HISTOPATHOLOGICAL CHARACTERISTICS AND EXPRESSION OF CDV-NP ANTIGEN IN THE BRAIN OF SEROLOGICALLY POSITIVE SPONTANEOUSLY INFECTED RED FOXES (VULPES VULPES) IN WESTERN SERBIA

ANIČIĆ Milan1*, VUČIĆEVIĆ Ivana1, VASKOVIĆ Nikola2, RADOJIĆIĆ Sonja3, JUNTES Polona4, NEŠIĆ Sladan1, ALEKSIĆ-KOVAČEVIĆ Sanja1, MARINKOVIĆ Darko1

1Department of Pathology, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia; 2Veterinary Specialist Institute “Kraljevo”, Kraljevo, Serbia; 3Department of Infectious Animal Diseases and Diseases of Bees, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia; 4Institute of Pathology, Wild Animals, Fish and Bees, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia

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Canine distemper virus (CDV) is a worldwide distributed RNA virus that can cause severe disease in carnivore and non-carnivore species. Red foxes are highly susceptible and may act as a reservoir of the virus. As in other wild species, distemper in red foxes can manifest as acute, systemic and chronic nervous form. In the present study, we detected antibodies against CDV among red foxes in Western Serbia, and analyzed histopathologically and immunohistochemically for CDV nuclear protein antigen (CDV-NP) brain samples derived from seropositive animals. Seroprevalence of CDV antibodies was 36.8%. Histopathological changes included gliosis, neuronal degeneration, satellitosis, mononuclear inflammation, demyelination and presence of inclusion bodies. Immunostaining showed a diffuse presence of CDV-NP antigen, mainly in the cytoplasm of astrocytes and neurons. Results of this work contribute to the opinion that red foxes act as a potential reservoir of CDV and underline the importance of routine vaccination of dogs that could come in close contact with these animals. Potential active surveillance program would give a better insight in the degree of CDV infection in wildlife.

Key words: brain, canine distemper virus, histopathology, immunohistochemistry, red fox

INTRODUCTION

Distemper is an infectious, highly contagious and often fatal disease caused by canine distemper virus (CDV, genus Morbillivirus, family Paramyxoviridae, order Mononegavirales). It is a pantropic virus with a non-segmented, negative stranded RNA
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CDV has six structural proteins of which haemaglutinin (H), the attachment protein, due to its high antigentic variability is used for genetic changes monitoring of the virus [2,3]. There is one serotype of CDV classified by H gene–based phylogenetic analysis into 14 genotypes (lineages) of different virulence and cell tropism [4]. The infection has been confirmed in the mammalian species belonging to six different orders: Carnivora, Rodentia, Artiodactyla, Primates, Proboscidea and Pilosa [5,6]. Dogs are primary reservoirs, while other families of the order Carnivora are natural hosts [7]. As a worldwide distributed disease it has been reported from most countries of the world [8]. Recent in vitro studies showing that CDV can possibly infect human cells have raised the importance of CDV as a potential zoonotic agent, especially considering WHO agenda for reducing measles virus vaccination [9,10].

Besides dogs, several wild species, including red foxes, have been proposed as reservoirs for CDV [11]. All fox species (Vulpes spp.) are considered susceptible to CDV infection [12]. Red foxes are susceptible with high mortality [13-16]. As in dogs, it is probable that red foxes get infected through aerosolized respiratory excretions or contact with oral, respiratory and ocular fluids and exudates containing the virus [17,18]. The majority of CDV infections are subclinical, while clinical infections manifest as an acute, systemic or a chronic, nervous form [19,20]. Infection of the CNS occurs hematogenously, either directly through fine blood vessels of brain parenchyma or indirectly via the choroid plexus and cerebrospinal fluid (CSF) [21-23]. Neuroinvasion via the olfactory nerves has been reported in ferrets [23]. Disease severity and mortality vary among different species. Considering dog’s related data Nouvellet et al. [17] suggest that around half of all CDV infected foxes will die because of this disease. Recent studies from Europe have found seroprevalence of CDV infection in red foxes ranged from 7.8 % in some regions of Spain (La Mancha) [18] up to 33.7% in parts of Germany (Saxony-Anhalt) [24].

Macroscopic changes in the brain of the animals infected with CDV are rare, while there is a number of described microscopic lesions characteristic for this disease [1]. Demyelinating encephalitis is one of the most dominant findings on the brain of red foxes affected with CDV [25]. Other brain lesions of CDV infected red foxes are multifocal to diffuse gliosis with the formation of nodules, neuronal degeneration, and perivascular cuffing, which according to many authors is rare and minimal to mild [26,27]. Intracytoplasmic and intranuclear eosinophilic inclusions were noted in many cells, most frequently in neurons and glial cells, and they are described by many authors [14,25-27].

The CDV-NP antigen is detected immunohistochemically in neurons, glial cells, ependymal cells, endothelial cells, as well as immune cells, mainly meningeal macrophages. An immunopositive reaction of CDV-NP antigen is diffuse, mainly cytoplasmic, and rarely nuclear, and it is also noted in the intracytoplasmic and intranuclear inclusions [14,25,26].
Even though many wild mammals are susceptible and reports of it are constantly increasing, the epidemiology of CDV in free-ranging carnivores remains poorly understood [28].

The aim of this study was to detect the exposure to CDV among red foxes (Vulpes vulpes) in Western Serbia and, in the case of serologically positive animals, to describe histopathological changes and expression and distribution of CDV-NP antigen in the brains of infected animals.

**MATERIAL AND METHODS**

Red fox carcasses intended for rabies surveillance and monitoring activities were brought to the Veterinary Specialist Institute “Kraljevo”, in Kraljevo. The age of the animals was estimated based on body size measurements and tooth eruption and wear [12]. From September 2016 until September 2017 a total of 285 fox carcasses were submitted. All examined foxes were classified as adults (more than 1 year of age). The blood was obtained directly from the heart and allowed to clot, centrifuged, the serum carefully removed, frozen and stored at temperature lower than -15 °C until used. Detection of rabies virus antigen in the brain samples was performed by a fluorescent antibody test.

Foxes sera were serologically examined using indirect enzyme-linked immunoassay (ELISA) for detection of specific IgG antibodies against CDV (Ingezim Moquillo IgG 15.CDG.K1, Ingenasa, Madrid, Spain). The sera samples were diluted 1/100 and examined following manufacturer’s instructions. The cut off value was calculated as optical density (OD) of positive control (OD pc) x 0.2. Positive samples were divided as follows: low titer (samples show OD values between 0.2 x OD pc and 0.4 x OD pc), medium titer (0.4 x OD pc and 0.8 x OD pc) and high titer (OD values higher than 0.8 x OD pc). Based on criteria such as non-hemolyzed serum, absence of advanced post mortal changes and usability of brain tissue for morphological examination 68 out of 285 animals were tested serologically.

Brain samples of foxes, which were negative for rabies and serologically positive for CDV were macroscopically examined and fixed in 10% neutral buffered formalin. Coronal sectioning of the brain parenchyma was performed, and four samples comprising the frontal cortex, thalamic region, hippocampal region and cerebellum were taken. After standard processing in an automated tissue processor, tissue samples were embedded in paraffin, blocks were cut in 5 µm thick sections, dewaxed in xylol, rehydrated through a series of ethanols of decreasing concentrations (100%, 96%, 70%) to distilled water, and stained with hematoxylin and eosin (HE) or used for immunohistochemistry, respectively.

In the process of immunohistochemical staining endogenous peroxidase was inhibited using 3% hydrogen-peroxide for 30 minutes. Unmasking of antigen was achieved by heating the tissue sections in a microwave oven at 560 W for 20 minutes in a commercial
sodium citrate buffer pH 6.0. After cooling down to room temperature (22 ± 3 ºC) for 30 minutes tissue sections were incubated with normal goat serum (2%) for 20 minutes at room temperature and then with primary CDV-NP (nucleoprotein CDV specific monoclonal antibody, Ingenasa, Madrid, Spain) antibodies (diluted 1:2200) overnight in a humid chamber at 4 ºC. After that, samples were incubated with biotinylated secondary (rabbit/mouse) antibodies for 30 minutes at room temperature. The immunoreaction was visualized using DAB+kit (3,3'-diaminobenzidine tetrahydrochloride, DAKO) for up to 3 minutes. Mayer's hematoxylin was used as a counterstain. Intensity of positive staining was scored according to the amount of positive cells into three categories: + - weak (less than 10 %), ++ - moderate (10 – 50%) and +++ - strong (more than 50%). Brain sections, which were formerly confirmed positive for CDV-NP antigen, were used as positive controls, and brains from foxes serologically negative for CDV were used as the negative control. Brain samples from 12 animals with characteristic changes were immunostained for detection of CDV-NP antigen.

The results of histochemical and immunohistochemical staining were analyzed by light microscope (BX51, Olympus Optical, Japan). Pictures were taken with Olympus Color View III® digital camera.

**RESULTS**

**Serological findings**

Using fluorescent antibody test no rabies antigen was detected in brain samples of submitted animals. Antibodies against CDV were detected in 25 (36.8%) out of 68 serum samples examined with indirect ELISA. Of those, 17 (25.0%) samples had low antibody titers while 8 (11.8%) had medium titers. None of the animals had a high titer of antibodies.

**Macroscopic and histopathological findings**

Macroscopic examination of brains derived from seropositive animals revealed no gross changes.

There were a number of significant histopathological changes noted in serologically positive animals. The accumulation of glial cells - gliosis was noted in 21/25 (84.0%) brains, sometimes with the formation of glial nodules. Degeneration of neurons was noted in 20/25 (80.0%) brains. Neuronal swelling with nuclear pyknosis, hypereosinophilia and central chromatolysis were most commonly observed in the frontal cortex. These changes were often associated with the accumulation of glial cells – satellitosis, which was present in 14/25 (56.0%) examined brains. In the same number of examined brains (14/25) degeneration and loss of Purkinje cells were noticed. Inflammatory lesions, which consisted of cerebral perivascular mononuclear infiltrate and meningitis were present in 12/25 (48.0%) examined brains, and were most easily noticed and sometimes restricted to the frontal cortex (Figure 1B). The
described lesions varied from mild to moderate, and in 2/25 (8.0%) cases moderate perivascular infiltrates throughout all brain sections comprising more than three layers of mononuclear inflammatory cells were seen. Demyelination was evident in 9/25 (36.0%) animals with the most prominent changes in the cerebellar white matter (Figure 1C). Edema was present in 8/25 (32.0%) examined brain samples. Intracytoplasmic and intranuclear eosinophilic inclusions characteristic for CDV were noted in glial cells, mostly astrocytes (5/25 animals) (20.0%) and neurons (4/25 animals) (16.0%) (Figure 1D). Round to oval, well demarcated cytoplasmic inclusion-like formations morphologically consistent with inclusions that lacked eosinophilia were noted in 6 animals (24.0%). Inclusions were also seen in ependymal cells along with syncytia formation. Occasionally, besides the described lesions, gemistocytes and binucleated astrocytes, discrete foci of calcification and perivascular hemorrhages were seen.

**Figure 1.** Histopathological changes in the brains of CDV seropositive red foxes, HE. a) gliosis and neuronal degeneration, b) mononuclear perivascular cuffing, c) demyelination of cerebellar white matter, d) neuronal intracytoplasmic inclusion (arrow).

**Immunohistochemical findings**

Immunolabeling of CDV antigen was detected in all samples with a moderate to strong positive reaction. The antigen was detected in neurons and glial cells, primarily in the cytoplasm and less frequently in the nuclei (Figure 2). Also, ependymal cells showed
signs of immunopositivity and occasionally the same could be seen in endothelial cells of small blood vessels.

**DISCUSSION**

Serological detection of antibodies against CDV in the serum is a sound proof of the contact of an unvaccinated animal with the virus. Although many wild mammals are susceptible to the CDV and reports of it are constantly increasing, the epidemiology of this disease in free-ranging carnivores is not entirely understood [25]. There are several serological studies throughout Europe that have described a seroprevalence of CDV infection in red foxes from 7.8% in some regions of Spain (La Mancha) [18] up to 33.7% in part of Germany (Saxony-Anhalt) [24]. Comparing serological results obtained in our study, 36.8% seropositive samples, 11.8% with medium and 25.0% with low antibody titers, to other studies from Europe [18,24] these values represent a high percentage of serologically positive animals.

Macroscopic examination of brains derived from seropositive animals from our study revealed no gross changes, which is similar to findings of other authors, while there were
a number of described microscopic lesions characteristic for this disease [1]. Similar to our findings Orrigi et al. [26] also described that the majority of lesions were present in the cerebral cortex while other parts (cerebellum, thalamus, hippocampus, nucleus caudatus, periventricular areas) were less affected. The most consistent histopathological change seen in the brain parenchyma of serologically positive animals was gliosis (84.0% brains). Sometimes it was very prominent with the formation of glial nodules. These findings are consistent with literature data, since many authors describe multifocal to diffuse gliosis with the formation of nodules in the brain of red foxes affected with CDV [26,27].

In our study, neuronal degeneration characterized by swelling of the neurons, nuclear pyknosis, hypereosinophilia and central chromatolysis were seen in different brain sections, most commonly in the frontal cortex. Neuronal degeneration was often associated with the accumulation of glial cells – satellitosis, and all mentioned changes are consistent with literature data [26].

According to some studies inflammatory lesions are characterized by congestion of the meningeal and cerebral blood vessels with rare and minimal to mild perivascular cuffing [25,26]. López-Peña et al. [25] did not notice any perivascular cuffs. The results of our study are somewhat different from these described in the literature, with inflammatory lesions present in just under half of examined brains (48.0%). These were almost exclusively in the form of discrete perivascular mononuclear infiltrates and mild meningitis, commonly noticed and sometimes restricted to the frontal cortex. Only 2 animals (8.0%) showed signs of moderate multifocal, mainly perivascular infiltrates throughout the brain parenchyma. Pope et al. [27] reporting of a CDV infection in grey foxes observed a minimal lymphoplasmacytic perivascular infiltrate, indicating an acute stage. Origgi et al. [26] found demyelinating encephalitis in 10% of cases while López-Peña et al. [25] described this lesion as the most dominant and found it in the brains of all red foxes examined. In our study it was present in 36.0% of animals. Similar to their findings most prominent changes were in the cerebellar white matter. Inclusion bodies were not common in our samples. We observed intracytoplasmic and intranuclear cosinophilic inclusions characteristic for CDV in glial cells, mostly astrocytes, neurons and occasionally ependymal cells. Other authors found these inclusions to be more frequent in neurons, Zhao et al. [14] in cytoplasm and Origgi et al. [26] mostly intranuclear. While degenerative changes of the astrocytes could be a consequence of viral presence, other uncommon, subtle changes that were seen, including calcification and hemorrhage are probably nonspecific.

A moderate to strong diffuse positive reaction for CDV-NP antigen was seen in all immunostained sections. The reaction was mainly cytoplasmic, also noted in the intracytoplasmic and intranuclear inclusions of the neurons and glial cells. Many authors reported similar results with a marked immunopositive reaction of CDV-NP antigen in neurons, glial cells, ependymal cells, endothelial cells, as well as immune cells, mainly meningeal macrophages [14,25,26].
CONCLUSION

In this study, we serologically detected antibodies against CDV and immunohistochemically viral antigen in the brain sections of red foxes in Western Serbia. The high prevalence of CDV infection in red foxes contributes to the current opinion that these animals act like potential reservoirs of the virus. Considering the natural behavior of red foxes, with emphasis on possible frequent contacts with hunting, as well as dogs in suburban and rural areas, high prevalence signifies the importance of routine vaccination against CDV. While histopathological findings were somewhat specific, immunolabeling of the CDV antigen is still one of the most reliable methods for definitive diagnosis. Although passive surveillance has its benefits, we think that the potential future active surveillance program would give better insight in CDV infection of wildlife in Serbia.

Authors’ contributions:

AM carried out gross and histopathological examination, acquired literature data, wrote the initial text and took photography. MD performed histopathological examination and participated in drafting the manuscript. VN was involved in sampling and carried out the serological testing. AM, VI and JP carried out immunohistochemical analysis. NS participated in histopathological examination. AKS, RS and JP made substantial contributions to the writing of the manuscript and critically revised the article for important intellectual content. All authors read and approved the final manuscript.

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HISTOPATOLOŠKE KARAKTERISTIKE I EKSPRESIJA CDV-NP ANTIGENA U MOZGU SEROLOŠKI POZITIVNIH PRIRODNO INFICIRANIH CRVENIH LISICA (VULPES VULPES) U ZAPADNOJ SRBIJI

ANIČIĆ Milan, VUČIĆEVIĆ Ivana, VASKOVIĆ Nikola, RADOJIČIĆ Sonja, JUNTES Polona, NEŠIĆ Slađan, ALEKSIĆ-KOVAČEVIĆ Sanja, MARINKOVIĆ Darko

Virus štenečaka (canine distemper virus, CDV) je virus rasprostranjen u celom svetu koji može izazvati ozbiljno oboljenje ne samo mesojeda već i drugih vrsta životinja. Crvene lisice su visoko prijeminive i potencijalno predstavljaju rezervuar virusa. Slično drugim vrstama divljih životinja, štenečak se kod crvenih lisica manifestuje u akutnoj, sistemskoj i hroničnoj, nervnoj formi. U ovom radu, utvrdivi smo prisustvo antitela protiv virusa štenečaka kod crvenih lisica u Zapadnoj Srbiji, i uzorke mozga serološki pozitivnih jedinki analizirali histopatološki i imunohistohemijski na antigen nuklearnog proteina