal necrosis and in the areas of perivascular infiltration. Clinical neurological findings are assumed to be induced by neuronal degeneration and neuronophagia observed in 45% of the birds and by demyelination observed in 68.75% of the pigeons in the study.

The primary histopathological lesions observed in the lymphoid organs were congestion, hemorrhage and lymphoid depletion (Mishra *et al*., 2000b; Zanetti *et al*., 2001). In the study, lymphoid atrophy of the spleen and bursa of Fabricius was one of the most common finding. Splenic hyperplastic lesions observed in pigeons' spontaneous infections were associated with the birds' recovery period (Maeda *et al*., 1987). In the study, 14 out of 20 birds with histopathologically confirmed splenic lymphoid hyperplasia revealed positive results with IHC, while no immunoreaction was demonstrated in 6 birds.

Twenty-one out of 49 immunoreactive birds were adults, while 28 were young birds. The collected data pointed out the infection's high prevalence in various districts of Istanbul. Both adults and young birds were susceptible to the disease. In the study, a monoclonal antibody (mAb 617/161) developed against HN protein, specific to pigeon isolates, was utilized to demonstrate the immunoreactions. In our study, polyclonal antibodies developed against NDV strains might have rendered more intense immunoreactions; however, it was highly likely to occur a cross-reaction with other paramyxovirus strains (particularly APMV-3), which – in return- might have caused interference and falsified the results. Vindevogel and Duchatel (1988) indicated that it was not feasible to isolate *pPMV-1* from the affected pigeons'

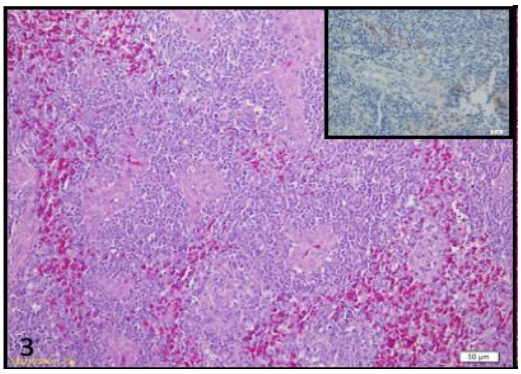


**Fig 3:** Lymphoid hyperplasia, spleen. Even though the splenic tissue maintained its typical architecture and pattern, lymphoid follicles were enlarged due to lymphoid proliferation and secondary lymphoid follicles were formed. 3-month-old. H&E. 200X Bar=50 µm. *Inset.* Disseminated immunolabeling in the cytoplasm of lymphoid cells in the spleen.

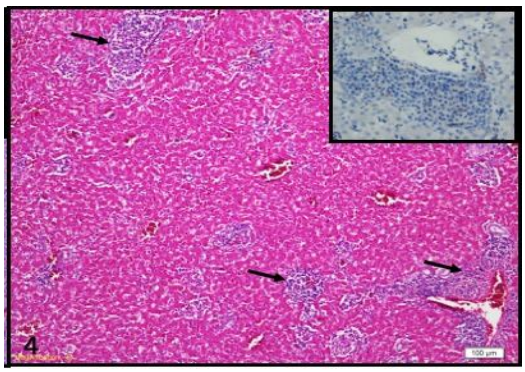


**Fig 4:** Liver. 6-month-old. Sinusoidal dilatation and dense congestion and disseminated parenchymatous degeneration and vacuolisation in the hepatocytes accompanying focal inflammatory cell infiltration (arrows). H&E. 100X Bar=100 µm. Inset. Immunolabeling of *pPMV-1* antigen in the cytoplasms of inflammatory lenfoid cells in the liver.

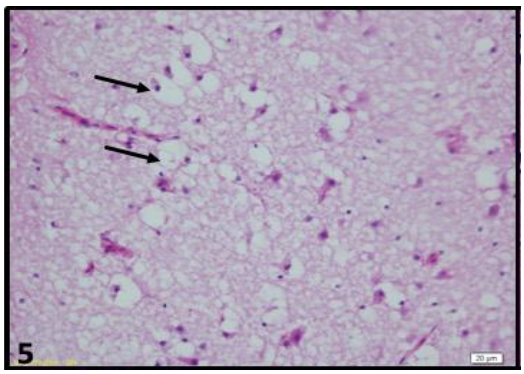


**Fig 5:** Brain. >1-years-old. Demyelination in the white matter, neuronal degeneration and neuronophagia (arrows). 400X Bar= 20µm.

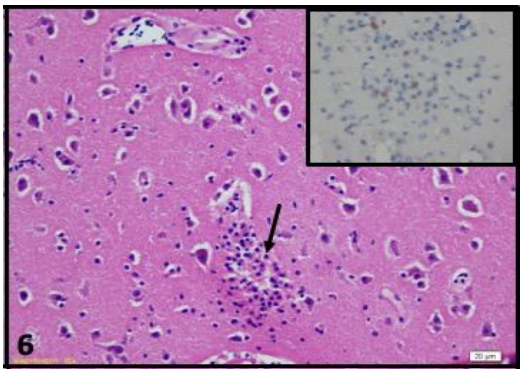


**Fig 6:** Brain. 4-month-old. Brain. Lymphocytic infiltration and focal gliosis (arrow). H&E. 400X Bar = 20 µm *Inset:* Immunolabeling of *pPMV-1* antigen in the focal gliosis area in the brain.

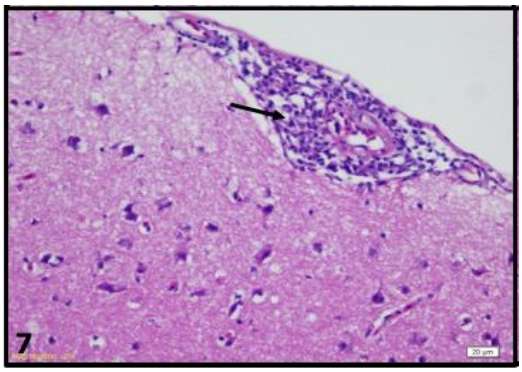


**Fig 7:** Brain. 4-month-old. Leptomeningeal lymphohistiocytic infiltration (arrow). H&E. 400X Bar = 20µm.

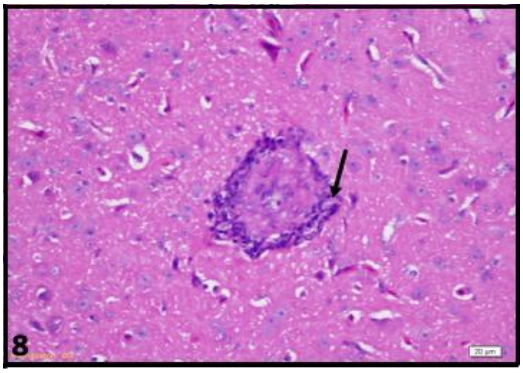


**Fig 8:** Brain. 6-month-old. Proliferative alterations in the vascular endothelial cells (arrow). H&E. 400X Bar = 20µm.

brains and intestinal tissues after 3-5 weeks of the infection since the neurological symptoms might persist for 2-6 months. Therefore, negative immunoreaction in birds afterward included in the study group, due to clinical symptoms, was associated with this phenomenon.

In the literature, NDV antigen was shown by IHC in the kidney's tubular epithelial cells in the cortex and medulla (Isidoro-Ayza *et al.*, 2017). The NDV antigen was also localized in necrotic tubular epithelial cells and mononuclear inflammatory cell cytoplasms within the areas of interstitial infiltration in different bird species and pigeons (Barton *et al.*, 1992). Ojok and Brown (1996) and Hamid *et al.* (1990) reported no stainingin the epithelial cells and viral antigen was only localized in mononuclear inflammatory cells. In this study, no positive staining was observed in the epithelial cells of any tissues. Viral antigen was mainly seen in the cytoplasm of mononuclear phagocytic cells. Diversity has been reported among NDV strains regarding necropsy findings and antigen distribution (Collins *et al.*,1994). Nevertheless, since mononuclear phagocytes are the most frequently involved cells, immunohistochemical evaluation of the tissues densely containing these cells can aid in more rapid and more straightforward diagnosis of the infection.

Once the serologic, histopathological and immuno histochemical findings were collectively evaluated, it can be deduced that all methods may offer assistance in the diagnosis, yet all should be concurrently utilized for a definitive diagnosis. Since the lesions may be localized within a limited tissue region, several histopathologic fields should be scanned and evaluated. IHC may be a utilized as a diagnostic tool to distinguish the lesions that might have developed due to other factors. However, none of the methods proved favorable for establishing the pathogenesis of the disease (Alexander and Senne, 2008).

## CONCLUSION

The *pPMV-1* infection that has become more prevalent in pigeons in recent years constitutes a threat for poultry since the pigeons are the causative agent's vectors. Asymptomatic pigeons, which were found to be serologically and immunohistochemically positive for the virus, may also play a role in the contagion. Therefore, it is of utmost importance to take measures in pigeon breeding by briefing the owners and taking necessary precautions against the spread of the infections, such as assuring quarantine in the suspected flocks.

The overall data revealed that -regardless of the occurrence of the clinical signs- having entered the body, the virus spreads to multiple organs, which result in pathological changes of varying severity and characteristics mainly localized in the kidney, pancreas, lymphoid organs, lever and brain. Further studies are necessary to elucidate the disease's pathogenesis and epizootiology in pigeons.

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**Conflict of interest:** None.

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