Detection of Coronavirus (CCoV) in Dogs by Transmission Electron Microscopy Techniques

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*—** *Coronaviruses are known for their ability to cause gastrointestinal, respiratory, and central nervous system diseases in various species of avian, mammalian, and human hosts. In dogs, it is one of the most important viral agents causing gastroenteritis. Canine coronavirus is an emerging infectious disease affecting animals of all ages. Canine Enteric Coronavirus (CCoV) belongs to the Coronaviridae family and the Nidovirales order, exerting a significant impact on veterinary activities in kennels and animal shelters due to the rapid spread of the virus, causing economic losses due to mortality and/or morbidity, especially in commercial production kennels. Pathogenic variants can cause severe disease, characterized by loss of appetite, vomiting, profuse bloody and watery diarrhea with a putrid odor, abundant serosanguineous fluid in the abdominal cavity, accompanied by fever, anorexia, vomiting, prostration, severe dehydration, and death in young animals. From 1994 to 2016, 643 samples of feces, rectal swabs, and organ fragments from dogs were submitted for viral agent research. The samples were processed for transmission electron microscopy by negative staining and immunoelectron microscopy techniques. Transmission electron microscopy examination by negative staining technique revealed the presence of pleomorphic, rounded, or spherical enveloped coronavirus particles containing typical radial projections in the form of a solar crown, with an average diameter of 140 nm in 287 (44.63%) out of 643 samples. In the immunoelectron microscopy technique, the antigen-antibody interaction was characterized by the aggregation of viral particles in 287 (44.63%) of the fecal samples, fecal swabs, intestinal mucosa, and intestinal fragments analyzed.*

***Keywords*— Coronavirus, Dogs, Gastroenteritis, Transmission Electron Microscopy.**

I. INTRODUCTION

Coronaviruses are known for their ability to cause gastrointestinal, respiratory, and central nervous system diseases in various species of avian, mammalian, and human hosts (Sun et al., 2020). Through an intermediate species, coronaviruses acquire zoonotic potential that allows them to move from a reservoir species to other species, including humans (Cui et al., 2019). This zoonotic ability is evidenced by their genetic plasticity that promotes a high frequency of genetic changes (mutation and recombination) (Decaro & Lorusso, 2020), which also influence tissue tropism and pathogenicity (Vijaykrishna et al., 2007). In dogs, CCoV is one of the most important viral agents causing gastroenteritis. Canine coronavirus is an emerging infectious disease affecting animals of all ages (Kong et al., 2007). Occurring worldwide, it has been detected in various countries, such as the United Kingdom (Radford et al., 2021), Japan (Takano et al., 2015), China (Tian et al., 2021), Turkey (Timurkan et al., 2021), Italy (Zobba et al., 2021), USA (Licitra et al., 2014), and Spain (Decaro et al., 2006). In Brazil, the first outbreak was detected in 1989 by Mitika et al., with viral particles identification by transmission electron microscopy at the Biological Institute of São Paulo, SP, Brazil. Subsequently, other cases were reported (Dezengrini et al., 2007; Castro et al., 2010; Guirao et al., 2013).

Canine Enteric Coronavirus (CCoV) belongs to the *Alphacoronavirus* genus, *Coronaviridae* family, and *Nidovirales* order. They are pleomorphic viruses, spherical to elongated, with a characteristic envelope containing surface projections from the viral membrane in the form of a club, solar crown, or petal, measuring 75-160 nm in diameter (Belouzard et al., 2012; Li, 2016). The CCoV genome is single-stranded RNA and measures from 27.6 to 31 kilobases in length. It has four fundamental proteins in its envelope essential for infection. The S glycoprotein is responsible for the entry of infectious virion particles into the target cell through interaction with the host cell receptors, as well as providing the virion with a crown-like appearance. By binding to the nucleocapsid, the M protein acts in viral assembly organization, and the E protein operates in pathogenesis, assembly, and virus release. The N protein facilitates the interaction of the M protein during virion assembly, promoting increased viral transcription efficiency (Dhama et al., 2020). CCoV has 3 subtypes, types I and II included in the *Alphacoronavirus* genus, and type III (CRCoV - canine respiratory coronavirus), in the *Betacoronavirus* genus. Type II can be divided into subtypes IIa and recombinant IIb. CCoV types I and II cause mild asymptomatic enteritis or self-limiting, but pathogenic variants of pantropic II-a CCoV can cause severe disease (Decaro et al., 2012), characterized by loss of appetite, vomiting, profuse bloody and watery diarrhea with a putrid odor, abundant serosanguineous fluid in the abdominal cavity, accompanied by fever, anorexia, vomiting, prostration, and severe dehydration (Hagiwara et al., 1989; Catroxo et al., 1998, Buonavoglia et al., 2006; Zapulli et al., 2008). Mortality is low but can occur, especially in co-infected puppies with parvovirus, canine distemper virus, Ehrlichia canis, Isospora, or other intestinal pathogens (Pratelli et al., 2008; Catroxo et al., 2023). Transmission occurs via the oral-fecal route, through ingestion of contaminated food or water and by direct contact with an infected animal. The incubation period is 1 to 4 days, and the duration of the disease is 2 to 10 days in most dogs, with cases where symptoms intermittency is observed, with periods of improvement and apparent cure, followed by diarrheal episodes. The viruses reach the duodenum within 48 hours, reach the small intestine, penetrate enterocytes, and begin their replication in the cytoplasm of villous epithelial cells, causing their atrophy. Dogs can be carriers and shed the virus in feces for up to 6 months post-infection (Mitika et al., 1989; Licitra et al., 2014).

Canine coronavirus (CCoV) has a significant impact on veterinary activities in kennels and animal shelters due to the rapid spread of the virus, mainly contaminating young animals aged 2 to 5 months (Decaro & Buonovoglia, 2008), causing economic losses due to mortality and/or morbidity, especially in commercial production kennels (Guirao et al., 2013).

This study aimed to detect the presence of coronavirus particles in feces, rectal swabs, and organ fragments from dogs using transmission electron microscopy techniques.

II. MATERIAL AND METHOD

2.1 Clinical cases:

During routine clinical research carried out at the Biological Institute of São Paulo, SP, Brazil, from October 1994 to July 2016, 643 samples of feces, fecal swabs and fragments of organs from dogs were sent for investigation of viral agents. The animals, of different breeds, aged between 23 days and 17 years, of both sexes, came from São Paulo, SP, Brazil. The dogs presented clinical symptoms and signs of apathy, weight loss, liquid and yellowish or bloody diarrhea, gastritis, prostration, anorexia, vomiting, dipsia, nausea, gastroenteritis, hemorrhagic gastritis, colitis, hematochezia, hypoglycemia, lymphopenia, bronchial pneumonia, pulmonary edema, cough, cardiac dilation, leukopenia, fever, nasal secretion, increased ALT and AST and hypovolemic shock.

2.1.1 Outbreak description:

In 1998, an outbreak of gastroenteritis occurred in a kennel belonging to the Military Police of the State of São Paulo, SP, Brazil, of German Shepherd breed, with black fur. Of the 130 animals in the kennel, 40 were affected by a sudden incidence of profuse, bloody and watery diarrhea, with a putrid odor, accompanied by fever, anorexia, vomiting, prostration and severe dehydration. Most of the affected animals were mainly puppies aged 6 to 8 months and the rest of the animals were adults aged between 1 and 5 years, of both sexes. All dogs had been immunized with V8 and anti-rabies vaccine, according to the vaccination schedule (first dose between 6-8 months and annual booster). Adult animals maintained contact with fomites and other animals during routine work around the city. Parasitological and bacteriological examinations of the feces were negative.

All samples from routine clinical cases and the occurrence of the outbreak were processed using the negative staining technique (rapid preparation) for transmission electron microscopy.

2.2 Transmission Electron Microscopy:

2.2.1 Negative staining technique (rapid preparation):

In the negative staining technique, feces, fecal swabs and intestine fragments were suspended in 0.1M phosphate buffer at pH 7.0. Drops of the viral suspension were placed in contact with copper screens previously covered with collodion film and metallized by carbon. After 10 minutes, the screens were removed from the viral suspension, contrasted with drops of 2% ammonium molybdate and subsequently dried on filter paper (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997). Observations were made using a Philips EM 208 transmission electron microscope under a voltage of 80 kV.

Samples positive for coronavirus particles using the negative staining technique were processed using the immunoelectron microscopy technique.

2.2.2 Immunoelectron microscopy technique.

In this technique, copper grids, previously prepared with collodion film and stabilized with carbon were first incubated with protein A (1ml/ml) placed in contact with the virus-specific antibody. After, grids were washed in PBS drops, incubated with the viral suspension of the 287 positive samples, washed with drops of water and negatively stained with 2% ammonium molybdate, pH 5.0 (Berthiaume et al., 1981; Katz & Kohn, 1984; Doane & Anderson, 1987; Hayat & Miller, 1990).

III. RESULTS AND DISCUSSION

3.1 Clinical cases

A total of 643 samples of feces, fecal swabs, and intestinal fragments were processed using the negative staining technique and examined under a transmission electron microscope. Of these, 287 (44.63%) were positive for coronavirus. Of the 287 CCoV-positive dogs, 107 (37.63%) were female, and 108 (37.63%) were male. In 72 (25.08%) samples, the gender of the animals was not identified. Regarding the age of the animals, it ranged from 23 days to 17 years, with 71 (24.73%) samples from younger animals aged less than 11 months and 65 (22.64%) from animals aged over 11 months, with no significant difference between these parameters. Concerning co-infection, 52 samples (18.11%) were mixed with other agents. A total of 20 samples (38.46%) were co-infected with paramyxovirus, 21 (40.3%) with parvovirus, 3 (5.76%) with parvovirus and mycoplasma, 4 (7.69%) with mycoplasma, 1 (1.92%) with herpesvirus, 1 (1.92%) with parvovirus and paramyxovirus, and 2 samples (3.84%) with Ehrlichia. Approximately 11 (3.83%) dogs died, with 7 animals infected solely with coronavirus, 2 having dual infection with coronavirus and parvovirus, and 2 presenting triple infection with coronavirus, parvovirus, and mycoplasma.

3.1.1 Outbreak

Animals from the outbreak received treatment with fluid replacement and antibiotic therapy, and complete remission of symptoms was observed in all animals after 10-15 days.

3.2 Transmission Electron Microscopy

3.2.1 Negative staining (rapid preparation) technique

In all 287 positive samples examined under the transmission electron microscope, the presence of coronavirus particles was observed, which were rounded, or spherical (Figs. 1, 2, arrow), pleomorphic (Fig. 3, arrow), enveloped, containing typical radial projections in the form of a solar crown, with an average diameter of 140 nm (Figs. 1,2, arrow). The spikes of the envelope measured between 16 and 20 nm in length (Fig.2, big arrow). An internal spherical core, measuring 60 to 85 nm in diameter, was visualized in some particles (Fig.3, big arrow).

3.2.2 Immunoelectron microscopy technique.

In 287 (44.63%) out of 643 fecal samples, fecal swabs, and intestinal fragments analyzed, the antigen-antibody interaction was characterized by aggregation of viral particles (Fig. 4, arrow).

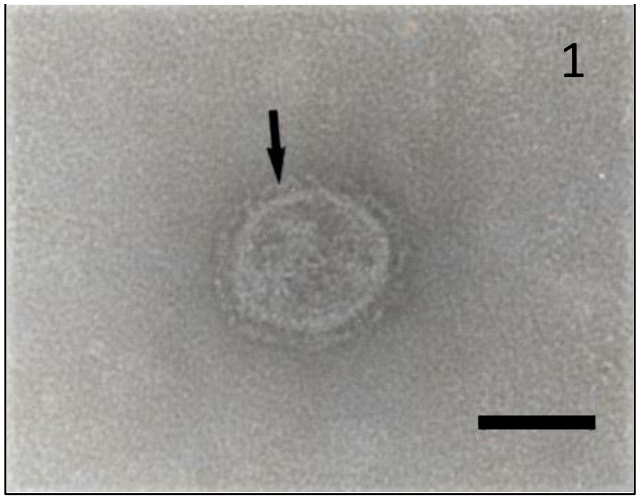

FIGURE 1: Negative staining of spherical coronavirus particle in dog feces suspension, showing envelope in the shape of a solar crown or petal (arrow). Bar: 80 nm.

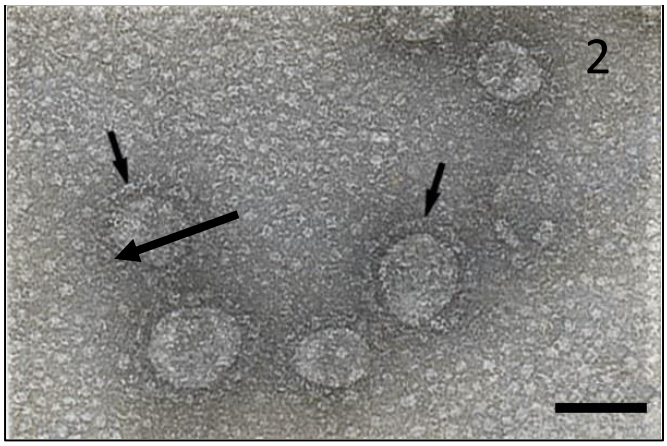

FIGURE 2: Negative staining of a group of spherical coronavirus particles, showing a solar corona-shaped envelope (minor arrow) and spikes of the envelope measured between 16 and 20 nm in length (big arrow). Bar: 100 nm.

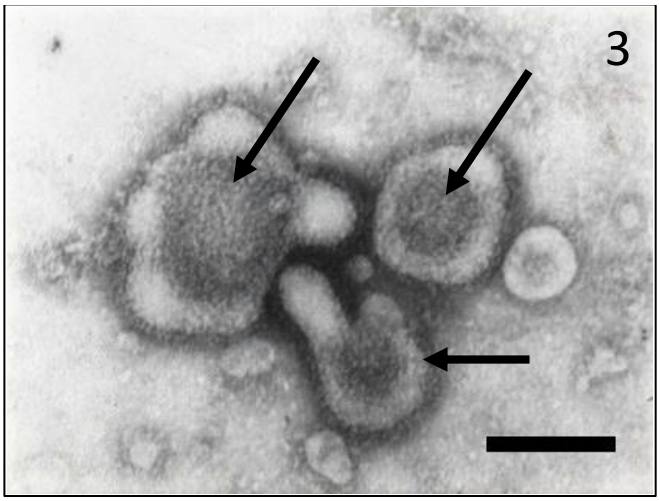

FIGURE 3: Negative staining of pleomorphic coronavirus particles (minor arrow), displaying an internal spherical core, measuring 60 to 85 nm in diameter (big arrow). Bar: 80 nm.

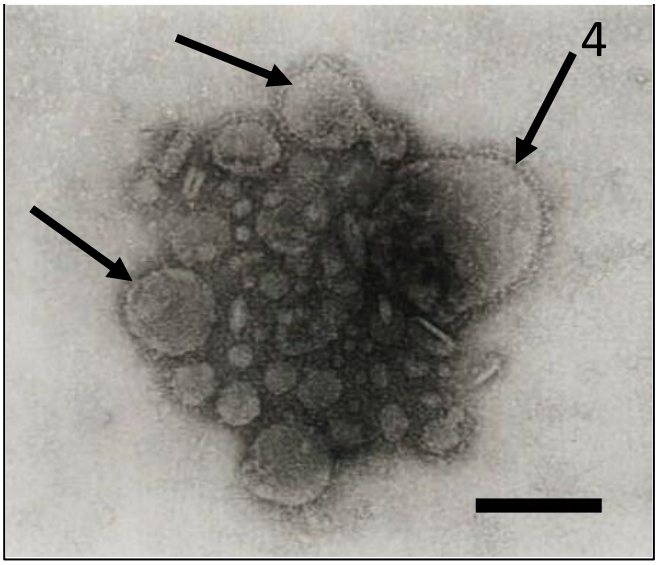

FIGURE 4: In the immunoelectron microscopy technique the coronavirus particles were aggregated by antigen-antibody interaction (arrows). Bar: 100 nm.

Canine coronavirus is an important disease that affects dogs in kennels, shelters or companions, causing everything from mild diarrhea to severe gastroenteritis (Zapulli et al., 2020). In this study, transmission electron microscopy techniques were applied to detect coronavirus particles in stool samples, fecal swabs and fragments of small intestine from clinical cases of dogs with diarrhea, sent to the electron microscopy laboratory of the Biological Institute to research the etiological agent. Around 287 samples were positive for coronavirus using the negative staining technique (rapid preparation), where the particles visualized were pleomorphic, rounded or elongated, enveloped, containing typical radial projections, in the shape of a solar corona, measuring on average 140 nm in diameter. According to previous reports, these ultrastructural aspects of particles from the *Coronaviridae* family were also reported by other researchers who used this technique in studies of canine coronavirus (Roseto et al., 1980; Vandenberghe et al., 1980; Williams, 1980; Hammond & Timoney, 1983; Mitika et al., 1989; Finlaison, 1995; Catroxo et al., 1998; del Amo, et al., 1999; Pratelli, 2008; Gan et al., 2021; Tian et al., 2021; Vieira et al., 2023). In some particles we were able to observe a spherical internal core, measuring 60 to 85 nm in diameter. Risco et al. (1996) reported the presence of this internal core, measuring around 65 nm in particles of swine transmissible gastroenteritis coronavirus and studies by immunogold mapping and protein analysis of purified cores showed that they consist of M and N proteins. The coronavirus particles in our study had envelope spikes measuring between 16 and 20 nm in length. According to Escors et al. (2001) and Locker et al. (1992), the long spikes of the 20 nm, which consist of the S glycoprotein, are present on all coronaviruses, whereas the short spikes, which consist of the HE (hemagglutinin-esterase) glycoproteins, are present in only some coronaviruses.

In the immunoelectron microscopy technique, a positive antigen-antibody reaction was detected in 287 samples of feces, fecal swabs and fragments of small intestine as a result of agglutination of a high number of viral particles. This technique was used previously by other researchers (Williams, 1980; Pensaert et al., 1981; Risco et al., 1996).

We found 44.63% positivity for coronavirus in dog samples from our study. Close percentages (40.8%) were reported in research conducted by Naylor et al. (2001) in kenneled dogs in Australia and 43.3% by van Nguyen et al. (2017) in Vietnam. In Brazil Castro et al. (2010) reported 47.8% of vaccinated dogs, 45.5% of the unvaccinated and 43.3% of the dogs with unknown historical vaccination. In other countries, CCoV positivity was quite variable, ranging from 4.3% in Bangladesh (Hossain et al., 2021), 4.8% in the Caribbean region (Navarro et al., 2017), 7% in Argentina (delAmo et al., 1999), 7.01% in Australia (Finlaison, 1995), 2.8% and 20.69% in United Kingdom (Staviski et al., 2010; Radford et al., 2021) respectively, 12.5% in France (Roseto et al., 1980), 33% in China (Dong et al., 2022), 34.61% in Italy (Decaro et al., 2004a), 62.5%-74.3% and 100% in Turkey (Yesilbag et al., 2004; Timurkan et al., 2021), respectively. In Brazil, positivity also varied between 22% (Guirao et al., 2013) and 50.4% (Dezengrini et al., 2007). Study carried out by Takano et al. (2016) in Japan showed that 88.9% of dogs were positive for CCoV-I and 7.4% for CCoV-II, while in Italy CCoV-IIa was found in 18% of dogs and CCoV -1 in 10.3% (Zobba et al., 2021). In another research carried out in a Zoological collection in United Kingdom, 100% of the *Speothos venaticus* studied were positive for coronavirus, showing that other canids are also susceptible (Rowland et al., 2021).

Considering age, the dogs in our study were between 23 days and 17 years old, 71 (24.73%) were animals aged up to 11 months and 65 (22.64%) were animals aged over 11 months, with no significant differences between these two parameters and confirming that dogs of all ages can be infected with CCoV, although puppies are more susceptible (Pratelli et al., 2008). Corroborating our results, Delamo et al. (1999) found a rate of 42.85% of positive animals between 6 weeks and 6 months and a similar percentage in dogs older than six months of age. Other authors reported the occurrence of higher percentages, such as 100% in adult animals (Hossain et al., 2021), 57% aged over 5 years (Dezemgrini et al., 2007), 52% over 3 years (van Nguyen et al., 2017) and 43.8% in dogs aged between 1 and 6 years (Castro et al., 2010). The average age of the animals studied by Radford et al. (2021) was 4 years. The highest number of occurrences, however, was mentioned in animals under 6 months old, although the percentage was lower (43%) (Dong et al., 2022), 46.4% under 1-year-old (Takano et al., 2016), between 1 to 6 months (Guirao et al., 2013), 4 days to 21 weeks (Licitra et al, 2014), 10 weeks old (Vandenberghe et al., 1980), and 2 weeks to 4 months (Yesilbaf et al., 2004).

We found that among the 287 positive samples, 108 (37.63%) were males and 107 (37.28%) were females, therefore there was no significant difference between these values, reinforcing the results of most authors who also did not report statistical differences (Dezemgrini et al., 2007; Castro et al., 2010; Zobb et al., 2021; Dong et al., 2022). Timurkan et al. (2021), however, found greater positivity (69.23%) in male dogs. Radford et al. (2021) also reported that the number of infected male dogs in their research was greater than that of female dogs.

The main clinical signs that we observed in the positive dogs in our study, such as apathy, prostration, weight loss, vomiting, gastroenteritis, dehydration and liquid or bloody diarrhea, were reported in other canine coronavirus studies (Vandenbergue et al., 1980; Tennant et al., 1991; Naylor et al., 2001; Stavisky et al., 2010; Licitra et al., 2014; Navarro et al., 2017; Radford et al., 2021; Rowland et al., 2021; Tian et al., 2021; Timurkan et al., 2021). Respiratory clinical signs such as bronchopneumonia and cough, as well as other less common signs, such as rhinitis, conjunctivitis, intestinal intussusception, and neurological signs, were mentioned by Vandenberghe et al. (1980) and Licitra et al. (2014).

Around 8 dogs (2.78%) in our research, aged between 1 day and 6 months, died. Licitra et al. (2014) reported 20% mortality in dogs 6 to 8 weeks old, while Tian et al. (2021) confirmed death in a 5-week- old dog and Timurkan et al. (2021) 1 month old. These data confirm that CCoV can cause mortality more frequently in puppies up to 21 weeks of age, especially when housed in kennels with a high population (Licitra et al., 2014).

Regarding mixed infections, a total of 20 samples (38.46%) were co-infected with paramyxovirus, however, Catroxo et al. (2023) found a rate of 5.51% and Zhao et al. (2016) 1.11%. Approximately 21 dogs in our survey (40.3%) were co-infected with parvovirus. Similar studies revealed a rate of 8.90% (Roseto et al., 1980); 5% (Delano et al., 1999) and 11.11% for CCoV II and CPV-2 and 2.77% for CCoV1a and CPV-2 (Zobba et al., 2021). Decaro et al. (2006) also reported the simultaneous detection of CCoV and CPV-2 in 40-day-old pups and Licitra et al. (2014) in a dog from their study. 7.69% of the dogs in our research were co-infected with Mycoplasma and two animals that died had a triple infection with coronavirus, parvovirus and Mycoplasma. Triple infection represented by the simultaneous presence of paramyxovirus, coronavirus and adenovirus was found in a dog, in the study by Decaro et al. (2004a). No occurrence reports the joint infection of coronavirus and Mycoplasma, however the concomitant presence of parvovirus and Mycoplasma has already been demonstrated by transmission electron microscopy, in young dogs, during an outbreak of hemorrhagic gastroenteritis with mortality (Cappellaro et al., 1995). Coronavirus facilitates transmission by other agents and these mixed, double or triple infections tend to worsen the clinical course of the disease and can lead to the death of infected animals (Decaro et al., 2006).

The animals from the Military Police kennel of the State of São Paulo, SP, Brazil, affected by the outbreak, were immunized with the V8 vaccine, which includes coronavirus. Little is known, however, about the immunological mechanisms involved in protection against CCoV enteritis (Decaro et al., 2004b). Several factors can contribute to vaccine failure, including deficient animal immune response, administration to immunocompromised, immunosuppressed or passively immune animals, use of ineffective vaccines or those with inadequate conservation or expired validity, among others (Pratelli et al., 2003).

Treatment for canine coronavirus is supportive, which includes maintaining hydro-electrolyte balance, control of secondary infections, prevention measures, through the institution of improvements in hygiene, efficient and adequate vaccination, intake of maternal colostrum, exemption from crowds in kennels, quarantine and isolation of sick animals (Dezengrini et al., 2007; Hass et al., 2008; Strottrmann et al., 2008).

Transmission electron microscopy applied to fecal suspensions, swabs and intestinal fragments can reveal the multiplicity of primary pathogens and predisposing factors involved in the installation of gastroenteric conditions (Hagiwara et al., 1989).

Through its various techniques, it is especially useful for quickly diagnosing agents in samples, allowing veterinary clinics to take immediate prophylactic and disease control measures (Goldsmith & Miller, 2009; Wolff & Bárcena, 2021).

Conjecturing that previous studies prove that several species of wild animals can be contaminated with coronaviruses (Catroxo et al., 2023), other research must be conducted to assess the impact of infections caused by these viruses in companion animals, kennels or wild animals and propose preventive and protective measures.

IV. CONCLUSIONS

Considering that canine coronavirus is an emerging infectious disease with an important impact on veterinary activities in kennels and animal shelters, causing economic losses due to mortality and/or morbidity, the use of transmission electron microscopy techniques allows for rapid and safe diagnosis, contributing to the immediate implementation of prophylactic measures, prevention and control of the disease, during routine procedures or in the event of outbreaks.

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