Universidade Estadual de Londrina

FERNANDA LOUISE PEREIRA LAVORENTE

MOLECULAR DETECTION OF PARAMYXOVIRUSES IN WHITE-EARED OPOSSUM (*Didelphis albiventris*) FROM CENTRAL NORTH OF PARANÁ STATE.

Londrina
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Dissertação de mestrado apresentada ao Progama de Pós Graduação em Ciência Animal da Universidade Estadual de Londrina como requisito parcial à obtenção do título de Mestre em Ciência Animal.

Orientadora: Profa. Dra. Alice Fernandes Alfieri

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"O saber a gente aprende com os mestres e os livros. A sabedoria se aprende é com a vida e com os humildes."

Cora Coralina

LAVORENTE, Fernanda Louise Pereira. **Detecção molecular de paramixovírus em gambás de orelha-branca (*Didelphis albiventris*, Lund 1840) da região norte central do estado do Paraná**. 2019. 78 f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

O Morbilivírus Felino (FeMV) foi detectado pela primeira vez em amostras de felinos domésticos provenientes de Hong Kong em 2012 e foi associado com nefrite tubulointersticial e doença renal crônica nesta espécie, embora tenha sido detectado em gatos assintomáticos em estudos posteriores. Portanto, não está claro o envolvimento FeMV com doenças renais e outras informações epidemiológicas sobre o agente ainda são desconhecidas, além disso, até o momento estudos reportaram a presença do FeMV exclusivamente em felinos domésticos. Este estudo relata pela primeira vez a detecção molecular de RNA de FeMV e isolamento *in vitro*, associado a achados patológicos e imuno-histoquímicos, em um marsupial sinantrópico, o gambá de orelha branca (*Didelphis albiventris*), habitando áreas periurbanas no norte do Paraná. Dos 23 animais coletados 6 (26%) foram positivos em ensaios de RT-PCR e RT-SNPCR para amplificação do gene N e L de FeMV nos tecidos do pulmão, enquanto que nos rins apenas o gene N foi detectado (2/6). Análises das sequencias de nucleotídeos e aminoácidos mostraram alta similaridade entre a cepa de FeMV do gambá e cepas de gatos domésticos do Japão, demonstrando não haver uma correlação positiva com a distribuição geográfica. Além disso, observou-se que na árvore filogenética, apesar de estarem agrupadas com as cepas de FeMV já detectadas, as cepas dos gambás formaram um novo ramo, evidenciando uma correlação positiva com hospedeiro. Os principais achados histopatológicos foram pneumonia intersticial, nefrite linfocítica e necrose tubular. No ensaio de imuno-histoquímica a proteína N do FeMV foi detectada nos tecidos do pulmão (5/6) e do rim (5/6). Além disso, uma das cepas de FeMV de gambá foi isolada em uma linhagem celular felina, a *Crandell Rees feline kidney* levando a formação de sincícios e morte celular. Assim, esses resultados evidenciam a habilidade do FeMV em infectar outras espécies de mamíferos e reforça a possibilidade dos gambás atuarem como disseminadores do vírus entre animais domésticos e selvagens.

Palavras-chave: Imuno-histoquímica. Isolamento. *Morbillivirus*. Pulmão. Rim.

LAVORENTE, Fernanda Louise Pereira. **Molecular detection of paramyxoviruses in white-eared opossum (*Didelphis albiventris* Lund, 1840) from central north of Paraná state,** 2019. 78 p. Dissertation (Master Degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2019.

ABSTRACT

Feline Morbillivirus (FeMV) was firstly detected in 2012 in domestic cats from Hong Kong and was associated with tubulointerstitial nephritis and chronic kidney disease although, in subsequent studies in other countries had been detected in healthy cats. Therefore, it is unclear the FeMV involvement with renal diseases and other epidemiological information on the agent are still unknown, besides this, until now studies have reported the presence of FeMV exclusively in domestic cats. This study shows the first molecular detection of the FeMV RNA associated with pathological and immunohistochemical findings in a synanthropic marsupial, the white-eared opossum (*Didelphis albiventris*), inhabiting peri-urban areas of northern Parana. Out of 23 animals collected 6 (26%) were positive in RT-PCR and RT-SNPCR assays for amplification of the N and L gene of FeMV in lung tissues, while in the kidneys only the N gene can be amplified (2/6). Nucleotide and amino acids analyzes showed high similarity between the FeMV strain of the possum and FeMV domestic cats strains from Japan, evidencing that there is no positive correlation with the geographical distribution. In addition, in the phylogenetic tree, although FeMV from opossums are clustered with the other FeMV strains, formed a new branch, evidencing a positive correlation with the host. The main histopathological findings were interstitial pneumonia, lymphocytic nephritis, and tubular necrosis. In the immunohistochemistry assay, FeMV N protein was detected in tissues of the lung (5/6) and the kidney (5/6). A FeMV opossum strain was isolated in Crandell Rees feline kidney lineage cell showing syncytia formation and cell death. Therefore, these results evidence the ability of FeMV in to infect other mammals species and reinforces the possibility of the opossum to be a disseminator of this virus among domestic and wild animals.

Keywords: Immunohistochemistry. Isolation. Kidney. Lung. *Morbillivirus*.

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
aa	Amino acid
CD	Canine Distemper
CDV	Canine Distemper Virus
CeMV	Cetacean Morbillivirus
CKD	Chronic kidney diseases
CPE	Cytopathic effects
CPV	Canine Parvovirus
CRFK	Crandell feline kidney cell
DMV	Dolphin Morbillivirus
FeMV	Feline Morbillivirus
FFPE	Formalin Fixed Paraffin-embedded
FPaV	Feline Paramyxovirus
H&E	Hematoxylin and Eosin
IHC	Immunohistochemistry
Kb	Kilobases
Kg	Kilograms
MEM	Minimal essential medium
Min	Minutes
mL	Milliliters
mRNA	Messenger ribonucleic acid
MV	Measles Virus
N: RNP	Ribonucleoprotein
nt	nucleotide
ORFs	Open reading frames
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PPRV	Small ruminant morbillivirus
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
RPV	Rinderpest Virus

RT-PCR	Reverse transcriptase-polymerase chain reaction
RT-snPCR	Reverse transcriptase semi-nested polymerase chain reaction
spp	Several species
TBE	Tris-borate-EDTA
TIN	Nephrite tubulointerstitial
UV	Ultraviolet

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1. INTRODUCTION

Anthropic impacts can change the composition and interaction of the environment with the wildlife communities (DAZAK; CUNNINGHAM; HYATT, 2000; KEESING et al., 2010). Biodiversity disturbances such as resource competition, altered trophic interactions, and emerging of diseases, beyond biodiversity loss and increase in the abundance of species that thrive in urban areas, is a consequence of urbanization process (BRADLEY; ALTIZER, 2007). Synanthropic animals are species adapted to survive or prosper in the environments modified by humans, benefiting from their dwellings and the remains of food (MCFARLANE; SLEIGH; MCMICHAEL, 2012). The cities serve as significant hubs of pathogen introduction, so synanthropic species may be essential components in the transmission ecology of different pathogenic microorganisms, once its transit between different ecological niches increasing the risk of infectious diseases exposure (BERMÚDEZ et al., 2017; BRADLEY; ALTIZER, 2007; DASZAK; CUNNINGHAM; HYATT, 2001).

Rodents, opossums, and mid-sized wild carnivores are examples of synanthropic mammals species easily found in urban areas (BERMÚDEZ et al., 2017). The opossums are highly adapted to urban environments and are able to remain in very devastated areas (JANSEN, 2002). The white-eared opossum (*Didelphis albiventris* Lund, 1840) belonging to the order Didelphimorphia, family Didelphidae, subfamily Didelphinae, genera *Didelphis* (ROSSI; BIANCONI; PEDRO, 2006) are the most commonly marsupial found in Brazil. The generalist diet of the white-eared opossum, classified as omnivorous, favors its adaptation in areas disturbed by humans (ROSSI; BIANCONI; PEDRO, 2006). Beyond, currently the opossum is considered the definitive host or reservoir of several parasites, bacteria, and viruses (MULLER et al., 2005), and its synanthropic habit highlights its great potential as a source of infectious diseases among different animal species.

Around the world microorganisms like parasites (COSTA-NETO et al., 2018; FEHLBERG et al., 2018; FERNANDEZ et al., 2018; FORNAZARI et al., 2011; GONDIM et al., 2017; ROMAN et al., 2018; SILVA et al., 2017b; TORRES-MONTOYA et al., 2018), bacteria (GUIMARAES et al., 2018; JORGE et al., 2012; LOPES et al., 2018b) and viruses (BOSCO-LAUTH et al., 2014; LINHARES; PEREIRA; NAKAUTH, 1986; OROZCO et al., 2014; PERES et al., 2018; SOARES; BENSABATH; TRAVASSOS DA ROSA, 1987; TRUJILLO et al., 2010) have been reported in white-eared opossum as well in different species of opossums. However, until now, studies with viral agents are still scarce in this

species and can provide important ecological and epidemiological data, mainly in relation to viruses of interest in human and animal health, such as the paramyxoviruses.

The paramyxoviruses belong to the *Negarnaviricota* phylum, *Haploviricotina* subphylum, *Monjiviricetes* class, *Mononegavirales* order and comprise 7 genus: *Aquaparamyxovirus*, *Avulavirus*, *Ferlavirus*, *Henipavirus*, *Morbillivirus*, *Respirovirus,* and *Rubulavirus* (ICTV, 2018). The paramyxoviruses are pleomorphic viruses with lipid envelope, nucleocapsid, and a matrix protein with 60 to 300 nm of diameter (MACLACHLAN; DUBOVI, 2017; WANG et al., 2011). The genome is composed by a single-stranded RNA non-segmented of negative polarity, measuring around 15 to 16 kilobases (kb) and encoding 7 to 8 structural and non-structural proteins (N, P/V/C, M, F, H, and L proteins) (MACLACHLAN; DUBOVI, 2017; WANG et al., 2011).

Morbillivirus is one of the most important genus in the *Paramyxoviridae* family, which shelter highly infection viruses of importance to human and animal health as Measles and Canine Distemper Virus (CDV), respectively. These viruses showed high morbidity and mortality rates and are known as trigger off great outbreaks and the ability to break interspecies barriers (COSBY, 2012; NAIM, 2015).

Canine distemper virus is one of the most important infectious diseases, responsible for a severe multisystemic and globally distributed infectious disease able to infect most species of terrestrial carnivores and other wildlife (DEEM et al., 2000; MCCARTHY; SHAW; GOODMAN, 2007). The main spread route of this virus is the respiratory tract through bodily secretions, mainly oral, respiratory, and ocular fluids (MARTELLA; ELIA; BUONAVOGLIA, 2008). However, the CDV is not stable in the environment and need close contact between the host and a susceptible, so the proximity among wildlife species and domestic animals increase the opportunities for cross-species infections (COSBY, 2012; WILLIAMS, 2001).

The emergence of new species and genotypes of morbilliviruses may be attributed to recombinant events, mainly in the region of genes responding to the entry in the cell host, increasing the host range of the virus (HAN; LIU; LI, 2008; PARK et al., 2014). Besides this, it is believed that the morbilliviruses may have evolved from a common ancestor and adapted to its hosts which justify its ability to adapt to new hosts and the great similarity between different species (NAMBULLI et al., 2016). In the last decades, this genus has grown as new species able to infect new hosts have been discovered, including marine mammals such as *Phocid Distemper Virus* (PDV) in seals, *Cetacean morbillivirus* (CeMV) in dolphins, whales,

and porpoises (BARRETT et al., 1993) and, *Feline morbillivirus* (FeMV) in domestic cats (WOO et al., 2012).

The FeMV was first time detected in domestic cats from China in 2012 and since then, has been associated with the presence of uropathies and the development of Chronic Kidney Disease (CKD). However, a positive correlation between this virus infection and the development of uropathies was not found in some studies (DAROLD et al., 2016; SHARP et al., 2016; WOO et al., 2012). The FeMV RNA was detected in other tissues from infected cats such as: lung, liver, spleen, and lymph nodes (YILMAZ et al., 2017). Nevertheless, the real implications of the infection to the health of cats are still unknown.

Natural recombination events in the F and H genes of FeMV had been reported and may be favored by its chronic infection condition. As a consequence, an increase in the host range of susceptible to FeMV infection might occur (PARK et al., 2014). So, to our better knowledge of the virulence, pathogenicity, and epidemiology aspects of the FeMV infection, more studies are necessary as it may become a threat to other species, including the wild species.

Therefore, considering the importance of paramyxoviruses as a threat to animal and human health and also the role of synanthropic animals as disseminators of pathogens among different ecological niches, the objective of this study was to investigate the presence and the consequence of the infection by paramyxoviruses in white-eared opossums inhabiting peri-urban areas in counties of northern Parana. In addition, this study aimed to evaluate the importance of the opossums as source and disseminator of the virus.

2. LITERATURE REVIEW

Emerging and reemerging viral diseases transmitted between humans, wild, and domestic animals can have significant impacts on public health, livestock economics, domestic animals health, and wildlife conservation (CLEAVELAND; LAURENSON; TAYLOR, 2001). Anthropic impacts as the advance of farming and the increase of urban centers are responsible for destroying and fragmenting forest areas and consecutively for the increasing of the proximity between humans, domestic, and wild animals (BRADLEY; ALTIZER, 2007; DASZAK; CUNNIGHAM; HYATT, 2000; DASZAK; CUNNINGHAM; HYATT, 2001; SHOCHAT et al., 2006).

The proximity between these different species can be a determinant factor in the transmission cycle of infectious diseases, once cities serve as significant hubs of pathogen introductions and sources of infection for wildlife that exist at the periphery of urban centers (BRADLEY; ALTIZER, 2007; KNOPS et al., 1999; LAFFERTY; GERBER, 2002; SCOTT, 1988). Though little is known about the distribution and risks of infectious diseases among host wild species and the ecological factors that drive those (PEDERSEN et al., 2007), the impacts of urbanization may alter the epidemiological process and bring further challenges for wildlife populations (BRADLEY; ALTIZER, 2007). Studies suggest infectious diseases as a cause of the decline in wild populations (KEESING et al., 2010; PEDERSEN et al., 2007; SMITH; SAX; LAFFERTY, 2006). According to Smith et al. (2006), from 833-animal species known to be extinct in the world, 3.7% are directly related to infectious diseases.

The wild animals, mainly synanthropic species able to survive or prosper in the environments modified by humans (MCFARLANE; SLEIGH; MCMICHAEL, 2012), may be the main source of reemerging infectious diseases, such as yellow fever (DASZAK; CUNNIGHAM; HYATT, 2000). Rodents, opossum, and mid-sized wild carnivores which inhabit or migrate throughout ecotones that contain forest, pasture, and human dwellings are examples of synanthropic species which may be important components of the transmission ecology of different pathogenic microorganisms (BERMÚDEZ et al., 2017).

2.1. THE SYNANTHROPIC OPOSSUM: *DIDELPHIS* SPP.

The opossums are highly adapted to urban environments and their interaction with the human is ancient (BERMÚDEZ et al., 2017). The opossums are commonly found sheltering in house linings and hollow of trees, besides to find in the trash a source of food

(CÁRCERES, 2000; FONSECA et al., 1996). Among mammals, the opossums are known as the synanthropic animal most adapted to remain in very devastated areas (GONDIM et al., 2017; JANSEN, 2002).

The order *Didelphimorphia* is the largest of the three American orders (*Didelphimorphia* Gill, 1872, *Paucituberculata* Ameghino, 1894, and *Microbiotheria* Ameghino, 1889), which is represented by the family *Didelphidae* Gray, 1821. The family *Didelphidae* is widely distributed throughout the American continent with more than 70 species (SILVA et al., 2017a). The didelphis occurs from southeastern Canada to southeastern Argentina including 6 species: *Didelphis marsupialis* Linnaeus, 1758, *Didelphis aurita* (Wied-Neuwied, 1826), *D. albiventris* (Lund, 1840), *Didelphis virginiana* Kerr, 1792, *Didelphis imperfecta* Mondolfi & Pérez-Hernández, 1984, and *Didelphis pernigra* J.A. Allen, 1900 (AUSTAD, 1988; IUCN, 2018).

In Brazil, the most common marsupials are the white-eared opossum (*Didelphis albiventris*) and common-opossum (*Didelphis marsupialis*). The black-eared opossum (*Didelphis aurita*) is also native of Brazil, however, is restricted to areas with remnants of primary and secondary forests of the Atlantic Forest and the Araucaria Forest (COSTA et al., 2015) (Figure 1).

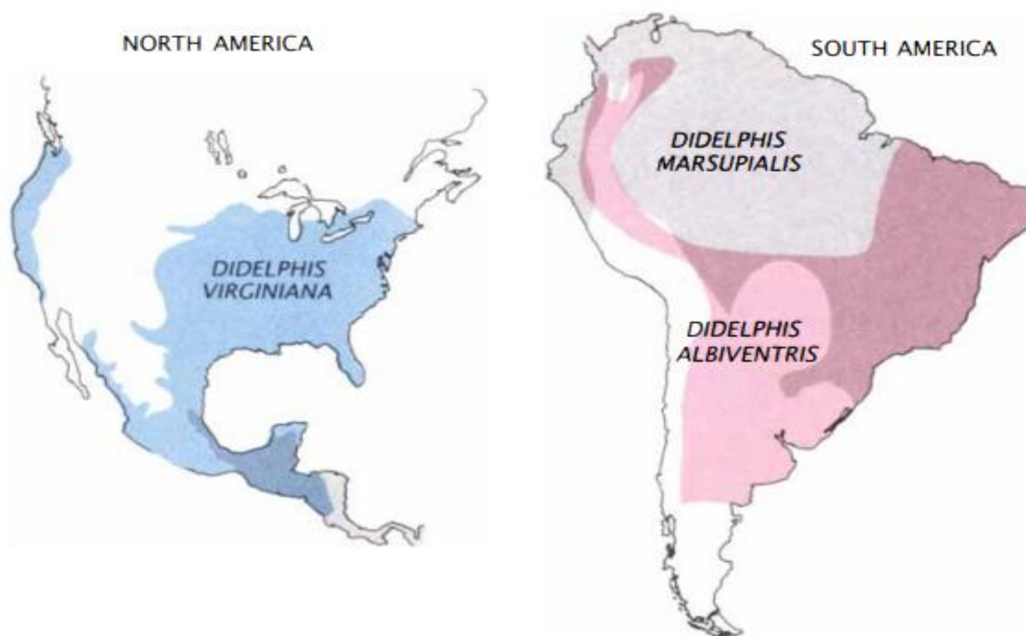

Figure 1. A schematic illustration showing the geographic distribution of the main species of opossum found around the American continent. Virginia opossums (*D. virginiana*) have a wide range throughout Central and North American, while common opossums (*D. marsupialis*) and white-eared opossum (*D. albiventris*) are widely distributed in South America. Although in lesser quantity, the black-eared opossum (*D. aurita*) are also found in Brazil.
Source: AUSTAD, 1988.

2.2. WHITE-EARED OPOSSUM (*DIDELPHIS ALBIVENTRIS*)

2.2.1. General Aspects

The white-eared opossum (*D. albiventris*) can be found in several countries of South America, including Argentina, Bolivia, Brazil, Paraguay, and Uruguay (COSTA et al., 2015). In Brazil, these animals are present in gallery forests and open spaces as the cerrado, moreover have been registered in transition zones of different biomes, such as Atlantic and Amazon Forest with the Cerrado (ROCHA et al., 2011).

The white-eared opossum is an animal of medium size measuring around 30.5 to 89 cm and weighing around 0,5 to 2,750 kg (CARCERES; MONTEIRO-FILHO, 1999; GONZÁLEZ et al., 2011; ROSSI; BIANCONI; PEDRO, 2006). There are considerable variations in coloration, with a predominance of grayish and black streaks on the face and the ears are black at the base and white-pink at the distal half (ROSSI; BIANCONI; PEDRO, 2006) (Figure 2).

The white-eared opossum is a solitary animal, in most of the time, excluding the reproductive period which can occur one to three times every year (RIGUEIRA et al., 1987). The gestational period has a duration of 12 to 14 days and the female give birth to up to 14 p joeys, which will stay on the pouch until approximately 70 days when will start the weaning process and independence (ROSSI; BIANCONI; PEDRO, 2006). These marsupials mammals are terrestrial and usually seeking shelter in the hollows of trees, among its roots or under fallen logs (FONSECA et al., 1996), although it can climb trees easily using the prehensile tail (ROSSI; BIANCONI; PEDRO, 2006).

The opossum is a nocturnal animal and *D. albiventris* is classified as omnivorous, including fruits and small vertebrates, like rodents, small birds, insects, and frogs in the diet (MULLER et al., 2005; ROSSI; BIANCONI; PEDRO, 2006). This generalist diet favors the adaptation of this species in areas disturbed by humans, being considered therefore as synanthropic (JANSEN, 2002). In urban environments can be found easily resources, and in cases of food storage use the forest only as a hall between different areas (CÁRCERES, 2000).

2.2.2. Infectious Diseases

The opossums due to the synantropic habits, transit between different environments which make it a potential disseminator of infectious diseases to humans, wild, and domestic

Figure 2. White-eared opossum (*Didelphis albiventris*) evidencing the grayish coloration of the pelage, the black streaks on the face, and the white-pink eared whit the black base.
Source: SENS, 2012.

animals (MCFARLANE; SLEIGH; MCMICHAEL, 2012; MULLER et al., 2005). The opossums are known to be the definitive host or reservoir of several parasites, bacteria, and viruses (MULLER et al., 2005). *Didelphis* spp. are known as an ancient reservoir of the protozoan *Trypanosoma cruzi*, responsible to a vector-borne zoonosis, endemic in South America, called Chagas disease (ROMAN et al., 2018). The protozoan is transmitted by a Triatomine and can infect several species of small mammals, responsible for its maintenance in the sylvatic environment including the white-eared opossum (ACOSTA et al., 2017; ROMAN et al., 2018).

The white-eared opossum is the wild host of *Leishmania* spp. that result to the most important emerging and reemerging infectious diseases around the world, transmitted by an phlebotominae, the visceral and cutaneous Leishmaniosis (DONALISIO et al., 2017). Although domestic dogs are considered the main reservoir in the urban environment, in human disturbed areas the proximity to wildlife increase the importance of wild and synanthropic hosts for the maintenance of the *Leishmania* spp (ROQUE; JANSEN, 2014).

Domestic animals and several wild animal species can develop toxoplasmosis and be important fonts of infection (FORNAZARI et al., 2011). Furthermore, reports have shown the circulation of the *Toxoplasma gondii* (*T. gondii*) in white-eared opossum inhabiting peri-urban areas of São Paulo state, Brazil. The *T. gondii* is a worldwide distributed intracellular parasite of importance in public health leading to encephalitis, eye disorders, and mental retardation in immunosuppressed individuals or pregnant women's (FORNAZARI et al., 2011). Beside those, molecular assays detected the parasites *Neospora caninum* and *Sarcocystis* spp DNA in tissues fragments, and *Hepatozoon canis* DNA in blood sample from

Brazilians *Didelphis*. These protozoan parasites induce important diseases in domestic and wild animals (GONDIM et al., 2017; SILVA et al., 2017b).

The opossum is also, frequently related as the potential host of bacterial agents, such as *Leptospira* spp. (JORGE et al., 2012) and *Ehrlichia* spp. (SILVA et al., 2017b). In relation to viruses, there are few reports. Serological surveys conducted in Argentina evidenced the circulation of Canine parvovirus (CPV) among white-eared opossums (OROZCO et al., 2014) and vaccinia virus, with zoonotic potential was isolated from blood of *Didelphis albiventris* from Brazil (PERES et al., 2018).

Several microorganisms were reported in other species of opossum, beyond those described above in white-eared-opossum, including parasites such as *Eimeria* spp. (FEHLBERG et al., 2018), helminths nematodes (COSTA-NETO et al., 2018; TORRES-MONTOYA et al., 2018), *Trypanosoma* spp. (LOPES et al., 2018a; ROMAN et al., 2018), *Leishmania* spp (ROQUE; JANSEN, 2014), and bacteria as *Erlichia* sp. (GUIMARAES et al., 2018). Furthermore, reports with viral agents in *Didelphis* are scarce. Serological surveys evidences the circulation of Hepatitis A (SOARES; BENSABATH; TRAVASSOS DA ROSA, 1987) and West Nile virus (BOSCO-LAUTH et al., 2014) among *D. marsupialis* and *D. virginiana*, respectively. Beyond, Rotavirus was detected in *D. marsupialis* through enzyme-linked immunosorbent assay (LINHARES; PEREIRA; NAKAUTH, 1986).

Therefore, the opossum may represent a potential source of infection to other animals. Furthermore, due to the synanthropic status of these animals and the shortage of reports viruses, mainly with zoonotic potential, more studies are necessary to evaluate its importance in the maintenance and dissemination of infectious diseases.

2.3. ETIOLOGIC AGENT

2.3.1. *Paramyxoviridae* Family

The *Paramyxoviridae* family includes pathogens that cause some of the most devastating human and veterinary diseases (LAMB; PARKS, 2013). This family includes the most infectious viruses currently known that have been targeted by the World Health Organization for eradication such as the measles (NAIM, 2015). Viruses of importance in veterinary medicine, with high rates of morbidity and mortality such as Rinderpest (RPV), Canine Distemper (CDV), and Newcastle belong to this family (ARNS et al., 2012).

Other paramyxoviruses also cause disease in a wide variety of mammals, birds, fishes, and reptiles (Table 1). As wildlife species are more in contact with humans and domestic animals through changes in habitat, the opportunities increase for cross-species infections by these and additional, yet unidentified paramyxoviruses.

2.3.2. Structure and Genome

Paramyxovirus belongs to the *Negarnaviricota* phylum, *Haploviricotina* subphylum, *Monjiviricetes* class, *Mononegavirales* order, which features other 10 families of enveloped virions and single-stranded RNA non-segmented of negative polarity as a genome, such as *Filoviridae, Rhabdoviridae,* and *Pneumoviridae* (COX; PLEMBER, 2017; ICTV, 2018). The *Paramyxoviridae* family comprises 7 genus of importance in humans (Table 1) and morphologically these virions consist in a lipid envelope, a nucleocapsid, and a matrix protein (WANG et al., 2011). The virus with 60 to 300 nm of diameter are pleomorphic or spherical, although filamentous forms are common (MACLACHLAN; DUBOVI, 2017; WANG et al., 2011) (Figure 3A and 3B).

The *Paramyxoviridae* genome measure around 15 to 19 kb of extension and to the correct replication is essential genomes with lengths multiples of 6 (WANG et al., 2011). Moreover, the RNA does not contain 5' cap and the 3' end is not polyadenylated. Around 7 to 10 proteins are encoding for the genome: a RNA-binding protein (N), a phosphoprotein (P), and a large polymerase protein (L) associated to nucleocapsid; a matrix protein (M), a fusion protein (F), and an attachment protein (G, or H, or HN) (MACLACHLAN; DUBOVI, 2017) (Figure 3C).

These proteins are common to all members in the *Paramyxoviridae* family, although variable proteins, structural and non-structural, derived of overlapping in the open reading frames (ORFs) can be synthesized depending on the virus (ARNS et al., 2012). From P gene, using RNA editing mechanism, all members of *Paramyxoviridae* family produces a non-structural protein V and using the same transcript, the *Respirovirus* and *Morbillivirus* genus produces an additional protein named C (ARNS et al., 2012). The envelope are derived directly from the host cell plasma membrane and has two membrane glycoproteins as projections of 8 to 20 nm of extension (LAMB; PARKS, 2013). The glycoprotein of attachment (H, HN or G) is responsible for the binding to the host cell receptor and facilities the entry of the virus (ARNS et al., 2012). The H protein is a hemagglutinin, so this can cause red blood cells agglutination, while HN (hemagglutinin-neuraminidase) cleaves the sialic receptor and prevents the viral biding in infected host cells. To the virus without H or HN

proteins, the G glycoprotein is responsible to aid the binding of the virus to the cell receptor (ARNS et al., 2012).

Table 1. Classification of the members of the *Paramyxoviridae* family.

Family	Genus	Species
Paramyxoviridae	*Respirovirus*	*Bovine respirovirus 3* *Human respirovirus 1* *Human respirovirus 3* *Murine respirovirus* *Porcine respirovirus 1*
	Morbillivirus	*Canine morbillivirus* *Cetacean morbillivirus* ***Feline morbillivirus*** *Measles morbillivirus* *Phocine morbillivirus* *Rinderpest morbillivirus* *Small ruminant morbillivirus*
	Rubulavirus	*Achimota rubulavirus 2* *Bat mumps rubulavirus* *Human rubulavirus 2* *Human rubulavirus 4* *Mammalian rubulavirus 5* *Mapuera rubulavirus* *Menangle rubulavirus* *Mumps rubulavirus* *Porcine rubulavirus* *Simian rubulavirus* *Sosuga rubulavirus* *Teviot rubulavirus* *Tioman rubulavirus* *Tuhoko rubulavirus 1* *Tuhoko rubulavirus 2* *Tuhoko rubulavirus 3*
	Henipavirus	*Cedar henipavirus* *Ghanaian bat henipavirus* *Hendra henipavirus* *Mojiang henipavirus* *Nipah henipavirus*
	Avulavirus	*Avian avulavirus 1 to 19*
	Aquaparamyxovirus	*Salmon aquaparamyxovirus*
	Ferlavirus	*Reptilian ferlavirus*

Source: Adapted from ICTV (ICTV, 2018).

Fusion glycoprotein (F) makes the entry of the virus in the host cell membrane through the fusion of its membranes (ARNS et al., 2012). In order to turn F protein active, the

primordial form of the protein called F0 is cleaved in F1 and F2, under neutral pH, on the trans portion in the Golgi complex (LAMB; PARKS, 2013). The amino-terminal sequence of the F1 protein has a hydrophobic domain inserted in the membrane of the target cell to initiate pore formation after the fusion process has been initiated, so membrane glycoproteins have key roles in the pathogenesis of all paramyxovirus infections (ARNS et al., 2012; LAMB; PARKS, 2013). Neutralizing antibodies specific for the attachment glycoprotein and to F protein are able to inhibit the adsorption of the virus to the cellular receptors and neutralize viral infectivity respectively (LAMB; PARKS, 2013).

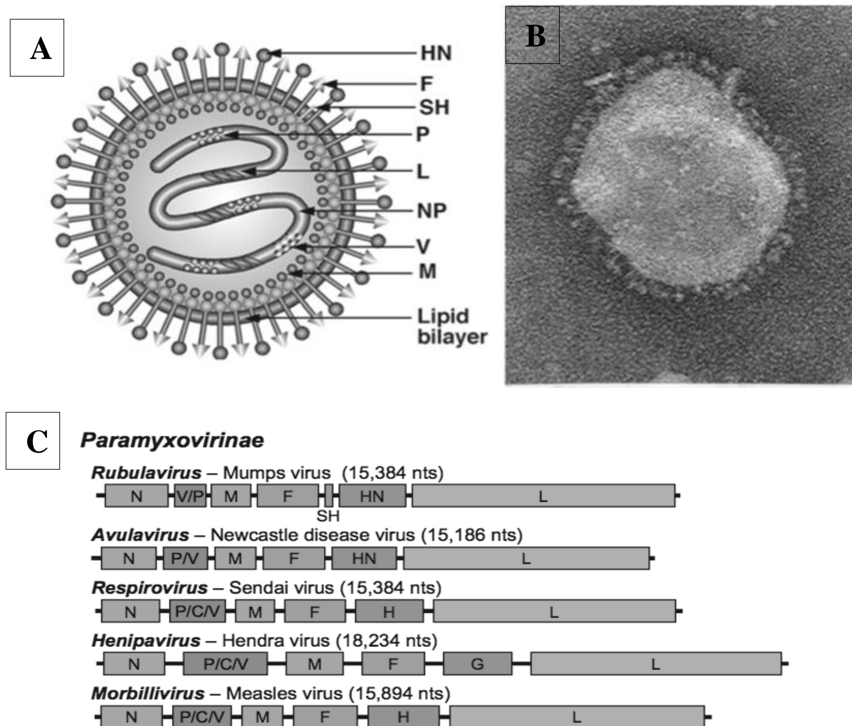

Figure 3. Virions of *Paramyxoviridae* family. A) Schematic illustration of a viral particle and its components. B) Electronic microscopy of a human paramyxovirus. C) Schematic illustration of the structure and RNA genome organization of Paramyxoviruses. (N) (formerly NP), nucleocapsid; P, phospho-protein; L, large polymerase protein; V, cysteine rich protein; M, matrix or membrane protein; F, fusion protein; HN, hemagglutinin-neuraminidase; SH, small hydrophobic protein).
Source: Adapted from ICTV, 2018.

The matrix protein (M) is the most abundant in the viral particle, besides, be important on the organization of viral morphogenesis. A layer of M protein is located between the envelope and the nucleocapsid, serving as anchorage to surface glycoproteins H and F (MACLACHLAN; DUBOVI, 2017). The RNA-binding protein, nucleoprotein (N), is highly conserved among the paramyxoviruses (more than 80%) and is responsible to control the transcription and replication of the genome (ARNS et al., 2012). When associated with the viral genome form a nucleocapsid of helical symmetry, measuring 13 to 18 nm of diameter

and 600 to 1,000 nm of extension (LAMB; PARKS, 2013). This ribonucleoprotein (N: RNP) complex is responsible to the protection of genome against nucleases and participates of the transcription and replication as a template of the RNA-dependent RNA-polymerase complex, formed to the association of large protein (L) and phosphoprotein (P) (LAMB; PARKS, 2013).

The L protein is the less abundant in the virion particle and is widely utilized in molecular diagnostics because is high conserved (ARNS et al., 2012). This protein when binding to the N: RNP through the P-carboxy, on the C-terminal portion of the P exercises the catalytic activity of the viral transcriptase, therefore, together the L and P proteins regulate transcription, replication, and the efficiency with which nucleoprotein is inserted and the nucleocapsid is assembled (LAMB; PARKS, 2013). Furthermore, the cytoplasmic aggregates known as inclusion corpuscles are formed by the N and P proteins (ARNS et al., 2012).

The additional non-structural proteins are not essential to viral replication, although are important in the virulence, evasion of the innate immune response, and survive of the virion in vitro. Furthermore, C protein helps in the regulation of RNA genomic synthesis (ARNS et al., 2012).

2.3.3. Replication

The replication of the paramyxovirus genome, as well as the viruses classified in the V class according to Baltimore, occurs integrally in the cytoplasm of the host cell. The infectious viral particles through glycoproteins of attachment in its surface bind to compatible receptors in the host cell and active form of F proteins (F1 and F2) responsible to fuse its envelope with the cytoplasmic membrane of the host cell. The fusion occurs in physiologic pH, because of this the paramyxoviruses are called as pH-independent virus. Syncytial formations are characteristic of paramyxoviruses infection and are formed as a resulting of the fusion of nearby cells. *In vivo* can cause tissue necrosis, once a multinucleated cell is less functional and walk gradually to death (ARNS et al., 2012; LAMB; PARKS, 2013).

The viral polymerase complex (L and P) transcribe the RNP and release it into the cytoplasm after the fusion to mRNA encoding the viral proteins. Each gene has initiation or termination signals and is transcribed with a sequential interrupted-synthesis mechanism to an individual polyadenylated mRNA with 5’ cap in the extremity. The mRNA synthesized is traduced by the host cell ribosome until having enough protein in the cytoplasmic. When the concentration of the N protein reaches a critical level, a promoter sequence at the 3’ end of the genomic RNA is transcribed and N protein binds to the emergent RNA chain. So, a complete

positive-sense antigenome is synthesized once the complex of N-RNA leads the polymerase to ignore the message-termination signals. This new product is the template that enabling the production of genomic RNA, thus amplifying dramatically the synthesis of viral proteins (ARNS et al., 2012; LAMB; PARKS, 2013).

Finally, the virus maturation begins with the incorporation of viral glycoproteins into patches on the host-cell plasma membrane. So, matrix protein (M) and other no glycosylated proteins associated with this altered membrane leads to the alignment of the RNP beneath the M protein and mature virions are released via budding (COX; PLEMBER, 2017).

2.4. *MORBILLIVIRUS* GENUS

Morbillivirus is an important genus in the *Paramyxoviridae* family, which shelter highly infection viruses of importance to both human and animal health (Table 1). The main spread route of this virus is the respiratory tract, beyond the morbilliviruses infection leads to severe immune suppression and has a capacity to cause outbreaks with high morbidity and mortality in previously unexposed populations (APPEL; SUMMERS, 1995; DE VRIES; PAUL DUPREX; DE SWART, 2015). The susceptibility of organisms to infection is defined through the distribution of receptors, e. g. Measles virus (MV) uses primate CD46 and CD150 as the receptor, while CDV and Ruminant pest virus use the carnivore and bovine CD150 receptor, respectively (NAMBULLI et al., 2016).

An important threat to human health is a morbillivirus known as MV that was described by the physician Rhazes (860-932bc) and is the prototype of morbilliviruses and is associated with a contagious viral disease in humans, the Measles. This virus was responsible for high rates of morbidity and mortality until an effective vaccine became available (NAIM, 2015). Domestic and wild animals are largely affected by morbilliviruses. Cattle and small ruminants are susceptible to RPV and Small ruminant morbillivirus (PPRV), respectively (COSBY, 2012). Several species of seal animals are vulnerable to Phocine Distemper Virus (PDV) infection which leads to distemper disease, besides this, *Cetacean morbillivirus* (CeMV) cause disease in dolphins and whales (BARRETT et al., 1993; DUIGNAN et al., 2014; VISSER et al., 1993). CDV is known to cause distemper in dogs and several species of wild carnivores and non-carnivores and recently, another morbillivirus named as *Feline Morbillivirus* (FeMV) was described in domestic cats (MARTELLA; ELIA; BUONAVOGLIA, 2008; WOO et al., 2012).

2.4.1. Morbillivirus: a Threat to Wild Animals

Since its discovery, morbilliviruses infections represent a serious threat to health of human and animals. Among domestic dogs and wild carnivores, the CDV is one of the most important infectious diseases, being the second major cause of death, losing to rabies only (DEEM et al., 2000). In domestic dogs and some captive species of carnivores, the CDV is controlled by vaccination and its worldwide distribution impossibilities the eradication (BARRETT, 1999). Recently, FeMV was reported and may be associated with CKD in domestic cats, although the origins of this virus and the potential for cross-species infection are still unclear (COSBY, 2012).

2.5. CANINE DISTEMPER VIRUS

Canine distemper virus (CDV) was first isolated by Carré in 1905 and is the etiologic agent of a severe multisystemic and globally distributed infectious disease which threats terrestrial carnivores and other wildlife species (Table 2), the canine distemper (CD) (APPEL; SUMMERS, 1995). The shedding of the virus occurs through bodily secretions, as urine, feces, and the skin, although aerosols and the contact with oral, respiratory, and ocular fluids containing the virus is the main form of transmission (DEEM et al., 2000; MARTELLA; ELIA; BUONAVOGLIA, 2008; SILVA et al., 2014). Although the viral shedding may occur up to 90 days after infection, the CDV is not stable in the environment and needs new hosts to persist among animal populations (BARRETT, 1999; DEEM et al., 2000).

Outbreaks in wildlife occur and usually have high morbidity and mortality rates, although the pathogenicity varies between species (MCCARTHY; SHAW; GOODMAN, 2007; SEIMON; MIQUELLE; CHANG, 2013). Factors related to viral and animal characteristics, including immune status, immune response, and age interfere in the complex pathogenesis of CDV which may be similar between domestic and non-domestic species (DEEM et al., 2000). Systemic infection with viremia is often present and after the first contact the virus spreads to the lymphatic system and can lead to pneumonia, enteritis, and encephalitis (WILLIAMS, 2001).

Terrestrial carnivores of all families are susceptible to CDV infections and this success of worldwide spread is due to the ability of the virus to break the barrier between carnivores hosts (VAN MOLL et al., 1995; WILLIAMS, 2001). *Canidae* are the most affected family in the order Carnivore and several species were reported as susceptible to CDV infection.

Table 2. Order, family, and species susceptible to CDV infection.

Order	Family	Species
Carnivora	*Canidae*	*Canis adustus, Canis aureus, Canis latrans, Canis lupus, Canis lupus baileyi, Canis lupus dingo, Canis lupus signatus, Canis mesomelas, Canis simensis, Cerdocyon thous, Chrysocyon brachyurus, Lycalopex culpaeus, Lycalopex griseus, Lycalopex gymnocercus, Lycalopex vetulus, Lycaon pictus, Nyctereutes procyonoides, Nyctereutes procyonoides viverrinus, Urocyon littoralis catalinae, Urocyon cinereoargenteus, Vulpes chama, Vulpes lagopus, Vulpes macrotis mutica, Vulpes velox, Vulpes vulpes, Vulpes zerda*
	Felidae	*Acinonyx jubatus, Felis lynx, Felis silvestris, Felis silvestris catus, Leopardus geoffroyi, Leopardus pardalis, Lynx Canadensis, Lynx lynx, Lynx pardinus, Lynx rufus, Panthera leo, Panthera leo persica, Panthera onca, Panthera pardus, Panthera pardus japonensis, Panthera tigris, Panthera tigris altaica, Panthera tigris tigris, Panthera uncial, Puma concolor*
	Mustelidae	*Enhydra lutris kenyoni, Galictis vittata, Galictis cuja, Gulo gulo, Lontra Canadensis, Lutra lutra, Martes foina, Martes martes, Martes melampus, Martes pennanti, Meles meles, Melogale moschata subauantiaca, Mustela erminea, Mustela itatsi sibirica, Mustela lutreola, Mustela nigripes, Mustela nivalis, Mustela putorius, Mustela putorius furo, Mustela vison, Neovison vison, Taxidea taxus*
	Procyonidae	*Nasua nasua, Potos flavus, Procyon lotor, Procyon pygmaeus*
	Ursidae	*Ailuropoda melanoleuca, Ursus americanus, Ursus americanus floridanus, Ursus arctos, Ursus arctos horribilis, Ursus arctos marsicanus, Ursus maritimus, Ursus thibetanus*
	Viverridae	*Arctictis binturong, Gennetta gennetta, Paguma larvata, Paradoxurus hermaphroditus, Viverricula indica*
	Hyaenidae	*Crocuta crocuta, Hyaena hyaena, Hyaena brunnea*
	Ailuridae	*Ailurus fulgens*
	Mephitidae	*Mephitis mephitis*
	Odobenidae	*Odobenus rosmarus*
	Otariidae	*Zalophus californianus*
	Phocidae	*Halichoerus grypus, Hydrurga leptonyx, Lobodon carcinophagus, Phoca caspica, Phoca sibirica, Phoca vitulina, Pusa caspica*
Rodentia	*Muridae*	*Mus musculus*
	Cricetidae	*Mesocricetus auratus*
	Sciuridae	*Marmota caudata*
	Caviidae	*Cavia porcellus*
Primates	*Cercopithecidae*	*Macaca fascicularis, Macaca fuscata, Macaca mulatta*
	Cebidae	*Saimiri sciureus*
Artiodactyla	*Suidae*	*Sus scrofa domestica, Sus Scrofa*
	Tayassuidae	*Tayassu tajacu*
	Cervidae	*Cervus nippon*
Pilosa	*Megalonychidae*	*Choloepus didactylus*
	Myrmecophagidae	*Tamanduá tetradactyla*
Proboscidea	*Elephantidae*	*Elaphas maximus*

Source: Adapted from Martinez-Gutierrez and Ruiz-Saenz, 2016.

2.6. FELINE MORBILLIVIRUS

Feline Morbillivirus (FeMV) is a new species of *Paramyxoviridae* family classified in the *Morbillivirus* genus, able to infect domestic cats. Until 2012 no paramyxoviruses specific to felids had been found (DEEM et al., 2000; HARDER et al., 2018; NAGAO et al., 2012; NAVA et al., 2008). Nambulli et al. (2016) believe that morbilliviruses may be evolved from a common ancestor and adapted to its hosts, therefore, justifies the ability of these viruses to adapt to new hosts and the emergence of new morbillivirus species.

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2.6.1. Biological Characteristics

The complete genome of FeMV has 16,050 bp and encodes 8 non-structural and structural proteins (N, P/V/C, M, F, H, and L) and obeys the rule of 6, similar to other Morbilliviruses (SIEG et al., 2018). The amino acid (aa) lengths of the 6 structural proteins coded by the FeMV genome are N, 519aa; P, 491aa; M, 337aa; F, 543aa; H, 595aa, and L, 2.202aa (MARCACCI et al., 2016). However, although classified as morbillivirus, the FeMV possesses peculiar characteristics such as, the great genome length in relation to the other members of the genus, the capacity of infect kidney cells, and lack of known severe acute signs upon its infection (NAMBULLI et al., 2016).

The FeMV can be cultured *in vitro* in all cat cell lineages including CRFK (Crandell-Reese feline kidney), beyond in Vero cell lineage of non-human primate. In optimum conditions of incubation, this virus causes characteristic cytopathic effects (CPE) to paramyxoviruses, as the syncytia formation and cell death (Figure 4A and 4B) (KOIDE; SAKAGUCHI; MIYAZAWA, 2015).

FeMV is relatively stable at environmental conditions, remaining infectious under a temperature of 37ºC for 12 days with a gradual decrease of the infectivity. However, in 4ºC the same period resulted in no reduction in infectivity. Thus, these characteristics may not only facilities the virus spread as its long-term storage. Heat treatments can reduce the viral titers quickly, at 60ºC for 10 min the viral titers are reduced to undetectable levels, while at 70ºC this time is reduced to 2 min. The FeMV infectivity is also destroyed when it is exposed to lipid solvents, non-ionic detergents, formaldehyde, and oxidizing agents (KOIDE; SAKAGUCHI; MIYAZAWA, 2015).

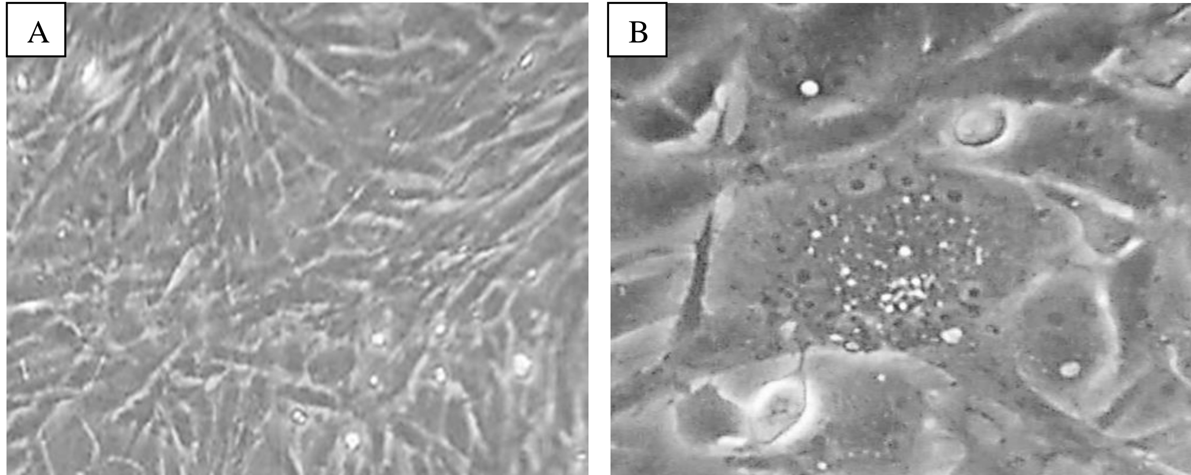

Figure 4. *Feline Morbillivirus* cytopathic effects. **A)** In vitro culture of *Crandell-Reese feline* kidney (CRFK) cells with normal structure and morphology. **B)** Presence of cytopathic effects with syncytia formation in a culture of CRFK cell, infected with *Feline Morbillivirus* strain from a domestic cat.
Source: Laboratório de Virologia – UEL.

2.6.2. Epidemiology

In 2012, occurred the first detection of FeMV in urine samples of cats in Hong Kong, China. The hypothesis of the group of researchers was based on the fact that until that moment it was not detected any paramyxovirus specific to felines (WOO et al., 2012). However, the biology and pathogenicity of FeMV are still not well understood, though studies suggest a correlation between the FeMV infection and the development of nephropathies such as tubulointerstitial nephritis (TIN) and CKD (LORUSSO et al., 2015; SIEG et al., 2015; WOO et al., 2012).

The FeMV was also detected in urine, blood, and kidney tissue of symptomatic and asymptomatic cats from different regions of Japan (FURUYA et al., 2014; SAKAGUCHI et al., 2014). Therefore, studies have suggested that FeMV are prevalent in cats from the Kyoto area and animals may be persistently infected (SAKAGUCHI et al., 2014). These results may also indicate a broad geographic distribution in the Asia continent.

The first report of the FeMV in Europe occurred in Italy, in a urine sample of a geriatric cat with nephropathy and none other viruses usually correlated with kidney diseases were detected (LORUSSO et al., 2015). Other studies conducted in Germany report a positive correlation between FeMV infection and the presence of various uropathies since only cats with uropathies were positive in the PCR assays (SIEG et al., 2015).

The description of the FeMV in the American continent occurred firstly in the United States where the presence of the virus in urine samples of symptomatic and asymptomatic domestic cats was detected (SHARP et al., 2016). The FeMV was also detected in South American cats, from Brazil, however, it was not possible to relate the presence of the virus RNA in urine and kidney diseases (DAROLD et al., 2016). FeMV was detected in others formalin-fixed tissues beyond the kidney, such as lung, liver, spleen, and lymph nodes in cats from Turkey and no relation was found between the FeMV infection and the presence of uropathy in the infected cats (YILMAZ et al., 2017). So, it is believed that the FeMV has a worldwide distribution, as well as the viruses that belong to the *Morbillivirus* genus (NAMBULLI et al., 2016).

Beyond FeMV another paramyxovirus closely related to rodents and bat paramyxoviruses was described in domestic cats, the species *Feline Paramyxovirus* (FPaV). The sequence similarity between the FeMV and the FPaV (larger than 85%) is accepted to define a new viral species, although it is possible that the FPaV represent only a possible genotype of FeMV (SIEG et al., 2015).

The emergence of new species or genotypes may be attributed to recombinant events, and in FeMV these events may be favored by chronic or persistent infection. Beside that recombination of genes related to entry of the viruses in the host cell may increase the host range of the virus. For example, the ability of CDV to infect different hosts is attributed to recombination in the H gene region of the, which was found in a sample from a giant panda (HAN; LIU; LI, 2008). So, natural recombinants events in F and H genes of FeMV have been reported and might result in changes in the host range of this virus (PARK et al., 2014), increasing the importance of epidemiological investigations of the FeMV, including wild mammals.

2.6.3. Diagnostic

RT-PCR and RT-SNPCR assays are the molecular techniques of choice for the diagnosis of FeMV, since it is sensitive and specific, allowing not only the detection of the virus but also molecular characterization through sequencing and phylogenetic analysis of amplified products (FURUYA et al., 2014; WOO et al., 2012). The L gene is widely used for molecular diagnosis because it is a conserved sequence in the genome (TONG et al., 2008). The *Real-time* PCR (*q*PCR) represent a good choice since in addition to amplifying is able to quantify the viral load in the sample (DE LUCA et al., 2018).

Beyond these conventional molecular techniques, the RT-LAMP (reverse transcription loop-mediated isothermal amplification) allows the extraction and the amplification in very little time, around 60 minutes, and was tested with success (KOIDE et al., 2016). Single-Primer Amplification (SISPA) that amplify a target independent in the genome, has been used associated with the Next-generation sequencing (NGS) to amplification of the complete genome of FeMV (MARCACCI et al., 2016). This combination provides the detection and the complete genomic sequences to genetic characterization of the virus (CHRZASTEK et al., 2017). To these analyses the urine, blood, rectal swab and kidney tissue from cats were used with success in molecular detection of FeMV (LORUSSO et al., 2015; PARK et al., 2016; WOO et al., 2012)

The FeMV isolation in the cell may be onerous, however, it is considerate the gold standard for virus detection. Multiples passages are requested to evaluate the cytopathic effect of the FeMV infects in cell culture and as well as another paramyxovirus in high passages, after 3-5 days is possible to visualize the CPE with syncytia formation, cell rounding, and dead cells in the supernatant. Studies have shown that CRFK and Vero cells are the most adapted to the in vitro growth of FeMV. (KOIDE; SAKAGUCHI; MIYAZAWA, 2015; SAKAGUCHI; KOIDE; MIYAZAWA, 2015).

The immunohistochemistry technique is an alternative to detect the FeMV in formalin-fixed tissues, utilizing monoclonal antibodies from guinea-pigs previously infected with N protein of FeMV (WOO et al., 2012). Indirect techniques can also be used in the FeMV diagnostic, such as the indirect immunofluorescence (IFI) and Western Blot technique to screen cat sera for anti-FeMV antibodies (WOO et al., 2012; SAKAGUCHI et al., 2014; SHARP et al., 2016; KOIDE; SAKAGUCHI; MIYAZAWA, 2015; YILMAZ et al., 2017).

Moreover, complementary techniques such as hematological and biochemical serum analyzes, urinalysis and renal histopathology can also provide additional information about animal clinical status and allow to relate the findings with the infection by the FeMV (WOO et al., 2012; YILMAZ et al., 2017).

3. CONSIDERATIONS

The proximity of the wild animals with the urban centers, due to anthropic impacts, increases their exposure and the possibility of interspecies transmission of infectious agents, such as viruses belonging to the *Paramyxoviridae* family. This family, besides shelter viruses of great importance for human and animal health has a great ability to break interspecies barriers and infect new hosts. Opossums due to these abilities to inhabit urban center and transit between different ecological niches are frequently exposed to several infectious pathogens and can become a host to several paramyxoviruses. Moreover, these animals can represents an important source of infection and dissemination of viruses between domestic animals, wild, and even humans.

Therefore, molecular investigations of the presence of paramyxovirus in opossums can provide relevant ecological and epidemiological data. The information may also aid in the control of epidemics and in eradication measures of paramyxoviruses among domestic and wild animals. In addition, knowledge of paramyxovirus infection in opossums may provide us information about the threat of this virus to the health and conservation of this species.

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4. OBJECTIVES

4.1. General objectives

To evaluate the presence of paramyxovirus in samples from synanthropic opossums collected in the central north region of the state of Paraná and, to evaluate their importance as a disseminator of the virus and the risk of the infection to their health.

4.2. Specific objectives

- To detect, by means of RT-PCR and RT-SNPCR assay, the N and L-protein gene, respectively, of paramyxoviruses in samples from white-eared opossums (*Didelphis albiventris*);

- To perform comparative phylogenetic analysis of viral strains found in the white-eared opossum with the Paramyxovirus strains available in public databases;

- To perform histopathological and immunohistochemical assays in the positive tissues to the Paramyxovirus L gene through RT-SNPCR in order to evaluate the presence of lesion and N-viral protein, respectively;

- To perform isolation in cell culture of paramyxovirus detected through molecular and histopathological assays in the opossum tissues;

- Georeferencing the collected animals in order to evaluate their proximity to urban centers and the possibility of acting as disseminators of paramyxovirus.

5. ARTICLE FOR PUBLICATION

5.1 *Feline Morbillivirus*: first molecular identification in white-eared opossums (*Didelphis albiventris*, Lund 1840) inhabitant peri-urban areas of northern Parana, Brazil

Abstract

Feline Morbillivirus (FeMV) was firstly detected in 2012 in domestic cats from Hong Kong and was associated with tubulointerstitial nephritis (TIN) and chronic kidney disease (CKD), although in subsequent studies in other countries had been detected in healthy cats. However, is not clear if FeMV plays a role as pathogens in kidney diseases of cats and other epidemiological data are still unknown. Until now, studies report the presence of FeMV exclusively in domestic cats. This study shows the first molecular detection of the FeMV RNA associated with pathological and immunohistochemical findings in a synanthropic marsupial, the white-eared opossum (*Didelphis albiventris*), inhabiting peri-urban areas of northern Parana. Molecular techniques identified the RNA and the immunohistochemistry (IHC) assays detected the N protein of FeMV in the lung and kidney tissues. The main histopathological findings were interstitial pneumonia, lymphocytic nephritis, and tubular necrosis. A FeMV opossum strain was isolated in Crandell Rees feline kidney lineage cell showing syncytia formation and cell death. Therefore, these results evidence the ability of FeMV in to infect other mammals species and reinforces the possibility of the opossum to be a disseminator of this virus among domestic and wild animals.

Keywords: immunohistochemical, lung, kidney, tubulointerstitial nephritis.

Archive in accordance to the to the publication standards of the journal Archives of Virology, Available in:
https://www.springer.com/biomed/medical+microbiology/journal/705?detailsPage=pltci_1060661

Introduction

The great scale of urbanization is changing the composition and interaction of the environment with wildlife communities, including the transmission ecology of pathogens [1]. Consequently, opportunistic species that inhabit in peri-urban areas, benefiting from the dwelling and remnants food from humans and domestic animals, known as synanthropic, may be important components in the dissemination of infectious diseases [2].

White-eared opossum (*Didelphis albiventris* Lund, 1840) are omnivorous marsupial with synanthropic habits, highly adapted to urban environments and able to remain in very devastated areas [3]. This species of opossum is largely distributed in South America and is found through Brazilian territory [4]. Currently, the opossums are considered the definitive host or reservoir of several parasites, bacteria, and viruses such as *Trypanosoma cruzi, Leptospira spp,* and *Vaccinia virus,* respectively [5–7]. Moreover, the proximity with cities, important hubs of pathogens, may increase the risk of exposure to infectious diseases and its potential as source and disseminator of infectious diseases, once it transits between different ecological niches [1, 8]. Due to its synanthropic status, the knowledge of pathogens in this species can provide important ecological and epidemiological data. However, studies with viral agents are still scarce, mainly with viruses that represent important threats to wildlife conservation and human health, due to the zoonotic potential, as well as the paramyxoviruses.

The *Paramyxoviridae* family belongs to the *Mononegavirales* order and comprises enveloped viruses with a non-segmented single-stranded negative RNA genome [9]. Currently, there are seven genus in this family, *Aquaparamyxovirus, Avulavirus, Ferlavirus, Henipavirus, Morbillivirus, Respirovirus*, and *Rubulavirus* [9]. Furthermore, among the RNA viruses that belong to the paramyxoviruses family shows one of the highest rates of cross-species transmission [10].

Among the paramyxoviruses, the *Morbillivirus* genus has highly infectious viruses worldwide distributed that represents a threat to humans, domestic animals, and the wildlife once is able to break interspecies barriers [11]. Canine distemper virus (CDV) is one of the most important morbilliviruses responsible for severe multisystemic infectious diseases of canids, able to trigger off outbreaks with high morbidity and mortality rates in all families of terrestrial carnivores, including small felids and other species of wild mammals [12]. However, in 2012, a research group from China detected in domestic cats a new virus species close related to morbillivirus, named *Feline Morbillivirus* (FeMV) [13]. After, the FeMV was

detected in domestic cats in other countries around the world, including Brazil [14], so, it is believed that the virus has a worldwide distribution as well as other viruses that belong to *Morbillivirus* genus [15].

However, although be classified as a morbillivirus, the FeMV possesses different characteristics, as a genome sequence length relatively distant from the other species of the genus [16]. The FeMV complete genome has 16,050 base pairs, the longest in *morbillivirus* genus and encodes 8 non-structural and structural proteins (N, P/V/C, M, F, H, and L) [9, 17]. Among paramyxoviruses the L and N genes are the most conserved, so due to it characteristic the L gene is used in broadly reacting PCR assays to diagnostic of several species of paramyxoviruses [18]. FeMV has lack of known severe acute signs upon infection, diverging of other species in the same genus. Furthermore, the FeMV is able to infect kidney tissues and may be associated with tubulointerstitial nephritis (TIN) and chronic kidney disease (CKD) in domestic cats [15, 16], although it has also been detected in cats without renal diseases [14].

The chronic infection condition of the FeMV in cats may favor recombinants events in the genome of the virus and consequently to increase in the host range of the virus[17, 19]. However, the biology, including virulence, pathogenicity, and host range of the FeMV is still not well understood. Therefore, the aim of this study was to investigate the presence of importance paramyxoviruses for both human or/and animal health in samples from white-eared opossums, from the central north region of Parana state, using a consensual primer for the Paramyxoviridae family. In addition, this study aims to understand the importance of these animals in the cycle of paramyxoviruses infection, as reservoir and disseminator.

Material and Methods

Ethics Committee

The methodology adopted in this study was approved by the Animal Experimentation Ethics Chamber of the Faculty of Veterinary Medicine and Animal Science of Universidade Estadual de Londrina-UEL (CEUA: 2764.2017.41) and SISBIO/ICBio nº553841. The opossums (Didelphis albiventris) evaluated in this study are part of a multidisciplinary project with road killed wild animals in the central northern mesoregion of Parana state/Brazil developed in Medicine Veterinary Preventive Department, Universidade Estadual de Londrina-UEL.

Animals and local of study

During the months of March 2017 to August 2018, free-living opossums road killed were collected in five cities of the central northern mesoregion of Parana state (Figure 1). The collections were performed in pre-established transects or connection of the population to our research group. The animals found in conditions of collect were sent to Universidade Estadual de Londrina-UEL for analysis. According to age, the opossums were further classified as adult, young, and joeys. During the autopsy, fragments of the cerebrum, lung, heart, tonsil, spleen, lymph nodes, kidney, urinary bladder, liver, and intestines, were collected for molecular and histopathologic analyses.

Georeferencing

Statistical analyses were performed with the spatial point distributions of the road killed and collected opossums and also of the confirmed infection-FeMV infected to obtain the kernel density estimation. The Kernel density is an interpolating and smoothing technique to generalize point locations in order to detect areas with high occurrences of infections.

Nucleic Acid Extraction, Molecular Analysis, and Sequencing

Nucleic acid extraction from tissues samples (tonsil, lymph nodes, kidney, urinary bladder, liver, and lung) was performed with 100mg of each fragment, pre-treated with proteinase K (Ambion, Kaufungen, Germany), using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate nucleic acid extraction methods [20]. In all procedures were included aliquots of sterile ultrapure water as a negative control.

A reverse transcription semi-nested PCR (RT-SNPCR) assay to amplify a partial fragment of the paramyxoviruses L gene was performed with a consensual primer able to amplify 25 species of the Paramyxoviridae family. In addition, reverse transcription PCR (RT-PCR) assay to amplify a fragment of morbilliviruses N gene were also performed. The primers used are shown in Table 1. The amplicons were analyzed through electrophoresis on a 2% agarose gel in TBE buffer, pH 8.4 (89mM Tris, 89mM boric acid, 2mM EDTA), containing ethidium bromide (0.5µg/ml) and visualized under UV light.

Amplicon with good quality in the RT-SNPCR to the L gene were purified using the commercial PureLink Quick Gel Extraction and PCR Purification Combo kit (Invitrogen Life Technologies, Carlsbad, CA, USA), quantified in Qubit Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA), and sequenced with BigDye Terminator v3.1 Cycle

Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in ABI3500 Genetic Analyzer automatic sequencer. The quality of the sequences obtained was evaluated in PHRED and the contig assembled was obtained in CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). The nucleotide (nt) sequences obtained were compared with nt sequences deposited in GenBank to verify the similarity. The phylogenetic tree based on the nt sequences was obtained using the maximum-likelihood method with the Kimura 2-parameter model with 1,000 replicates in the MEGA-X. The nt and amino acid (aa) sequence identity matrices were performed in BioEdit version 7.1.3.0.

Histopathologic and Immunohistochemical analyzes

Fragments of lung and kidney previously positive for FeMV in the RT-PCR or RT-SNPCR were fixed by immersion in 10% buffered formalin solution and routinely processed for histopathological evaluation with hematoxylin and eosin stain to identify possible histopathological patterns. Formalin fixed paraffin-embedded (FFPE) fragments of the lung and kidney were used in IHC assays [21] designed to identify a previously described FeMV [13] and CDV antigens [22]. An anti-FeMV N serum produced in immunized guinea pig, served as the primary antibody for FeMV [13] kindly given by Darold et al. (2016); Positive controls consisted of FFPE renal sections from domestic felines previously known to contain FeMV antigens. A commercially produced antibody (CDV-NP, VMRD, Pullman, Washington, EUA) was used to detect antigens of CDV. Positive controls consisted of FFPE cerebellar sections of dogs previously known to contain antigens of CDV [22]; for negative control the primary antibody was replaced by the commercial diluent of the primary antibody. Positive and negative controls were included in each IHC assay.

Viral Culture and isolation

Positive and sequenced-FeMV sample was selected for viral isolation. Briefly, to a macerated fragment of 100mg of the lung tissue sample was added 1.0ml of phosphate-buffered saline solution (PBS) and homogenized vigorously. Then, the preparation was centrifuged at 1,000xg for 5 min to clarify and the supernatant was collected and filtered. Further, to 500µL of the filtered, 10µL of streptomycin (2.5%) antibiotic and 400µL of eagle minimal essential medium (MEM) was added. The preparation was stored overnight at 4°C. Crandell Rees feline kidney (CRFK) cells culture bottles were used to inoculate the prepared. After 3h at 37ºC in slow stirring to adsorption, the inoculum was not washed out the bottles and was added 10ml of MEM. The cultures were incubated for 7 days at 37ºC in a humidified

atmosphere with 5% CO_2. The virus isolation was daily observed to identify the cytopathic effect (CPE) by light microscopy. Aliquots of the cell culture isolation were frozen at -80ºC and thawed to harvest cell lysates and an aliquot was used to RT-SNPCR with the protocol previously described in this study.

Results

Epidemiological data

Twenty-three opossums were collected during the 2017 and 2018, provided of the roads of Central northern of Parana state in forest patches in peri-urban areas. The highest number of animals collected occurred in the autumn (n=12, 52%) and in the winter (n=6, 26%) seasons. Out of 23 animals, 17 were males (73.9%) and 6 were females (26.1%). According to age, the animals were classified as a joeys (n=1), young (n=12), and adults (n=10) (Table 2).

Georeferencing

The kernel density estimation analysis based on the distribution of the road killed opossums show a larger cluster of collects in the Londrina (Fig 2A). A positive correlation between the infected opossums and the sites of the collection was not established (P=0.1772) (Fig 2B). Proximity between the infected opossums and the urban centers was observed (Fig 2C).

Molecular and Phylogenetic analyses

The L and N genes of paramyxoviruses were detected for molecular assays in 26% (6/23) of the lung tissues from opossums analyzed. While in the kidney tissues only two samples were positive to the N gene and none sample was positive to the L gene (Table 2). None other analyzed tissue was positive to the L and N genes of paramyxoviruses.

Through nt identity analyses it was possible to determine the paramyxovirus found in opossums samples: Feline Morbillivirus. The FeMV opossum strains herein this study exhibited high nt (99.6%) and aa (99.4%) similarity each other and high nt (90.2% to 96%) and aa (96.3% to 97.9%) similarity with other FeMV strains deposited in GenBank (Table 3). With other morbilliviruses species the FeMV opossum strains showed lower nt and aa similarity (66.1% and 71.5% to 72% with measles virus; 67% to 67.1% and 72% to 72.5%

with rinderpest virus; 66.6% to 66.8% and 72.5% to 73% with CDV; 68.7% to 69% and 71.5% to 72% with phocine distemper virus; 69% to 69.4% and 73.5% to 74% with dolphin morbillivirus, and 69.2% to 69.5% and 73.5% to 74% with cetacean morbillivirus), respectively. In the phylogenetic tree the FeMV opossum strains clustered with Feline Morbillivirus strains, however, formed a new branch (Fig 3).

Histopathology and Immunohistochemical FeMV infection

The immunohistochemical results and frequency of the main histopathological lesions observed in the lungs and kidneys are shown in table 2. In the lungs, the main histopathological lesion observed was interstitial pneumonia (66,6%; 4/6) - characterized by marked thickening of the alveolar septa, due to hyperplasia of type II pneumocytes and accumulation of alveolar macrophages (Fig. 4A). In the kidneys, mononuclear interstitial nephritis (33.3%; 2/6), mainly lymphocytic, and tubular necrosis (33.3%, 2/6) were observed. Positive immunoreactivity for FeMV antigens was observed in 83.33% (5/6) of the kidneys, mainly in the tubular necrosis region. Similarly, in the lungs 83.33% (5/6) the immunostaining occurred in the bronchial epithelia and in mixed glands.

Viral isolation

The BRA013/2018 strain from lung led to CPE with syncytia formations and cell death after six passages in CRFK (Fig 5). The aliquot of viral isolation cell was positive for the L gene in RT-SNPCR assay.

Discussion

FeMV is an emerging virus detected for the first time in domestic cats (*Felis catus*) from Hong Kong, China, and subsequently in other several countries, including Brazil [13, 14, 19, 23–26]. This study reports the first detection of FeMV in a non-cat host, the synanthropic mammal white-eared opossum, from Brazil. Therefore, we suggest that the FeMV may be able to infect other mammals species.

In molecular assays, the FeMV L gene was detected only in the lung tissues; on the other hand, the N gene was detected in the lung and kidney tissues. These results suggest that although the FeMV are able to infect the kidney tissue of the opossums, this organ probably is not the main site of replication and maintenance of the virus in this species. In addition, the

respiratory tract may be not only the primary site of replication but also the main route of the FeMV virus elimination when infecting opossums.

The FeMV has been associated with uropathies such as TIN, and CKD in domestic cats, although there are divergences in this regard [13, 14, 26, 27]. The TIN is a disorder in tubules and kidney interstitium appointed as the main cause of the development of CKD, which in turn is characterized by the loss of more than 66% of the functional units of the kidney [26, 28]. In the kidney tissues from FeMV- positive opossums herein analyzed the lymphocytic tubular nephritis, tubular necrosis, and inflammatory cell infiltrations were found. However it was not possible to establish a statistical correlation between the FeMV infection and the lesions, due to the small number of samples. Therefore these findings are in accordance with that found in kidney tissues of domestic cats infected with FeMV, mainly the inflammatory condition [29]. In the other hand, histopathological findings in the lungs tissues of FeMV-positives opossums, characterized as lymphocytic interstitial pneumonia, are compatible with viral infections, including morbilliviruses infection such as CDV [30], however, there are no data about histopathological findings in lung tissues of domestic felids FeMV infected.

Furthermore, five of the 6 opossums previously positives to FeMV showed FeMV-N protein in the inflammatory lesions in kidney tissues, mainly in necrotic tubular cells through immunohistochemical staining. Therefore, the necrosis in tubular cells may be related to FeMV infection. In contrast with other reports, in this study, FeMV-N protein-positive mononuclear cells were not found [13, 24]. In the lung tissue from five opossums, bronchi epithelial cells and mixed glands were immunoreactivity to FeMV-N protein, reinforcing the epitheliotropic characteristic of morbilliviruses [15]. In addition, immunohistochemical against CDV N protein in the kidney and lung tissues resulted in no staining.

In the nt and aa analysis, the BRA003/2018 and BRA013/2018 FeMV strains revealed high similarity to each other and with FeMV strains from Japan [27] so we suggest that there is no positive correlation with the geographic distribution. On the other hand, we observed a correlation with the host species, once the opossum FeMV strains formed a new branch in the phylogenetic tree a part to the FeMV strains detected in domestic cat hosts. A lower similarity were observed among Brazilian FeMV strains described herein with FeMV strains from Thailand, China, Italy, and USA [13, 19, 31]. Comparison with the other Brazilian FeMV strains from domestic cats already described [14] was not possible because the analyses were realized using different regions of the L gene.

The transmission mechanisms and the host range of the FeMV are still obscure. *In vitro* studies using cell infection demonstrated that FeMV is able to infect only cell lineages

originated from cat and non-human primates [32]. However, in the present study the presence of FeMV RNA, in a natural infection, from a non-cat host, the white-eared opossum was detected. It can be explained due to the known ability of the morbilliviruses to break the interspecies barrier and to adapt in new mammal hosts since it is likely that they evolved from a common ancestor [11]. Furthermore, recombinants events have been reported in the region of FeMV genome that encodes proteins related to entry of the virus in the host cell (F and H) and may increase the host range of the virus [17]. Therefore, the possibility of this virus infects other mammals species cannot be discarded [32].

Infected cats can shed the virus from several days and until months through the urine and may represent an important source of environmental contamination [19]. Sharp et al (2016) detected the FeMV RNA in a urine sample from a healthy cat for 15 months after the first identification. However, due to the biological characteristics common to all morbilliviruses, the FeMV is susceptible to the environmental conditions and direct contact appears to be the main form of virus dissemination [33]. Beyond the direct transmission it is suspected that other species of mammals can serve as a vector, important in the epidemiology of the infection. Due to the high similarity found in phylogenetic analyses among strains isolated from cats from China and Japan, the authors suggested that synanthropic mammals, which are able to migrate very long distances, like bats and rodents, may be important vectors [27].

The opossums are known to be the definitive hosts or reservoirs of several infectious pathogens with zoonotic potential, e.g. *Trypanosoma cruzi, Leptospira* spp., and *Vaccinia virus* [5–7]. Besides that, due to its synanthropic habits have the capacity to live in degraded environments, near of the urban centers increasing their exposure to infectious agents and make them a potential disseminator of infectious diseases to humans, wild, and domestic animals [2].

In this study, it was possible to observe the proximity of the FeMV-positive white-eared opossums with peri-urban fragments of forest, although in accordance with the georeferencing and kernel density estimate, there is no correlation between the location of opossums collects and the presence of FeMV. Beyond, adult males were the main captured animals possibly due to a peculiar characteristic of mammals, in which the male performs larger detachments when in comparison with the females [34]. This habit may have favored not only road kill but also the greater contact with domestic animals in domiciled regions and therefore the exposure to infectious agents such as FeMV.

In the city of Londrina there are approximately 30,336 domestic felines according to the last survey, equivalent to 1 cat for every 17.9 human inhabitants of the city [35] that although are domiciled, have some access to the street. Therefore, in view of these information, the previously knowledge of nocturnal habits of both, domestic felids and opossum, and that the opossums eventually seek food in residences [4], it is clear the possibility of the transmission of infectious pathogens between these species.

In domestic cats, data indicates that the infection with FeMV is closely related to kidney tissue injuries, leading to TIN and CKD [13]. Furthermore, the FeMV RNA has already been found in kidney, lung, intestine, spleen, liver, and lymph nodes tissues of infected cats in Turkey [24]. An *in vitro* study was demonstrated the ability of FeMV to infect epithelial, fibroblastic, lymphoid, and glial cells indicating that the receptors to the virus are expressed in all of these cat's cells [32]. So, is probable that the FeMV may cause not only nephropathy in infected cats, as also multisystemic infection including encephalitis, common in CDV infections.

Additional *in vitro* analysis was performed to evaluable the behavior of the opossum FeMV strain (BRA013/2018) and the cytopathic effects observed were in accordance with other studies conducted with domestic cats samples [13, 32, 33]. These results reinforced the ability of the opossum be a reservoir and disseminator of this virus among domestic cats, considering that the opossum FeMV strain was able to infect *in vitro* a cat cell lineage.

Conclusion

This is the first molecular detection of FeMVstrains from a synanthropic mammal, the white-eared opossum from Brazil, closely related to the FeMV strains previously detected in domestic cats. The immunohistochemical assay demonstrated the presence of N protein of the virus in the kidney and lung tissues and the histopathological findings were compatible with the FeMV infection. Moreover, the FeMV strain from opossum is still able to infect cell lineage from cats once the isolation in CRFK cell culture was performed, reinforcing the possibility of this species to serve as a disseminator of the virus. However, more studies are necessary to be performed to better understanding the pathogenicity of the FeMV in opossums and to evaluable the risks to its health

Table 1. Set of primers used to amplify L and N genes of *Paramyxoviridae* family and *Morbillivirus* genera, respectively.

Primer	Target	Sequence (5' - 3')	Position	Amplicon size	Reference
Par-R	*Paramyxoviridae*	GCTGAAGTTACIGGITCICCDATRTTNC	4233 - 4260	662bp	(TONG et al.,
Par-F1	(L gene)	GAAGGITATTGTCAIAARNTNTGGAC	4869 - 4894		2008)
Par-F2		GTTGCTTCAATGGTTCARGGNGAYAA	4790 - 4816	584bp	
PI-1	*Morbillivirus*	ACAGGATTGCTGAGGACCTAT	769 - 789	287bp	(FRISK et al.,
PI-2	(N gene)	CAAGATAACCATGTACGGTGC	1055 - 1035		1999)

Table 2. Detection of *Feline Morbillivirus* from tissue samples from opossums according to season, age, gender, and city.

ID[a]	Season	Age	Gender[b]	City	RT-SNPCR (L)[d]		RT-PCR (N)[d]		IHQ[e]		Histopathology[f]	
					Lung	Kidney	Lung	Kidney	Lung	Kidney	Lung	Kidney
1	Autumn	Young	F	Londrina	-	-	-	-	.	.	.	.
2	Autumn	Young	F	Uraí	+	-	+	-	+	+	I.P.	T.I.N.
3[*]	Autumn	Adult	M	Cambé	+	-	+	+	+	+	.	T.I.N.
4	Autumn	Adult	M	Londrina	-	-	-	-	.	.	.	-
5	Autumn	Adult	M	Faxinal	-	-	-	-	.	.	.	.
6	Autumn	Young	M	Londrina	-	-	-	-	.	.	.	.
7	Autumn	Adult	M	Londrina	-	-	-	-	.	.	.	.
8	Autumn	Young	M	Londrina	-	-	-	-	.	.	.	.
9	Autumn	Young	M	Londrina	-	-	-	-	.	.	.	.
10	Autumn	Young	F	Londrina	-	-	-	-	.	.	.	.
11	Winter	Young	M	Londrina	-	-	-	-	.	.	.	.
12	Winter	Adult	M	Ibiporã	+	-	+	-	+	-	I.P.	T.N.
13[*]	Winter	Joyes	M	Londrina	+	-	+	+	+	+	I.P.	.
14	Winter	Adult	M	Londrina	-	-	-	-	.	.	.	.
15	Winter	Adult	M	Londrina	-	-	-	-	.	.	.	.
16	Winter	Joeys	M	Londrina	-	-	-	-	.	.	.	.
17	Spring	Adult	F	Londrina	+	-	+	-	+	+	I.P.	-
18	Spring	Adult	F	Londrina	-	-	-	-	.	.	.	.
19	Spring	Adult	M	Londrina	-	-	-	-	.	.	.	.
20	Spring	Young	M	Londrina	+	-	-	-	.	+	.	T.N.
21	Summer	Young	M	Londrina	-	-	-	-	.	.	.	.
22	Autumn	Young	M	Londrina	-	-	-	-	.	.	.	.
23	Autumn	Young	F	Londrina	-	-	-	-	.	.	.	.

[a] * Strains BRA003/2018 and BRA013/2018

[b] F, female; M, male.

[d] +, positive; -, negative.

[e] IHQ, Immunohistochemical assay anti-FeMV N protein; +, positive; -, negative; ., not tested.

[f] T.I.N., Tubulointerstitial Nephritis; I.P., Interstitial Pneumonia; T.N., Tubular Necrosis.

Table 3. Pairwise comparison of partial nucleotide (bottom left) and amino acid (upper right) sequences of the FeMV L gene.

Strains	Similarity in percentage										
	BRA003/ 2018*	BRA013/ 2018*	SS1	OtJP001	Thai-U16	MiJP003	761U	M252A	Piuma/2015	SS3	US1
BRA003/2018*		99.4	97.4	97.4	97.4	96.3	97.4	97.4	96.8	97.4	97.4
BRA013/2018*	99.6		97.9	97.9	97.9	96.8	97.9	97.9	97.4	97.9	97.9
SS1	95.7	96.0		1	98.9	97.9	98.9	98.9	98.4	98.9	98.9
OtJP001	95.5	95.8	99.6		98.9	97.9	98.9	98.9	98.4	98.9	98.9
Thai-U16	90.7	91.0	92.7	92.4		98.9	1	1	99.4	1	1
MiJP003	90.7	91.0	92.9	92.6	98.4		98.9	98.9	98.4	98.9	98.9
761U	90.5	90.8	92.9	92.9	93.9	94.1		1	99.4	1	1
M252A	90.5	90.8	93.4	93.4	97.9	98.1	94.3		99.4	1	1
Piuma/2015	90.3	90.5	92.0	92.0	94.3	94.1	95.3	94.3		99.4	99.4
SS3	91.0	91.4	93.2	92.9	98.7	99.3	94.5	98.4	99.4		1
US1	90.2	90.5	92.6	92.2	93.6	93.8	98.9	93.6	99.4	1	

Strain followed by the access number in GenBank: SS1 (AB910309); OtJP001 (AB924120); Thai-U16 (MF627832); MiJ003 (AB924121); 761U (NC_039196); M252A (JQ411016); Piuma/2015 (KT824132); SS3 (LC036587); US1 (KR014147).

*FeMV strains from this study.

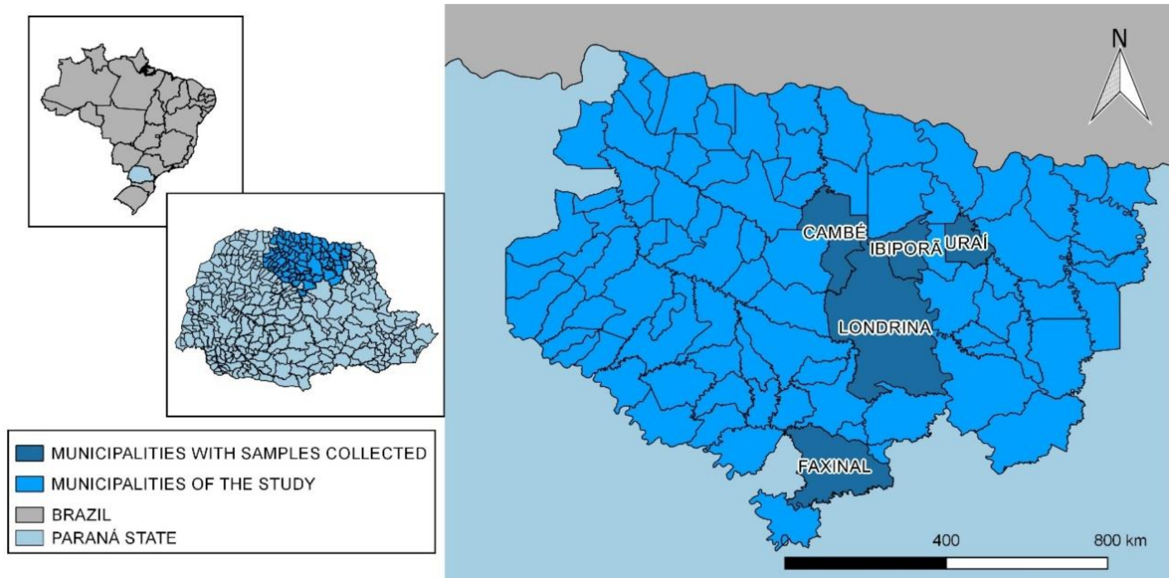

Fig 1 Maps showing the counties of Paraná state included in the study.

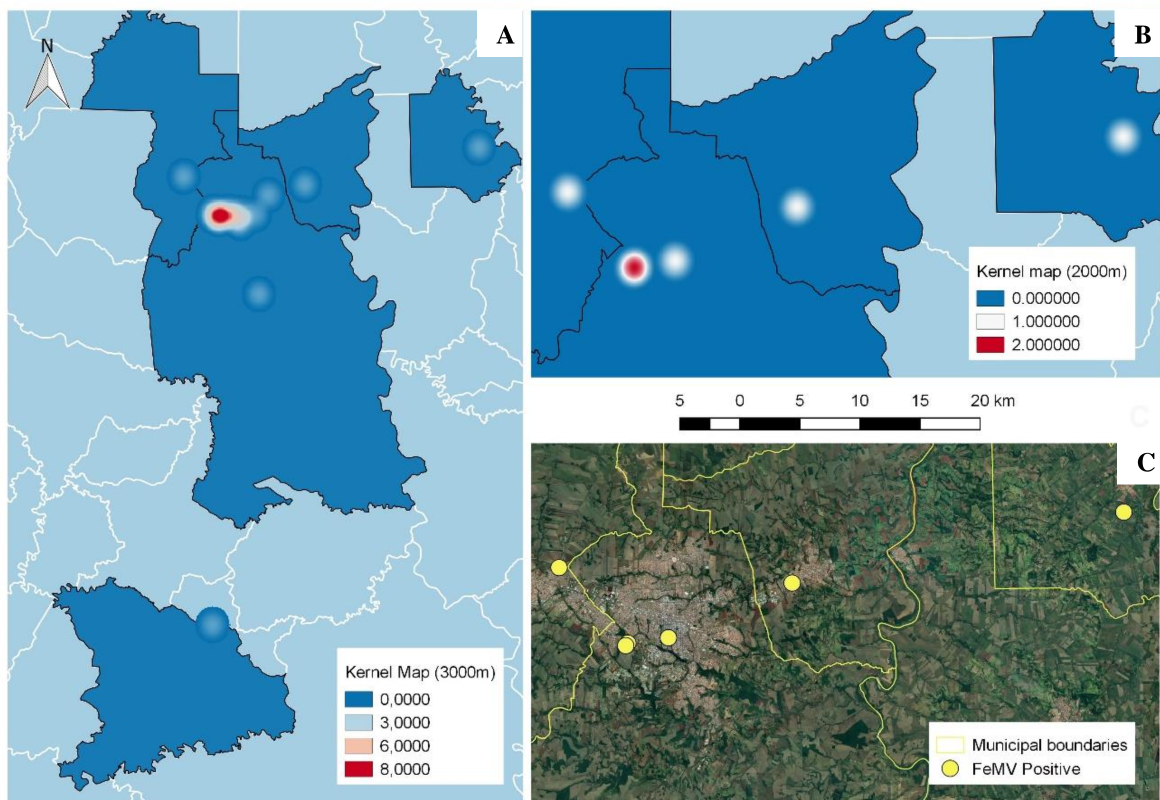

Fig 2 A) Kernel map showing the location and concentration of collected samples.
B) Kernel map with site and concentration of positive samples for *Feline Morbillivirus*.
C) Satellite image showing the proximity the *Feline Morbillivirus* infected white-eared opossum with urban areas.

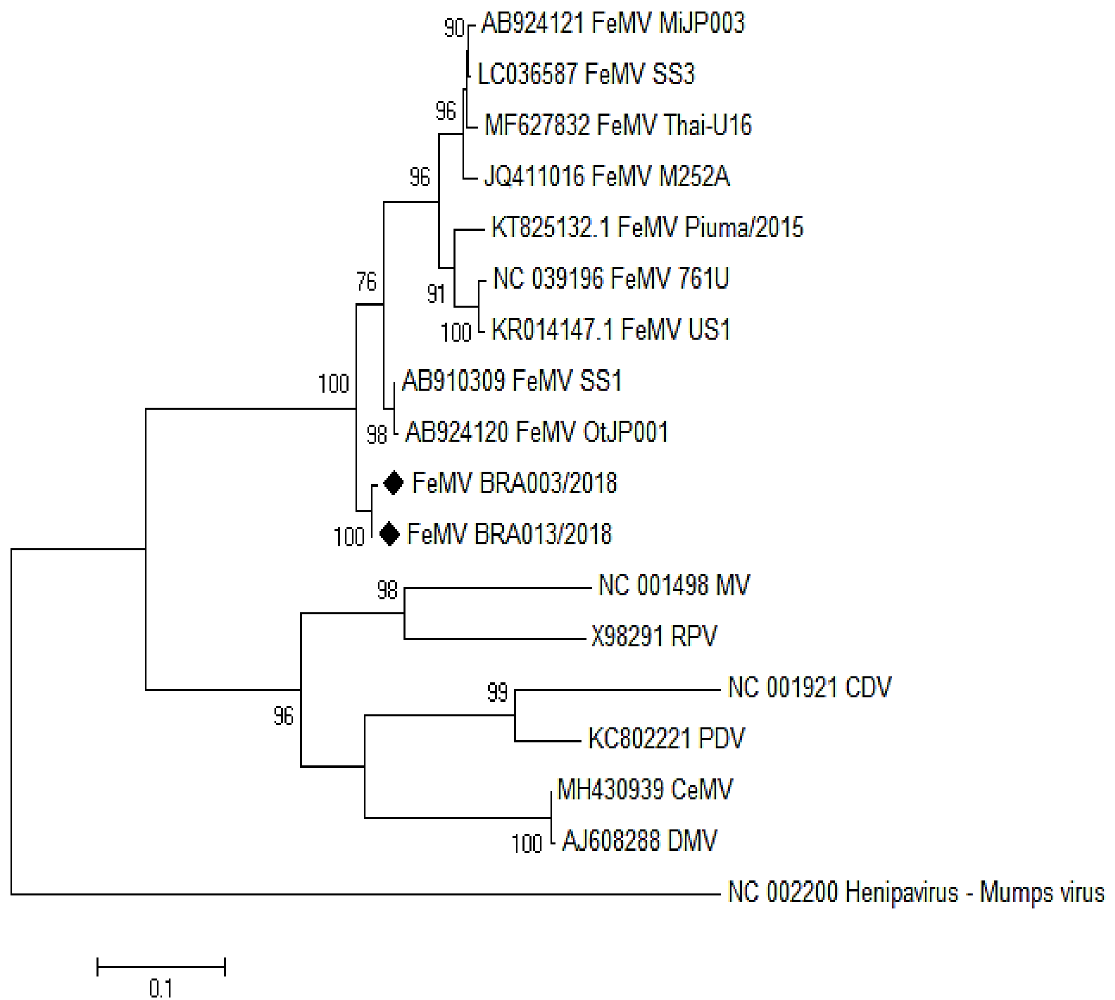

Fig 3 Phylogenetic analysis of the paramyxovirus strains from Brazilian opossums (BRA003/2018 and BRA013/2018) based on the L gene. The phylogenetic tree was constructed using the maximum-likelihood method with the kimura-two parameter model. Bootstrapping was statistically supported with 1,000 replicates. Bootstrap values of less than 60 are not shown. The scale bar indicates nucleotide substitutions per site. GenBank accession numbers are shown in front of the sequence name. The sequences from this study are marked with a filled diamond. A sequence from henipavirus genus (*Mumps virus*) was used as outgroup.

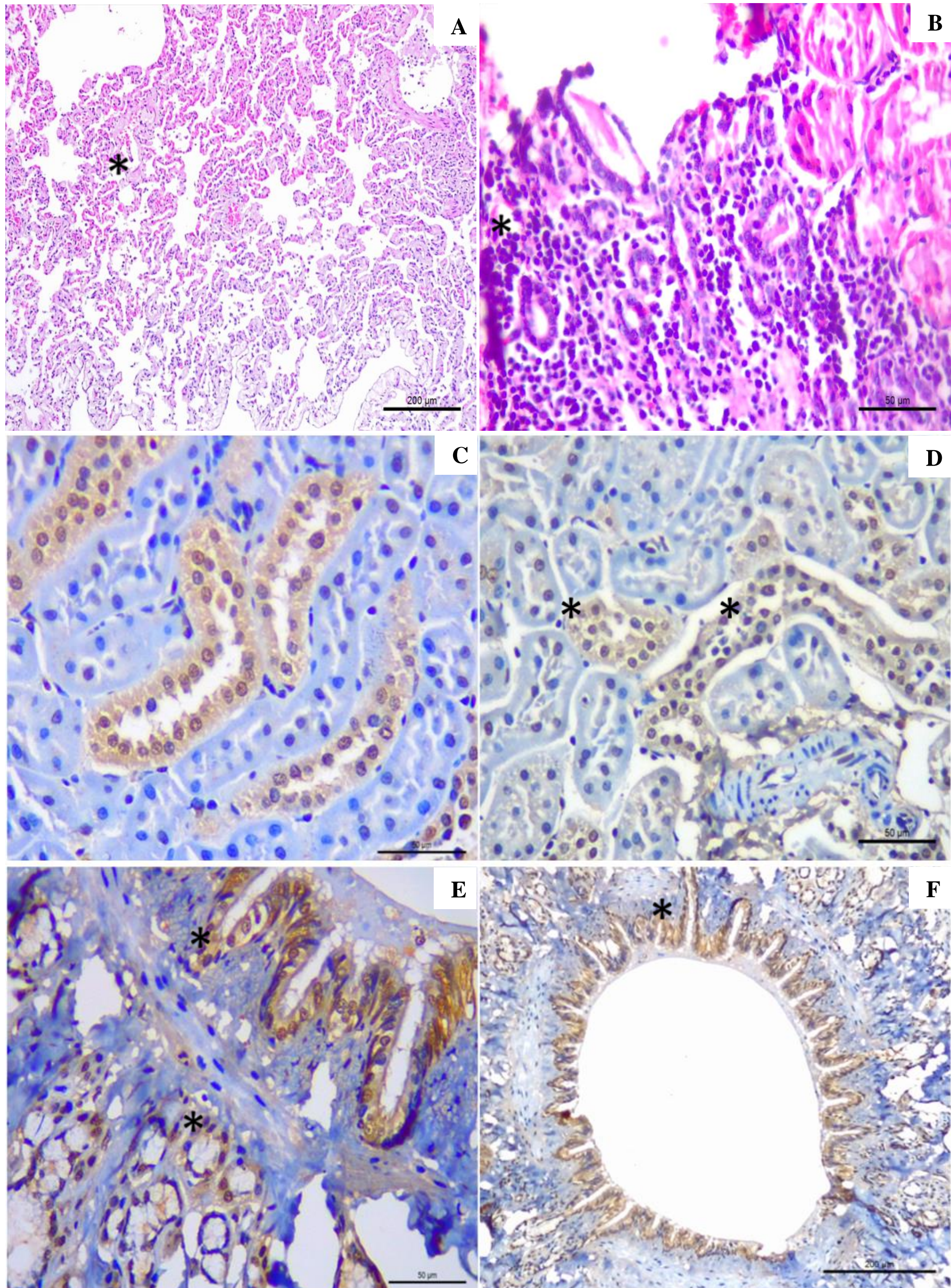

Fig 4. Histopathological and Immunohistochemical findings associated with FeMV in white-eared opossum. Lungs showing the interstitial pneumonia (A) and kidney with inflammatory infiltrate (B) (black arrow). In the kidneys, there was positive immunostaining in the epithelium of the necrotic renal tubules (black arrow) (C and D). In the lung, there was positive immunoreactivity to FeMV antigens in the region of bronchial epithelium and mixed glands (black arrow) (E and F). Immunoperoxidase stained with hematoxylin. Bar, A, B, C 50µm; D 200µm.

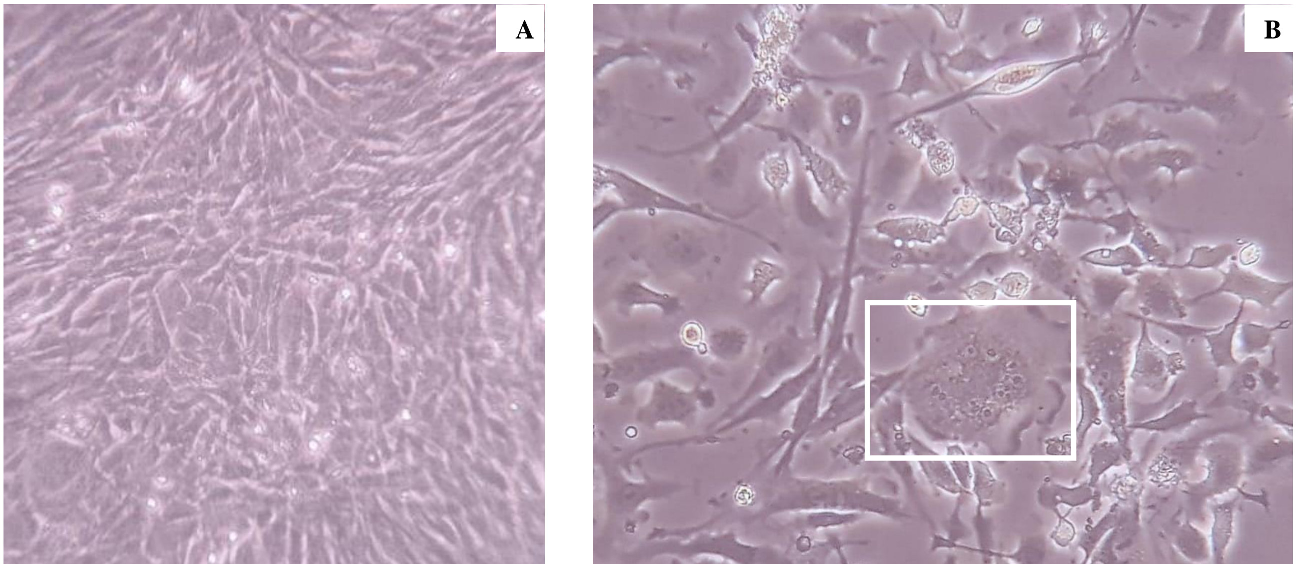

Fig 5 Microscopic findings in cell cultures inoculated with Feline Morbillivirus from white-eared opossum. (A) CRFK without virus inoculation. (B) Cytopathic effects of FeMV on CRFK cells. White squares show the syncytia formation.

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CONCLUSIONS

- The paramyxovirus RNA was detected in lung and kidney tissue samples of the collected white-eared opossum (*Didelphis albiventris*) inhabiting peri-urban areas in cities of northern Paraná.

- Through molecular analyzes it was possible to identify the paramyxoviruses from opossums as *Feline Morbillivirus*

- There is a circulation of the FeMV among the Didelphis albiventris and not only among domestic cats as have been reported.

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- The lung may be the main site of replication and maintenance of the FeMV, being, therefore, the airways the main virus excretion route, in this animal species.

- The molecular results were reinforced with immunohistochemical assay. Beyond, the histopathological finding suggest that the FeMV is able to replicate in lung and kidney tissues causing characteristic lesions of morbillivirus infection

- Brazilians opossum FeMV strains showed high similarity with the Japanese strains, therefore there is no positive geographic correlation

- The opossum may serve as a disseminator of the FeMV since the strain isolated was able to infect cat cell lineage *in vitro*.

ATTACHMENTS

ATTACHMENT A – REAGENTS LIST

1. Absolute ethyl alcohol (C 2 H 2 OH) M.W. 46.07 (Nuclear®)
2. Acetone P.A. (CH 3 COCH 3) M.W. 58.08 (Dynamic®)
3. Agarose (Gibco BRL®)
4. Anhydrous dibasic sodium phosphate (NA2 HPO4) M.W. 141.96 (Synth®)
5. BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems®)
6. Bromophenol Blue (Sigma®)
7. Diethyl pyrocarbonate water (DEPC) (Invitrogen Life Technologies®)
8. DNA molecular size standard 123 bp (Invitrogen™ Life Technologies®)
9. dNTP Set 100 mM, 4 x 250 mL; 25 mmol of each (100 mM of the dATP solutions, dCTP, dGTP, dTTP) (Invitrogen™ Life Technologies®)
10. Ethidium bromide (C21H20N3Br) M.W. 394.3 (Sigma®)
11. Ethylenediaminetetraacetic acid disodium salt (EDTA) P.A. (C10H14N2O8NA2. 2H20) M.W. 372.24 (Reagen®)
12. Guanidine isothiocyanate (Invitrogen™ Life Technologies®)
13. Hematoxylin (Sigma-Aldrich®)
14. Hydrochloric acid (HCl) M.W. 36.46 (Reagen®)
15. Hydroxyethyl amino methane TRIS 99% M.P. 121.14 (Inlab®)
16. Methanol (Methyl Alcohol) PA (Emsure Merck®)
17. M-MLV reverse transcriptase (200 units) (Invitrogen™ Life Technologies®)
18. Oligonucleotide initiator (primer) forward **Par-F1** (forward; 5´ GAAGGITATTGTCAIAAR NTNTGGAC-3´; nucleotide (nt) 4869 - 4894) Tong et al. (2008) - 200 pmol (Invitrogen™ Life Technologies®)
19. Oligonucleotide initiator (primer) forward **Par-F2** (forward; 5´- GTTGCTTCAATGGTTCA RGGNGAYAA-3´; nucleotide (nt) 4790 - 4816) Tong et al. (2008) - 200pmol (Invitrogen™ Life Technologies®)

20. Oligonucleotide initiator (primer) reverse **Par-R** (reverse; 5´GCTGAAGTTACIGGITCIC CDATRTTNC-3´; nucleotide (nt) 4233 - 4260) Tong et al. (2008) -200pmol (Invitrogen^{TM} Life Technologies®)

21. Oligonucleotide initiator (primer) forward **PI-1**(forward; 5´- ACAGGATTGCTGAG GACCTAT -3´; nucleotide (nt) 769-789) Frisk et al. (1999) – 200pmol (Invitrogen^{TM} Life Technologies®)

22. Oligonucleotide initiator (primer) reverse **PI-2** (reverse; 5´CAAGATAACCATGTACGG TGC-3´; nucleotide (nt) 1055- 1035) Frisk et al. (1999) - 200 pmol (Invitrogen^{TM} Life Technologies®)

23. PCR Buffer 10x (200mM Tris-HCl, pH 8.4, 500mM KCl) (Invitrogen^{TM} Life Technologies®)

24. Potassium chloride (KCl) m.p. 74.56 (Reagen®)

25. PureLink Quick Gel Extraction Kit (Invitrogen^{TM} Life Technologies®)

26. Quant-iT ™ quantification kit (Invitrogen^{TM} Life Technologies®)

27. Recombinant Polymerase Platinum Taq DNA (500units) (Invitrogen^{TM} Life Technologies®).

28. Sacarose /sucrose P.A. ($C_{12}H_{22}O_{11}$) MW 342.31 (Reagen®)

29. Silica (SiO_2) m.p. 60.08 (Sigma®)

30. Sodium Chloride (NaCl) m.p. 58.45 (Reagen®)

31. Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NO_4S$) M.W. 288.38 (BDH)

32. Sodium hydroxide P.A. (NaOH) M.W. 40.00 (Dynamic®)

33. Superscript TM III RNase H – Reverse Transcriptase – 200units/µL (Invitrogen^{TM} Life Technologies, EUA)

34. Triton x-100 (J.T. Baker®)

35. Xylol (Xileno) PA (Alphatec®)

ATTACHMENT B – SOLUTIONS AND BUFFERS

• Agarose Gel 2%

- 1.0g agarose
- 50mL of 1x TBE buffer
- 20µl of ethidium bromide

• Hydration of silica

- 6g silica (Sigma)
- Add 50mL of autoclaved ultrapure water
- Slowly shake and rest for 24h
- By suction, discard 44mL of the supernatant
- Suspend the silica in 50ml of autoclaved ultrapure water
- Keep at rest for 5h to sediment
- Discard 44ml of the supernatant
- Adjust the pH (pH 2.0)
- Aliquot and autoclave

• SDS 10%

- 5g sodium dodecyl sulfate - Sodium lauryl sulfate - SDS ($C_{12}H_{25}NaO_4S$)
- double distilled water q.s.p. 50mL

• Solution L6

- 120g of guanidine isothiocyanate (GUSCN)
- 100mL of 0.1M TRIS-HCl, pH 6.4
- 22mL of 0.2M EDTA pH 8.0
- 2.6g of Triton x-100

• Solution L2

- 120g of guanidine isothiocyanate (GUSCN)
- 100mL of 0.1M TRIS-HCl, pH 6.4

• Running Buffer - TBE (Tris - Boric Acid - EDTA) 10x

- 107.78g TRIS 0.89M

- 55.03g of 0.89M boric acid
- 7.45g of 0.02M EDTA
- twice-distilled water q.s.p. 1 liter
- adjust the pH (pH 8.4)

• **Sample buffer for agarose gel electrophoresis**

- 0.25g of bromophenol blue 0.25%
- 45g sucrose ($C_{12}H_{22}O_{11}$) 45%
- Twice-distilled water q.s.p.100mL

Saline Phosphate Buffer (PBS) pH7.2

- Disodium hydrogen phosphate heptahydrate 3.96 g
- Monosodium dihydrogen orthophosphate 0.72 g
- Sodium chloride 16.34g
- Water q.s.p. 2000mL
- Adjust the pH to 7.20 - 7.24

Endogenous Peroxidase Blocking Solution

- Solution to endogenous peroxidase blockade (36% of the hydrogen peroxide in methanol).
- Oxygenated water (20 vol) 40 mL
- Methanol 110 mL
- Use tub and glass crib.

Revelation Solution

- Distilled water 1 mL
- Solution 1 1 drop
- Solution 2 1 drop
- Solution 3 1 drop

Blocking solution of endogenous Biotin

- 5% milk powder solution
- Powdered milk 10g
- Washing solution q.s.p. 200mL

ATTACHMENT C – TECHNIQUE PROTOCOLS

• Extraction of nucleic acid by a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate methods

- 100mg previously macerated tissue

- Add 500µL of PBS buffer

- Homogenize in vortex

- Centrifuge at 3.000rpm/5min

- Collect the supernatant

- Add 10µL of proteinase K and 50µL of SDS 10%

- Homogenize in vortex

- Incubate at 56°C/30min

- Centrifuge at 13.000rpm/30s

- Add 500µL of phenol/chloroform/isoamyl alcohol (25:24:1)

- Homogenize in vortex

- Incubate at 56°C/15min

- Homogenize in vortex

- Centrifuge at 13.000/30s

- Collect the supernatant (in a new microtube)

- Add 500µL of L6 solution

- Add 25µL of silica

- Homogenize in vortex

- Shake at room temperature/30min

- Centrifuge 13.000rpm/30s

- Discard the supernatant in solution containing 10M NaOH

- Add 500µL of L2 solution

- Homogenize in vortex

- Centrifuge at 13.000rpm/30 s

- Discard the supernatant in solution containing 10M NaOH

- Add 500µL of L2 solution

- Homogenize in vortex

- Centrifuge at 13.000rpm/30 s

- Discard the supernatant in solution containing 10M NaOH

- Add 1000µL L of cold ethanol 70%

- Homogenize in vortex
- Centrifuge at 13.000rpm/30 s
- Discard the supernatant
- Add 1000µL L of cold ethanol 70%
- Homogenize in vortex
- Centrifuge at 13.000rpm/30 s
- Discard the supernatant
- Add 1000µL of cold acetone P.A.
- Homogenize in vortex
- Centrifuge at 13.000rpm/30 s
- Discard the supernatant
- Dry the pellet in thermo block at 60°C/2 – 5min
- Add 50µL of DEPC water
- Homogenize in vortex
- Incubate at 56 ºC/15 min
- Homogenize in vortex
- Centrifuge at 13.000rpm/4 min
- Collect the supernatant in a 500µL microtube.
- Store at -20°C until use.

· **RT-PCR Morbillivirus N gene**

Denaturation
- 9µL of extracted nucleic acid
- 20pmol *forward* primer

*Program: 70ºC/10 min and ice bath/5 min

Reverse transcription
- 1x RT *buffer* (250mM Tris-HCl, pH 8,3, 375mL KCl, 15mM $MgCl_2$)
- 1,3mM of each dNTP
- 0,1M of DTT
- 50units of reverse transcriptase M-MLV

*Program: 37°C/50 min; 70°C/15 min.

- **PCR**

- 1x PCR *buffer* (30mM Tris-HCl, pH 8.4 e 75mM KCl)
- 3mM de $MgCl_2$
- 0.8mM of each dNTP
- 20pmol of each *primer*
- 2.5units of *Taq* DNA polymerase
- 5µL of the cDNA
- DEPEC water q.s.p. 50µL

*Programa: 94°C/1 min; 35 cycles de 94°C/1 min, 59.5°C/1 min e 72°C/1 min; 72°C/5 min.

- **RT-SNPCR Paramyxovirus L gene**

Denaturation
- 9µL of extracted nucleic acid
- 20pmol *forward* primer (Par-F1)

*Program: 70ºC/10 min and ice bath/5 min

Reverse transcription
- 1x RT *buffer* (250mM Tris-HCl, pH 8.3, 375mL KCl, 15mM $MgCl_2$)
- 1.3mM of each dNTP
- 0,1M of DTT
- 50units of reverse transcriptase Super script III

*Program: 55°C/50 min; 70°C/15 min.

PCR
- 1x PCR *buffer* (30mM Tris-HCl, pH 8.4 e 75mM KCl)
- 3mM de $MgCl_2$
- 0.8mM of each dNTP
- 20pmol of primer forward (Par-F1)
- 20pmol of primer reverse (Par-R)
- 2.5units of *Taq* DNA polymerase
- 5µL of the cDNA
- DEPEC water q.s.p. 50µL

*Programa: 94°C/2min; 40 cycles de 94°C/30sec, 50°C/30sec and 72°C/45sec; 72°C/7min.

SN-PCR

- 1x PCR *buffer* (30mM Tris-HCl, pH 8.4 e 75mM KCl)
- 3mM de $MgCl_2$
- 0.8mM of each dNTP
- 20pmol of primer forward (Par-F2)
- 20pmol of primer reverse (Par-R)
- 2.5units of *Taq* DNA polymerase
- 2µL of the cDNA
- DEPEC water q.s.p. 50µL

*Programa: 94°C/2min; 40 cycles de 94°C/30sec, 50°C/30sec and 72°C/45sec; 72°C/7min.

· **Agarose gel (2%) electrophoresis**

- 1,0g of agarose
- 50mL TEB *buffer* (Tris 89 mM; acid boric 89 mM; EDTA 2mM) pH 8,4
- 20µL of ethidium bromide (0,5ug/mL)

5µL of the amplicon and 1µL of the sample buffer are used. Electrophoresis under voltage (100V) and amperage (80A) constants for approximately 50min.

· **Purificação de produto de PCR excisado do gel**

- Weigh the excised fragment of the gel into a 1.5mL microtube.
- Add 3 volumes of the gel solubilization buffer (L3) to each 1 volume of gel.
- Incubate the tube at 50°C/15 min, homogenizing every 3min.
- Transfer the dissolved gel with the amplified of interest to a column with the collector tube.
- Centrifuge at 13,000xg/1min.
- Discard the filtrate and replace the column in the same collector tube.
- Add 500µl of Wash buffer 1 (W1) to the column.
- Centrifuge at 13,000xg/1 min.
- Discard the filtrate and replace the column in the same tube.
- Centrifuge tube again at 13.000xg/3min
- Discard the collector tube and transfer the column into a 1.5ml microtube.
- Add 30µL of the Elution buffer 1 (E1) in the center of the column.

- Incubate at room temperature/1min
- Centrifuge at 13.000xg/1min.
- Store the DNA purified at -20°C.

- **Quantificação de produto de PCR**

- Prepare the Quant-iTTM Working Solution by diluting the Quant-iTTM reagent in Quant-iTTM Buffer 1: 200. 200µL of this solution is required per sample and for standards 0 and 100.
- Homogenize in vortex.
- In the microtube of the samples add 198µL of the Quant-iTTM Working Solution to 2µL of the purified DNA fragment.
- In the standard microtube 0 add 190µLL of the Quant-iTTM Working Solution to 10µL of the 0 standard.
- In the standard 100 microtube add 190µL of the Quant-iTTM Working Solution to 10µL of the 100 standard.
- Homogenize in vortex/2-3sec
- Incubate at room temperature/2min
- Perform the reading using Qubit TM Fluorometer (Invitrogen TM Life Technologies, USA)
- Multiply by the dilution factor to determine the correct sample concentration.

- **Sequencing by the sanger method**

- Prepare of the samples

Samples and primers should be in the concentration shown in the table below:

Fragment size (base pairs)	Samples concentration (ng/µL ou µg/mL)	Primer concentration (pmol/µL ou µM)
< 300	2	5
300 - 700	4	5
>700	10	10

- Sequencing mix

Reagent	Volume
BigDye Terminator v3.1	2.0µL
Buffer 5x	1.5µL
Ultrapure water autoclaved	0.5µL
Final volume	**4.0µL**

*BigDye Terminator v3.1 Cycle Sequencing Kit

The final volume of sequencing mix is added to 5µL of purified sample + 1µL of primer.

- Temperature cycles and temperature of sequencing reaction

Reaction	Temperature	Time	Cycles
Inicial denaturation	96°C	1min	1
Denaturation	96°C	15s	35
Annealing	50°C	15s	35
Extension	60°C	4min	35

*Recommendation of Applied Biosystens.

- Precipitation with EDTA and Ethanol

- Add the 10µL of the sequencing reaction in a well of a MicroAmp® Optical 96-Well Reaction (0.2mL) (Applied Biosystems)
- Add 2.5µl of 125mM EDTA (Ethylenediaminetetraacetic acid) pH 8.0
- Add 30µL of 100%
- Homogenize slowly the plate
- Incubate the plate for at room temperature/10min
- Centrifuge at 2720 x g at 20°C/30 minutes
- Discard the contents of the plate
- Centrifuge at 2720xg at 20°C/ min with the plate inverted onto paper
- Add 100µl of ethanol 70%
- Centrifuge at 2720xg at 20°C/1min
- Discard the contents of the plate
- Centrifuge at 2720xg at 20°C/1min with the plate inverted onto paper
- Cover the plate with paper and leave at room temperature/10min
- Add 10µL of formamide (HIDI)
- Add the septa
- Homogenize the vortex plate
- Submit to plate to spin on manipulate spinner
- Place the plate in thermocycler (lid open) at 95°C/5min
- Place the plate in cooler or ice bath/1min

After precipitation with EDTA and ethanol, the plate is inserted into the sequencer (ABI 3500 Genetic Analyzer - Applied Biosystems) to perform the capillary electrophoresis.

- **Histopathological slides staining:**

- **Preparation of histopathological slides**

- Alcohol 80% / 60min
- Alcohol 95% /60min
- Absolute alcohol I / 60min
- Absolute alcohol II / 60min
- Absolute alcohol III / 60min
- Xylol I / 60min
- Xylol II / 60min
- Xylol III / 60min
- Xylol I / 60min
- Paraffin I / 20min
- Paraffin II / 20min

- **Hematoxylin - Eosin (HE) staining**

- Xylol I / 20min
- Xylol II / 20min
- Absolute alcohol / 2min
- Absolute alcohol / 2min
- Ammonia alcohol / 10min
- Alcohol 95% / 2min
- Distilled water / 2min
- Hematoxylin / 4min
- Running water / 10min
- 95% alcohol / 1min
- Eosin / 1min 30sec
- 95% alcohol / 1min
- Absolute alcohol / 2min
- Absolute alcohol / 2min
- Allow the blades to dry at room temperature
- Xylol / 2min
- Xylol II / 2min

· **Immunohistochemical staining:**

- **Dewaxing**

- Put the slides in the stove at 60°C/ 10h
- Incubate the slides in Xylol I/ 20min
- Incubate the slides in Xylol II/ 20min

- **Rehydration**

- Incubate in alcohol 100% I/ 10 min
- Incubate in alcohol 100% II/ 10 min
- Incubate in alcohol 90%/ 5 min
- Incubate in alcohol 80%/ 5 min
- Incubate in Alcohol 70%/ 5 min
- Wash in running water

- **Antigen Recovery**

- Incubate in citrate pH 6.0 in the pressure cooker 86-90 ° C 3 min
- Remove from the pan with the aid of a kitchen glove
- Material temperature stabilization at room temperature 20 min
- Wash under running water for 10 minutes
- Diving in distilled water

- **Antigen Recovery**

- Incubate in methanol (110 ml) + hydrogen peroxide (40 ml) solution at room temperature / 30 min
- Protect from light
- Discard methanol solution in alcohol waste
- Wash under running water for 10 minutes
- Diving in Distilled Water
- When necessary, blocking with Mollico® Milk (10g of milk + 200ml of distilled water at room temperature for 10min)
- Rinse in distilled water (2x)

- **Primary Antibody Incubation**

- Dry around the cuts with paper towel
- Incubate the slide with diluted Primary Ac in diluent in Wet Chamber at 4 ° C / 22h

- Wash in PBS pH 7.2 / 5min (2x)

- **Secondary Antibody Incubation**

- Dry around the cuts with paper towel
- Add diluted Secondary Ac in moist chamber at room temperature/ 30min
- Wash in PBS pH 7.2 / 5min (2x)

- **Chromogenic Solution**

- Dry around the cuts with paper towel
- Add DAB to chapel and incubate at room temperature / 3min
- Stop the reaction under running water at room temperature.
- Discard in the adequate place
- Wash under running tap water / 10min
- Diving in distilled Water

- **Coloring and dehydration**

- Immerse in Harris Hematoxylin / 35sec
- Discard the 1st water, in dye discard.
- Incubate in alcohol 70% / 5min
- Incubate in alcohol 80/ 5min
- Incubate in alcohol 90% / 5min
- Incubate in alcohol 100% / 10min
- Incubate in Alcohol 100% II / 10min
- Allow the slides to dry in the hood so as not to moisturize the Xylol
- Incubate in Xylol I / 20min
- Incubate in Xylol II / 20min
- Mount with coverslip (suitable size + resin)

ATTACHMENT D – SOFTWERES LIST

- Electropherogram quality analysis - Phred e CAP3 (http://asparagin.cenargen.embrapa.br/phph/)
- BLAST The Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- MEGA package software version X (http://www.megasoftware.net/mega4/mega41.html)
- BioEdit software version 7.1.3.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html)