Occurrence of the Distemper Canine: Ultrastructural and Histopathological Aspects

Catroxo, M.H.B.[1*]; Martins, A.M.C.R.P.F.[2]; Pedroso, M.F.[3]

Electron Microscopy Laboratory, Research and Development Center in Animal Health, Biological Institute, São Paulo, SP, Brazil

Biological Institute, Avenida Conselheiro Rodrigues Alves, 1252, Vila Mariana, São Paulo, SP, Brazil

*Corresponding Author

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***Abstract*—** *Canine distemper caused by the paramyxovirus of the genus Morbilivirus is a highly contagious disease that mainly affects dogs of all ages, with an important socioeconomic impact on the Brazilian veterinary field due to the high cost of treatment, failures in vaccine immunization and the suffering of animals and owners. It presents itself in 2 forms, overacute, characterized by abrupt fever and sudden death and, acute, when the animals show signs of fever, prostration, lack of appetite, nasal and ocular secretions, conjunctivitis, vomiting, respiratory distress, anorexia, diarrhea, dehydration, and cutaneous rash, which may follow neurological symptoms such as paralysis, convulsions and death. From 2004 to 2015, approximately 622 samples from dogs with suspected distemper were sent to the Electron Microscopy Laboratory of the Instituto Biológico, São Paulo, SP, Brazil (oral and nasal swabs, feces, urine and organ fragments). for diagnosis of viral agents. The samples were processed using transmission electron microscopy (negative staining and resin embedding) and routine histopathological techniques. In the transmission electron microscope, 254 samples (40.83%) were visualized, pleomorphic, rounded or elongated, enveloped paramyxovirus particles containing helical herring-bone like nucleocapsid, measuring between 100 and 500 nm in diameter. In ultrathin sections of the brain, the presence of a nucleus with marginalized chromatin containing intranuclear inclusions was visualized. Intracytoplasmic granular amorphous inclusions, formed by viral nucleocapsids were also observed. Complete particles measuring 100 to 250 nm in diameter and incomplete particles, measuring on average 70 nm in diameter, budding from the plasma membrane could also be identified. Large areas of demyelination were also observed. Histological sections of the brain showed perivascular and focal mono and polyclonal encephalitis, monoclonal meningitis, congested blood vessels and parenchyma with areas of demyelination. The presence of eosinophilic inclusion bodies was also observed in several nerve cells (Corpuscles of Lentz).*

***Keywords*— Canine distemper, dogs, Transmission Electron Microscopy, Histopathology.**

I. INTRODUCTION

Canine distemper is a highly contagious disease that mainly affects dogs of all ages. The disease is caused by a paramyxovirus belonging to the *Paramyxoviridae* family of the genus *Morbilivirus*. This genus includes, in addition to canine distemper virus (CDV), rinderspest virus (RPV), peste des petits ruminants virus (PPRV) and measles virus (MV) (Grifin, 2007). Paramyxoviruses (PMVs) are enveloped particles, with nucleocapside helicoidal and negative-sense, single-stranded RNA viruses with genes coding for at least six major proteins, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion glycoprotein (F), receptor binding protein (RBP, formerly designated variously as HN, H, or G), and the large protein (L) that possesses RNA-dependent RNA polymerase (RdRp) activity (Rima et al., 2019). CDV has a tendency to cross the species barrier by infecting species other than *Canidae*, such as *Mustelidea*, *Procyonidae*, *Ursidae* and *Viverridae* (Greene et al., 2012).

Phylogenetic studies suggest that paramyxoviruses related to human mumps virus (MuV, *Rubulavirus*) and canine distemper virus (CDV, *Morbillivirus*) in host bats may have the potential to create spillover events and cause disease in other mammalian species (Drexler et al., 2012).

The disease has a high socioeconomic impact in the Brazilian veterinary field due to the high cost of treatment, failures in vaccine immunization and the suffering of animals and guardians (Headley et al., 2012). In the wild, it is considered an emerging disease, since wild canids are vulnerable (Jucá et al., 2022), such as lions, tigers, leopards (Appel et al., 1994) and monkeys (Sun et al., 2010). The disease has also been described in aquatic mammals and cetaceans such as dolphins (Stone et al., 2011). In the United States, the occurrence of contamination from domestic dogs to raccoons indicates that they can act as intermediate hosts (Kapil et al., 2008). In other countries such as Italy, distemper represents an immediate problem due to the illegal trade in puppies from east Europe (Dall'ara, 2020; Mira et al., 2018).

The disease presents in 2 forms, overacute, characterized by fever and sudden death, and acute, when the animals show signs of fever, prostration, lack of appetite, nasal and ocular secretions, conjunctivitis, vomiting, respiratory distress, anorexia, diarrhea, dehydration, and cutaneous rash, in addition to neurological symptoms such as paralysis, seizures and death (Oliveira et al., 2008).

Histologically, canine distemper virus produces necrosis of lymphatic tissues, interstitial pneumonia, and cytoplasmic and intranuclear inclusion bodies in respiratory, urinary, and gastrointestinal epithelium, neuronal degeneration, encephalitis, meningitis and intranuclear inclusion bodies (Origgi et al., 2012). The duration and severity of the disease depend on factors such as the animal immune status, strain virulence, affected organs, among others. The course can be short (10 days) or go on for weeks or months. The mortality rate ranges from 30 to 80% and surviving animals may recover normally or have permanent central nervous system sequelae or late complications such as demyelinating encephalitis (old dog encephalitis), ascending paresis, paralysis, convulsions and hyperkeratosis of the paws, which can lead to the death of animals. Young dogs are more susceptible, but everyone regardless of age can be affected. Dogs that recover from acute disease with persistent infection may shed virus in urine and through the skin on the foot pads. Transmission occurs through aerosols or contaminated food and objects (Beineke et al., 2009; Martella et al., 2008; Kapil & Yeary, 2011).

This work aimed to investigate the presence of paramyxovirus in samples of feces, urine, ocular and nasal swabs and organ fragment from dogs, using transmission electron microscopy and histopathology techniques.

II. MATERIAL AND METHODS

2.1 Clinical cases

During the period from 2004 to 2015 samples of feces, urine, ocular and nasal swabs and organ fragments from 622 dogs, from the State of São Paulo, SP, Minas Gerais, MG, Espírito Santo, ES and Rio de Janeiro, RJ, Brazil, were sent to the Electron Microscopy laboratory of the Biological Institute of São Paulo, SP, Brazil for research of viral agents. The dogs were of different breeds, of both sexes and aged between 05 days and 17 years. The symptoms and clinical signs most commonly presented by the animals were characterized by apathy, lack of appetite, progressive weight loss, nausea, prostration, fever, vomiting, severe abdominal pain, gastritis, gastroenteritis, diarrhea with blackened stools, pneumonia, anemia, leukopenia, neutrophilia, thrombocytopenia, corticosteroid blindness, paresis of hind and lower limbs, cerebellar ataxia, staggering gait, pedaling movements, convulsions, mental confusion, muscle spasms or myoclonus, nystagmus, tremors, salivation, loss of consciousness, tetany, liver with bleeding areas, alopecic wounds, hyperkeratosis of the paws, nutritional and vitamin deficiency, infestation by ecto and endoparasites (isospora, giardia, hookworm and enterobius) and death.

Samples were processed for transmission electron microscopy by negative staining (rapid preparation) and resin embedding techniques and for histopathology by hematoxylin and eosin (H&E) technique.

2.2 Transmission Electron Microscopy

2.2.1 Negative staining technique (rapid preparation)

In this technique, stool, urine, swabs nasal e ocular and cérebro fragments samples were suspended in phosphate buffer 0.1 M, pH 7.0. Drops of the obtained suspensions were placed in contact with metallic copper grids with carbon stabilized supporting film of 0.5% collodion in amyl acetate. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate, pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

2.2.2 Resin embedding technique

Brain fragments were fixed in 2.5% glutaraldehyde in 0.1 M, pH7.0 phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in cetonic series, the fragments were embedded in Spurr resin (González- Santander 1969; Luft, 1961). Ultrathin sections were cut on the LKB ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson, 1958; Reynolds, 1963).

All grids submitted to the above reactions were observed in a Philips EM 208 electron microscope, at 80 kV.

2.3 Histopathology

2.3.1 Routine histological technique

All brain fragments were fixed in 10% buffered formalin, dehydrated, diaphanized and embedded in paraffin. 5 mm thick sections were performed and stained with hematoxylin and eosin technique.

III. RESULTS AND DISCUSSION

3.1 Clinical cases

Of the 622 samples of feces, urine, nasal and ocular swabs and brain fragments from dogs, 254 (40.83%) were positive for paramyxovirus by the negative staining technique (rapid preparation). The age of the animals ranged from 05 days to 17 years, and the percentage of positive animals up to 11 months was higher (46.8%) than in dogs aged over 1 year (28%). With reference to sex, 114 (44.88%) were positive females and 108 (42.5%) were males. In 32 samples, the sex of the animals was not identified. About 14 samples (5.51%) were co-infected with coronavirus and 11 samples (4.33%) with parvovirus. Three samples had the association of paramyxovirus, parvovirus and isospora and 3 had paramyxovirus and ehrlichiosis.

3.2 Transmission Electron Microscopy

3.2.1 Negative staining (rapid preparation) technique

During examination under the transmission electron microscope using the negative staining technique, 254 samples of feces, urine, ocular and nasal swabs and brain fragments were observed to contain paramyxovirus particles, pleomorphic, spherical (**fig. 1, big arrow**) or filamentous (**fig. 1, minor arrow**), ranging in diameter from 100-500 nm, with a outer envelope containing fine surface projections or spikes (**fig. 2, big arrow**) approximately 9 nm long. A helical nucleocapsid **with a characteristic " herring-bone" appearance** (**fig. 2 minor arrow**) com 15 nm of diameter, was also observed.

3.2.2 Resin embedding technique

The ultrathin sections of brain fragments revealed the presence of nuclei with marginalized and densely packed chromatin (**fig. 3, big arrow**), containing intranuclear inclusions (**fig. 3, minor arrow**). Intracytoplasmic granular amorphous inclusions surrounded by membrane (**figs. 4,5 (big arrow**), formed by viral nucleocapsids were also observed. Complete particles measuring 100 to 250 nm in diameter (**fig. 6, big arrow**) and incomplete particles, measuring on average 70 nm in diameter, budding from the plasma membrane could also be identified (**fig. 6, minor arrow**). Large areas of demyelination were also observed **(fig.7, arrow**).

3.3 Histopathology

3.3.1 Routine histological technique (H&E).

Under a direct light microscope in routine histopathological examination of the brain, a very marked perivascular and focal mono and polyclonal encephalitis was observed, as well a monoclonal meningitis (fig. 8, 100x). The blood vessels were congested and the parenchyma showed areas of demyelination (Fig 9, 40x). There was the presence of eosinophilic inclusion bodies in several nerve cells (corpuscles of Lentz) (fig. 10, arrow, 100x).

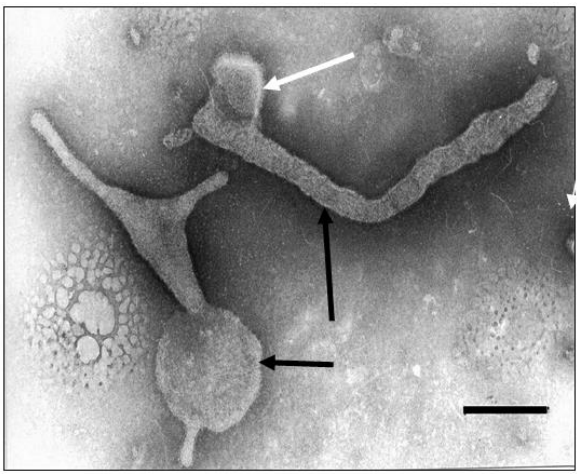

Figure 1: Negative staining of paramyxovirus in dog feces suspension, exhibiting pleomorphic and filamentous (big arrow) and rounded particles (minor arrow) and an envelope with club-shaped spikes (white arrow). Bar: 100 nm.

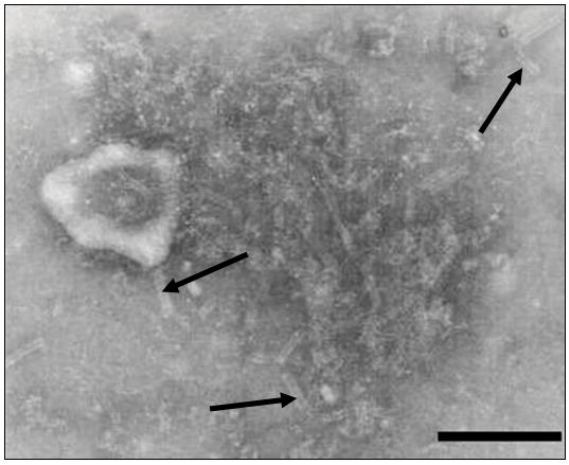

Figure 2: Negative staining of paramyxovirus particles in dog lung suspension, showing herringbone-like nucleocapsid (arrow). Bar: 180 nm.

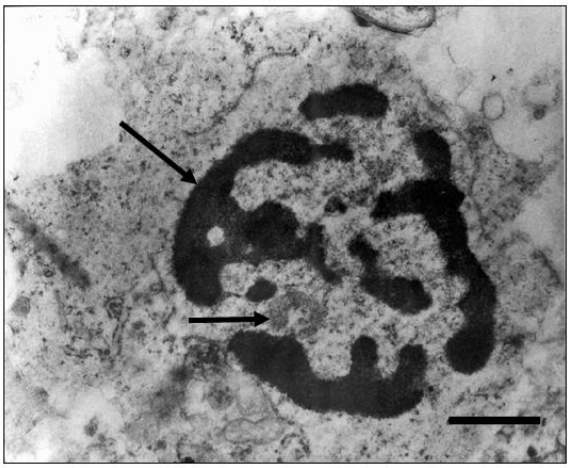

Figure 3: Ultrathin section of the brain showing a nucleus with marginalized and densely packed chromatin (big arrow) and intranuclear inclusion (minor arrow). Bar: 1600 nm.

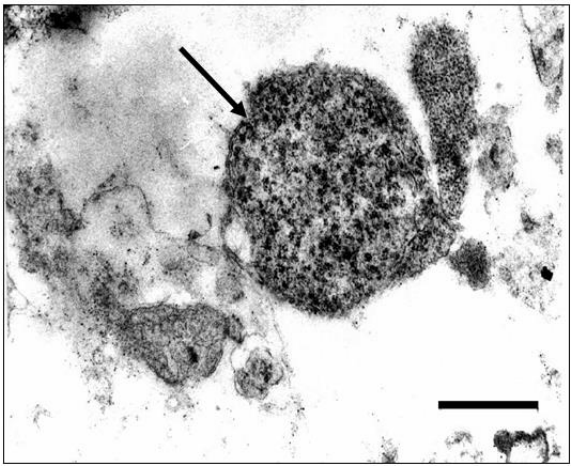

Figure 4: Ultrathin section of the brain showing Intracytoplasmic granular amorphous inclusions, formed by viral nucleocapsids (arrow). Bar: 900 nm.

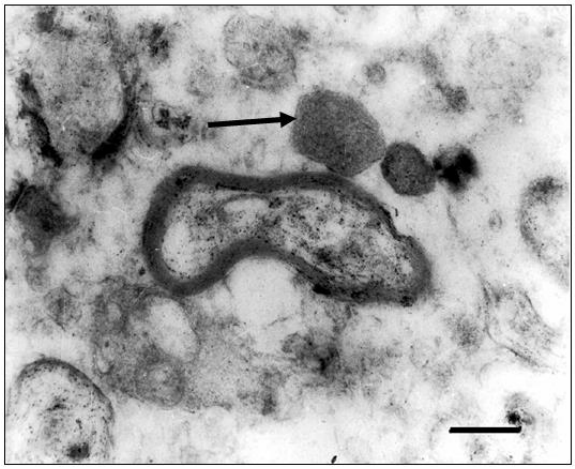

Figure 5: Ultrathin section of the brain, exhibing intracytoplasmic inclusions fomed by viral nucleocapsids (arrow). Bar: 500 nm

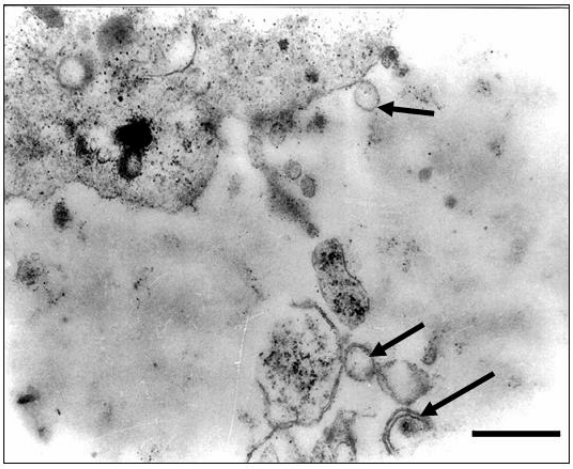

Figure 6: Ultrathin section of the brain evidencing complete particles (big arrow) and incomplete particles, budding from the plasma membrane (minor arrow). Bar: 400 nm.

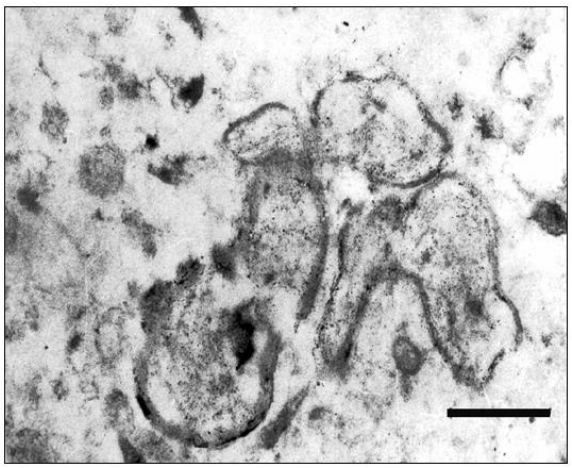

Figure 7: Ultrathin section of the brain, where large areas of demyelination are observed. Bar: 1000 nm.

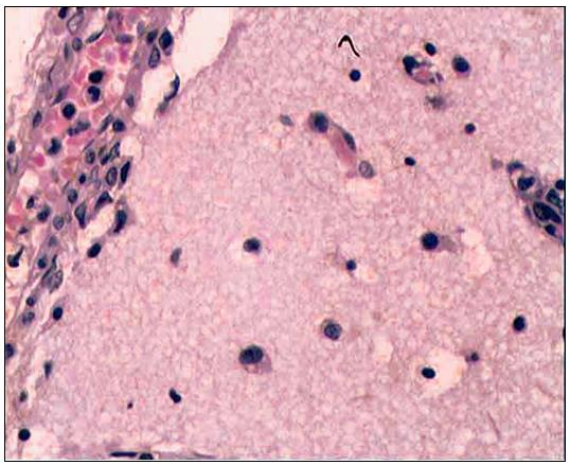

Figure 8: Histological section of the brain, where a very marked perivascular and focal mono and polyclonal encephalitis is visualized, as well a monoclonal meningitis (100x).

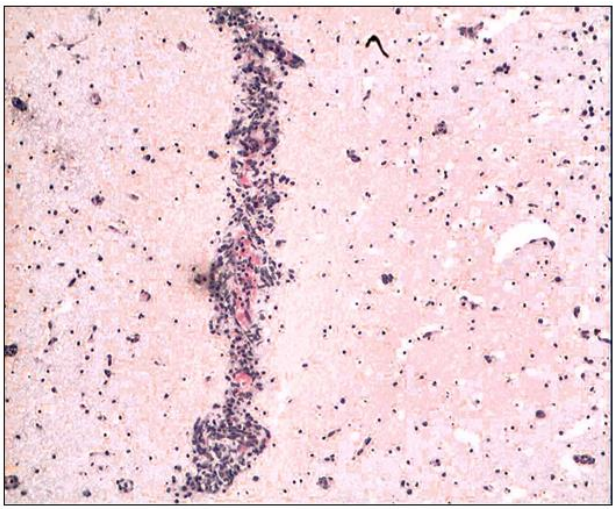

Figure 9: Histological section of the brain, showing congested blood vessels and parenchyma with areas of demyelination (40x)

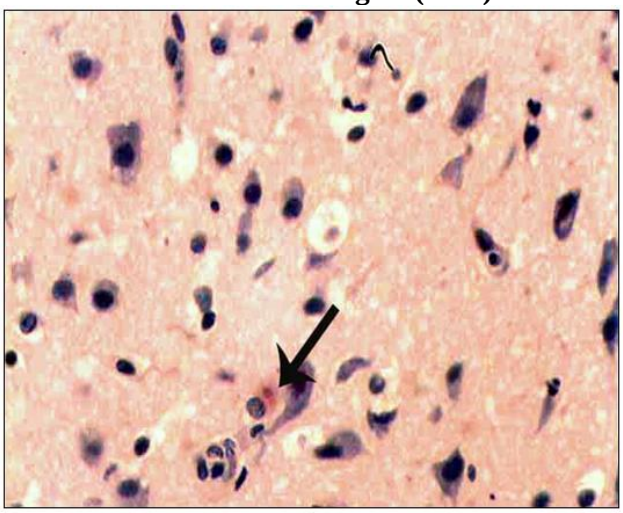

Figure 10: Histological section of the brain, showing the presence of eosinophilic inclusion bodies in several nerve cells (corpuscles of Lentz) (100x)

Transmission electron microscopy using the negative staining technique (rapid preparation) has been used for viral diagnosis, where the particles are visualized directly in the sample (Goldsmith & Miller, 2009). In this study, a total of 622 dog samples were sent to the Electron Microscopy Laboratory of the Biological Institute of São Paulo, SP, Brazil, for research of viral agents. Of these, 254 samples (40.83%) were positive for canine distemper using the negative staining technique (rapid preparation). Using this same technique associated with others, Schumaker et al. (2012) found 100% positivity during an outbreak of canine distemper that occurred in the USA and Li et al. (2013) reported about 96% in China. In other studies, on canine distemper, positivity showed to be quite oscillating, varying between 4.04 and 7.44 (Sarchahi et al., 2022) and 85% in Iran (Mousafarkhani et al., 2023); 6.9 and 29.62% in Iraq (Mohammad et al., 2022); 11.3% in the Himalayan (Dorji et al., 2020); 18% in India (Pranitha et al., 2022) and 60.9% in Portugal (São João et al., 2021). In Italy, Alfano et al. (2022) reported 18.4% in imported dogs, 74% in stray dogs and 3.9% in domestic dogs. In Brazil, positivity also varied between 2.63% (Santos et al., 2020); 34% (Costa et al., 2019) and 68.75% (Jesus et al., 2021). Menezes et al. (2023) described 82.61% mortality rate. Other authors, however, detected the presence of the virus in asymptomatic dogs (Del Puerto et al., 2010) and in healthy dogs (Mohammad et al., 2022).

Some of the different symptoms and clinical signs commonly presented by the animals in our study were also mentioned in other studies on distemper in dogs (Amude et al., 2006; Gowtage-Sequeira et al., 2009; Del Puerto et al., 2010; Schumaker et al., 2012; Li et al., 2013; Santos et al., 2020; Jesus et al., 2021; Johnson et al., 2021; São João et al., 2021; Silva et al., 2022; Menezes et al., 2023 and Mousafarkhani et al., 2023). Other symptoms have also been referenced, such as paralysis of the

mandible, urinary bladder and rectum (Catroxo et al., 2003), enamel hypoplasia, aggressiveness, vocalization, restlessness and palmigrade postures (Menezes et al., 2023), renal abnormalities in terminal patients (Silva et al., 2022) and metaphyseal sclerosis secondary to canine distemper virus (Johnson et al., 2022).

With regard to age, the dogs in our research ranged from 05 days to 17 years, and the percentage of animals infected up to 11 months was higher (46.8%) than in dogs aged over 1 year (28.0 %). Corroborating our data, other authors reported that 70% of the animals in their study were younger than 12 months (Mousafarkhani et al., 2022), higher positivity in puppies 8-14 weeks old (Schumaker et al., 2012), with less than 6 months (Braund, 1994) or that most were puppies (Alfano et al., 2022). Contrary to other studies, a higher incidence of the disease has been reported in animals aged over 12 months (19.04%) (Pranitha et al., 2022), over 3 years (22.73%) (Mohammad et al., 2022), between 1 and 8 years (Jesus et al., 2021) and 1 and 6 years (41.30%) (Menezes et al., 2023). Headley & Graça (2000) stated that the age of the animals most affected by CDV they studied was from 0 to 1.5 year and Silva et al., (2022) from 4 months to 2 years.

With regard to gender, we found 44.88% positivity in females and 42.51% in males, with no significant differences between them, which was also confirmed by Dorji et al. (2020), São João et al. (2021) and Menezes et al. (2023). Other studies, however, reported a higher percentage in infected female dogs (25.23%) (Mohammad et al., 2022) and 71.42% and 20% in males, respectively (Jesus et al., 2021; Pranitha et al., 2022). Mousafarkhani et al. (2022) mentioned that the female dogs in their study were more susceptible than male dogs, but the mortality rate from male dogs was higher than that of female dogs.

Fourteen animals in our study (5.51%) were coinfected with coronavirus and 11 (4.33%) with parvovirus. Zhao et al. (2016) reported a 1.11% rate of co-infection with coronavirus and 4.7% with parvovirus in dogs in their study, while Deng et al. (2023) reported 26.7% coinfection with coronavirus and Headley et al. (2018) 100% with parvovirus. Stilwell et al. (2019) reported dual infection with emergent strain of canine distemper virus and canine parvovirus in an Arctic wolf (*Canis lupus arctos*). Three animals were co-infected with *Erlichia canis*, a fact also reported in a young female during research by Santos et al. (2020). Considering that the canine distemper virus causes immunosuppression, concomitant infections may arise, as is the case with *Erlichia canis*, hindering the organism response to other parasites, which leads to aggravation of the clinical condition (Schneider et al., 2017).

Likewise, three other dogs had the association of paramyxovirus, parvovirus and isospora. Isosporosis has been associated with diarrhea, infecting young dogs subjected to stress factors, which leads to immunocompetence of the immune system (Rodrigues and Menezes, 2003) and predisposing animals to viral diseases that have tropism for the intestinal and respiratory epithelium and nervous system, as is the case with distemper virus, parvovirus and coronavirus (Silva et al., 2007). These multiple viral infections occur frequently and contribute to sudden death in puppies. Canine infectious morbillivirus continues to be one of the most important disease agents of puppies and due to its immunosuppressive effects can facilitate the development of other infectious disease pathogens (Headley et al., 2018).

Paramyxovirus particles, pleomorphic, rounded or elongated, measuring 100 to 500 nm in diameter, containing an envelope covered by spikes and helical herring-bone-like nucleocapsid, were visualized in all 254 positive samples from dogs, using the negative staining technique, under transmission electron microscope. These morphological aspects were also described by other authors in other research on distemper in dogs (Schulz et al., 2008; Woma et al., 2009; Tan et al., 2011; Schumaker et al., 2012; Li et al., 2013; Yang et al., 2020), in ferrets (Williams et al., 1988; Catroxo et al., 2010), in rhesus monkeys (Sun et al., 2009), and in a raccoon dog (Cheng et al., 2015). In ultrathin sections of brain fragments, intracytoplasmic inclusions formed by viral nucleocapsids were visualized, also observed in other investigations of canine distemper, in heart muscle cells (Higgins et al., 1981), in neutrophil cells (Mc Laughlin et al., 1985) , in adipose stem cell (Altamirano-Samaniezo et al., 2022), within the bronchiolar epithelial cells of the lions, tigers, and leopards (Appel et al., 1994), and, in pulmonary syncytial cells in dolphin (*Tursiops truncatus*) (Stone et al., 2011). Ultrathin sections revealed the frequency of intranuclear inclusions, as also reported by Chludzinski et al. (2023) and by Stone et al. (2011). The appearance of the nucleus with marginalized and densely packed chromatin that we visualized was observed by Altamiro-Samaniezo et al. (2022). The presence of complete particles measuring 100 to 250 nm in diameter and incomplete particles, measuring on average 70 nm in diameter, budding from the plasma membrane that we identified was not visualized in cardiac muscle cells from dogs infected with distemper, in a study carried out by Higgins et al. (1981). These ultrastructural aspects, however, were reported in another study of avian paramyxoviruses (Catroxo et al., 2023). According to Goldsmith & Miller (2009) paramyxoviruses can obtain their outer membrane by budding into cytoplasmic vesicles or out of the plasma membrane.

In the histopathological examination of the brain, a very marked perivascular and focal mono and polyclonal encephalitis was observed, as well a monoclonal meningitis, which were also visualized in other histopathological studies of distemper in dogs

(Headley & Graça, 2000; Johnson et al., 2022; Higgins et al., 1981), in lions, tigers and leopards (Appel et al., 1994); in jackals (Goutage-Sequeira et al., 2009); in dolphin (Stone et al., 2011), and in ferret (Williams et al., 1988). The presence of eosinophilic inclusion bodies (Lentz bodies), which we found in several nerve cells, was also reported by other authors, in dogs (Headley & Graça, 2000); in arctic wolf (Stilwell et al., 2019); in lions, tigers and leopards (Appel et al., 1994); in jackls (Goutage-Sequeira et al., 2009); in dolphin (Stone et al., 2011) and in ferret (Williams et al., 1988), being considered pathognomonic in the diagnosis of canine distemper (Thrall, 2015). Other authors found these Lentz inclusions in blood cells (Leal et al., 2011; Schneider et al., 2017; Castillo et al., 2019); skin (Areco et al., 2022), corneal epithelium (Headley et al., 2018), in osteoclasts (Johnson et al., 2022), and, in bronchial epithelial cells (Tamukai et al., 2020).

The techniques used were useful for rapid diagnosis, since they detected the viral presence directly in the samples, which allowed the immediate initiation of prophylactic measures and disease control when necessary. The different types of biological samples, nasal and oral swabs, urine, feces and organ fragments were adequate for the visualization of paramyxoviruses.

There is no specific treatment for canine distemper, but supportive therapies (fluid therapy, antibiotic therapy, use of vitamins, immunostimulants, anticonvulsants, antiemetics and analgesics) and complementary therapies (acupuncture, physiotherapy, hydrotherapy and application of stem cells) have been used to the decrease in symptoms (Dornelles et al., 2015; Greene & Vandevelde, 2015). Considering that CDV constitutes a danger both for animals in kennels, as well as for those belonging to private owners and wild carnivores, adequate vaccination prevents the spread and transmission of the disease, in addition to contributing to the preservation of wild species, avoiding events of spillover (Headley & Graça, 2000; Kapil & Yeary, 2011).

IV. CONCLUSIONS

Considering that canine distemper is a highly contagious disease that mainly affects dogs of all ages, causing a high socioeconomic impact in the Brazilian veterinary, in addition to being classified as an emerging disease also affecting vulnerable wild canids, the application of transmission electron microscopy and histopathology techniques in routine or during outbreaks of the disease may help to develop measures for prevention and control of canine distemper, in addition to assisting in the preservation of wild carnivorous species.

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