UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

MARIA ALEJANDRA ARIAS LUGO

INVESTIGATION OF RNA VIRAL PATHOGENS IN WILD ROAD-KILLED CARNIVORES ON HIGHWAYS OF SÃO PAULO STATE, BRAZIL

SÃO PAULO

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INVESTIGATION OF RNA VIRAL PATHOGENS IN WILD ROAD-KILLED CARNIVORES ON HIGHWAYS OF SÃO PAULO STATE, BRAZIL

Dissertation submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master’s degree in sciences.

Department:

Pathology

Area:

Experimental and comparative pathology

Advisor:

Prof. José Luiz Catão Dias, Ph.D.

São Paulo

2024

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Dissertação (Mestrado – Programa de Pós-Graduação em Patologia Experimental e Comparada – Departamento de Patologia) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2024.

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Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Comissão de Ética no Uso de Animais

São Paulo, 06 de dezembro de 2023
CEUAx N 3436120722
(ID 002285)

Ilmo(a). Sr(a).
Responsável: Jos? Luiz Cat?o Dias
Área: Patologia Experimental E Comparada

Título da proposta: "INVESTIGA??O DE PAT?GENOS (RNA V?RUS) EM CARN?VOROS SELVAGENS ATROPELADOS EM RODOVIAS DO ESTADO DE S?O PAULO".

CERTIFICADO (Alteração do cadastro versão de 11/agosto/2023)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 11/agosto/2023) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Devido a inconvenientes na amostragem de animais domésticos consideramos necesario realizar uma modificação do titulo do projeto".

Comentário da CEUA: Face a mudan?a referente a concession?ria que est? contribuindo com os animais mortos encontrados nas estradas, foi solicitado altera??o no t?tulo do projeto. N?o interfere na proposta inicial.

Camilla Mota Mendes

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Profa. Dra. Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 82263-1	**Data da Emissão: 30/03/2023 08:13:49**	**Data da Revalidação*: 30/03/2024**

De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

SISBIO

Nome: MARIA ALEJANDRA ARIAS LUGO	CPF: 090.725.331-86
Título do Projeto: Aportes da mortalidade em rodovias para o estudo do fluxo de patógenos virais selecionado entre carnívoros domésticos e selvagens.	
Nome da Instituição: UNIVERSIDADE DE SAO PAULO	CNPJ: 63.025.530/0001-04

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Recoleção de Carcaças	04/2023	12/2023

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	Deve-se observar as as recomendações de prevenção contra a COVID-19 das autoridades sanitárias locais e das Unidades de Conservação a serem acessadas.
3	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros).
4	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros)
5	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
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9	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
10	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.

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Nome da Instituição: UNIVERSIDADE DE SAO PAULO	CNPJ: 63.025.530/0001-04

Observações e ressalvas

11	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.

Outras ressalvas

1		CENAP Atibaia-SP

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Rodovias	São Paulo-SP	Mata Atlântica	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas in situ	Fora de UC Federal
2	Coleta/transporte de amostras biológicas ex situ	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de amostras biológicas in situ	Mustelidae	-
2	Coleta/transporte de amostras biológicas ex situ	Mustelidae	-
3	Coleta/transporte de amostras biológicas in situ	Procyonidae	-
4	Coleta/transporte de amostras biológicas ex situ	Procyonidae	-
5	Coleta/transporte de amostras biológicas in situ	Canidae	-
6	Coleta/transporte de amostras biológicas ex situ	Canidae	-
7	Coleta/transporte de amostras biológicas ex situ	Felidae	-
8	Coleta/transporte de amostras biológicas in situ	Felidae	-

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

A quantidade significa: por espécie X localidade X ano.

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Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Carnívoros)	Animal encontrado morto ou partes (carcaça)/osso/pele, Ectoparasita, Fragmento de tecido/órgão, Pêlo, Regurgitação/conteúdo estomacal

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	Faculdade de Medicina Veterinária e Zootecnia USP	Laboratório

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Nome da Instituição: UNIVERSIDADE DE SAO PAULO	CNPJ: 63.025.530/0001-04

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	**Qtde.**	**Tipo de Amostra**	**Qtde.**	**Data**

* Identificar o espécime do nível taxonômico possível.

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EVALUATION FORM

Author: ARIAS LUGO, MARIA ALEJANDRA

Title: Investigation of RNA viral pathogens in wild carnivores killed by vehicular collisions on roads highways in the state of São Paulo.

Dissertation submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master’s degree in sciences.

Date: _____/_____/_____

Committee Members

Prof. ____

Institution: _________________________ Decision: ________________________

Prof. ____

Institution: _________________________ Decision: ________________________

Prof. ____

Institution: _________________________ Decision: ________________________

DEDICATION

Esta tesis está dedicada a todos los investigadores en fauna silvestres que con sus aportes a la ciencia cada vez están dejando un granito de arena para preservar las especies silvestres y sus poblaciones.

This thesis is dedicated to all wildlife researchers who, with their contributions to science, are doing their bit to preserve wild species and their populations.

ACKNOWLEDGEMENTS

Since I was finishing my professional career as a veterinarian, I was excited to learn about wildlife pathology and think about being a great pathologist of these interesting species; During my college years, "Tratado de Animais Selvagens" was a book that I always kept as a treasure, and meeting professor Dr. Catão, the book's author, was one of my biggest dreams. I was thrilled when a professor mentioned a graduate, Dr. Pedro Navas, who was pursuing a doctorate under the direction of Dr. Catão himself, when I asked him about internships in Brazil in wildlife pathology. Later on, I began to email looking for an internship at LAPCOM; Fast forward six years from that moment, and here I am, writing this master's thesis – a dream come true. I am deeply grateful with God for the opportunity to learn from the exemplary professionals from LAPCOM. Reflecting on this journey, I realize thanking myself is really very valuable in this instance; If I had not been the persistent person that I was at that moment, none of this would be possible, in the same way I commend myself for the strength and bravery I showed embracing a new culture, people and language while I was developing this project exactly as I expected. The other paragraphs of gratitude will be written in the languages of those whom I will be eternally grateful.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001 baixo o programa de PATOLOGIA EXPERIMENTAL E COMPARADA (33002010057P1). Agradeço às agências de fomento Brasileiras.

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Migrar, salir de tu país para seguir preparándome profesionalmente no es nada fácil y más si dejas la mitad de tu corazón; es inevitable pensar en dar agradecimientos a una persona inmensamente importante para mí como lo es el amor de mi vida Guillermo, eres y serás lo más bonito que me pudo pasar y como todos los días te lo recordaba gracias por haber sido fuerte por los dos, por apoyarme y darme amor a lo lejos, por ser mi polo a tierra y mi más grande admirador; gracias por qué sé el sacrificio que hiciste al haber comenzado una vida juntos y tener que irme de un momento a otro fue algo muy duro para ti; quiero que sepas que estoy inmensamente orgullosa de ti, del hombre que eres, de lo inteligente y lo valiente y estoy segura que quiero compartir el resto de mi vida junto a ti y con nuestro perrito (pepinillo).

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RESUMO

ARIAS-LUGO, R.E. **Investigação de patógenos (RNA vírus) em carnívoros selvagens atropelados em rodovias do estado de São Paulo**. 2024. 79 p. Dissertação (Mestrado em ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2024.

Sessenta e nove espécies de mamíferos estão oficialmente ameaçadas de extinção no Brasil, o que representa 10,6% das 652 espécies dos mamíferos nativos. As ordens com as maiores proporções de espécies em categorias de ameaça são Primatas e Carnívora. O impacto das atividades humanas, junto com a atual taxa de extinção global de recursos naturais e as mudanças climáticas, influenciam a transmissão de diversos patógenos que podem causar doenças com resultados potencialmente devastadores. Diversos estudos já relataram o aumento do fluxo de patógenos entre animais domésticos, selvagens e humanos, levando a epizootias ou inclusive pandemias. Especificamente, entre carnívoros domésticos e silvestres algumas doenças já relatadas incluem: cinomose, parvovirose, raiva, leishmaniose e dirofilariose. Para aumentar o conhecimento sobre a ocorrência de patógenos em carnívoros silvestres, esta dissertação teve como objetivo estudar por métodos moleculares e anatomopatológicos a presença de quatro agentes RNA vírus: vírus da cinomose (CDV), SARS-CoV-2, coronavírus felino (FCoV) e vírus da raiva (RABV). Para isso, foram coletadas carcaças de carnívoros silvestres atropelados em rodovias do estado de São Paulo. Foi realizada necropsia buscando alterações macroscópicas sugestivas das doenças causadas pelos agentes estudados. Adicionalmente, foram coletados fragmentos de órgãos, fixados em formalina 10%, e processados de rotina para confecção de lâminas coradas com H&E para posterior análise histopatológica em busca de alterações microscópicas condizentes com os agentes virais estudados, naqueles casos positivos nos exames moleculares. Para cada agente viral, foram coletadas amostras do órgão alvo: CDV (pulmão, sistema nervoso central, rim e intestino), SARS-CoV-2 (pulmão), FCoV (intestino) e RABV (sistema nervoso central), e foram realizados testes de reação em cadeia da polimerase convencional (PCR) ou em tempo real (RT-PCR). No total, foram coletadas 33 carcaças representando quatro espécies de felídeos *(Herpailurus yagouaroundi, Leopardus Pardalis, Puma concolor, Leopardus guttulus*), três de mustelídeos (*Lontra longicaudis, Galictis cuja, Eira barbara*), duas de canídeos (*Cerdocyon thous e Chrysocyon brachyurus*), e duas de procioníideos (*Nasua nasua, Procyon cancrivorus*). Por meio da avaliação anatomopatológica não foram observadas alterações sugestivas das doenças causadas pelos agentes estudados. Igualmente a triagem molecular evidencio que todos os casos foram negativos para todos os testes realizados. Conclui-se que não houve evidência da presença desses vírus nos animais estudados; desta forma, nossa amostragem permite inferir que na área de estudo estes agentes virais não parecem ser, neste momento, uma ameaça à conservação dos carnívoros selvagens. No entanto, devido à diversidade de espécies neste estudo, consideramos que o atropelamento pode ser sim importante para a perda de indivíduos aparentemente sadios. Ainda, salienta-se a importância de aumentar a implementação de medidas de mitigação nas rodovias do estado de São Paulo.

Palavras-chave: Atropelamento. Carnívoros selvagens. Conservação. Vírus de RNA.

ABSTRACT

ARIAS-LUGO, R.E. **Investigation of RNA viral pathogens in wild carnivores killed by vehicular collisions on roads highways in the state of São Paulo.** 2024. 79p. Dissertação (Mestrado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2024.

Sixty-nine species of wild mammals are officially classified as threatened of extinction in Brazil, which represents 10.6% of 652 native species. The orders with the highest proportions of threatened species are Primates and Carnivora. Human activities, resource depletion, and climate change fuel a dangerous rise on transmission of several pathogens, increasing the risk of diseases with potentially harmful outcomes. Many studies have reported the flow of pathogens among domestic animals, wildlife, and humans, leading to consequences such as epizootics or pandemics. Specifically, among domestic and wild carnivores, some diseases have already been reported, including distemper, parvovirus, rabies, leishmaniasis and dirofilariosis. To enhance the knowledge about the occurrence of pathogens in wild carnivores, this dissertation aimed to study by molecular and anatomopathological methods the presence of four RNA virus agents: Canine Distemper Virus (CDV), SARS-CoV-2, Feline Coronavirus (FCoV) and Rabies Virus (RABV). For this, carcasses of roadkilled wild carnivores on roads in the state of São Paulo were collected. A necropsy was performed looking for gross changes suggestive of the diseases caused by the studied viruses. Additionally, fragments of organs were collected, fixed in 10% formalin, and routinely processed to make slides stained with H&E for subsequent histopathological analysis in search of microscopic changes consistent with the viral agents studied, for those cases where the molecular assays were positive. For each viral agent, samples from the target organ were collected: CDV (lung, central nervous system, kidney, and intestine), SARS-CoV-2 (lung), FCoV (intestine) and RABV (central nervous system), and conventional polymerase chain reaction (PCR) or real-time polymerase chain reaction (RT-PCR) tests were performed. In total, 33 carcasses were collected representing four species of felids (*Herpailurus yagouaroundi*, *Leopardus Pardalis*, *Puma concolor*, *Leopardus guttulus*), three of mustelids (*Lontra longicaudis*, *Galictis cuja*, *Eira barbara*), two of canids (*Cerdocyon thous* and *Chrysocyon brachyurus*), and two of procyonids (*Nasua nasua* and *Procyon cancrivorus*). Through anatomopathological analyses, no changes (gross or microscopic) suggestive of the diseases caused by the agents studied were observed. Likewise, molecular screening showed that all cases were negative for all tests carried out. It is concluded that there was no evidence of the presence of these viruses in the animals studied; therefore our sampling allows us to infer that in the study area these viral agents do not appear to be, at this time, a threat to the conservation of wild carnivores. However, due to the species diversity in this study, we consider that unnatural mortality by roadkills may be important for the loss of apparently healthy individuals. Finally, we highlight the importance of increasing the implementation of mitigation measures on roads in the state of São Paulo.

Keywords: Roadkill. Wild carnivores. RNA viruses. Conservation.

RESUMEN

ARIAS-LUGO, R.E. **Investigación de patógenos RNA virus en carnívoros silvestres atropellados en carreteras del estado de São Paulo.** 2024. 79p. Disertación (maestría en ciencias) – Facultad de Medicina Veterinaria y Zootecnia, Universidad de São Paulo, São Paulo, 2024.

Sesenta y nueve especies de mamíferos están oficialmente amenazadas de extinción en Brasil, lo que representa el 10,6% de las 652 especies de mamíferos nativas. Las órdenes con mayor proporción de especies en las categorías de amenaza son primates y carnívoros. El impacto de las actividades humanas, combinado con el actual ritmo de extinción global de los recursos naturales y el cambio climático, influyen en la transmisión de diversos patógenos que pueden causar enfermedades con resultados potencialmente devastadores. Diversos estudios ya describen el aumento del flujo de patógenos entre animales domésticos, silvestres y humanos, los cuales pueden llevar a epizoóticas o incluso pandemias. Específicamente, entre carnívoros domésticos y silvestres, entre las enfermedades compartidas ya relatadas se encuentran: moquillo, parvovirosis, rabia, leishmaniosis y dirofilariasis. En busca de aumentar el conocimiento sobre la presencia de patógenos en carnívoros silvestres, esta disertación tuvo como objetivo estudiar por métodos moleculares y anatomopatológicos la presencia de cuatro agentes virales RNA: virus del moquillo canino (CDV), SARS-CoV-2, coronavirus felino (FCoV) y virus de la rabia (RABV). Para ello, fueron colectados cadáveres de carnívoros silvestres atropellados en carreteras del estado de São Paulo. Se realizó necropsia en busca de alteraciones macroscópicas sugestivas de las enfermedades causadas por los agentes estudiados. Adicionalmente, fueron colectados fragmentos de órganos, posteriormente fijados em formalina 10%, y procesados de rutina para realizar laminas con la tinción H&E, para posterior estudio histopatológico en búsqueda de alteraciones microscópicas sugestivas de los agentes virales seleccionados. Para cada agente viral fueron colectadas muestras de los órganos diana: CDV (pulmón, sistema nervioso central, riñón e intestino), SARS-CoV-2 (pulmón), FCoV (intestino) y RABV (sistema nervioso central), para posterior realización de ensayos en cadena de la polimerasa convencional (PCR) y en tiempo real (RT-PCR). En total, fueron colectados 33 cadáveres representando cuatro especies de felinos (*Herpailurus yagouaroundi, Leopardus Pardalis, Puma concolor, Leopardus guttulus*), tres de mustélidos (*Lontra longicaudis, Galictis cuja, Eira barbara*), dos de canidos (*Cerdocyon thous, Chrysocyon brachyurus*), y dos de prociónidos (*Nasua nasua, Procyon cancrivorus*). La evaluación anatomopatológicos no evidencio alteraciones (macroscópicas o microscópicas) sugestivas de las enfermedades causadas por los patógenos estudiados. Igualmente, el tamizaje molecular evidencio que todos los casos fueron negativos en todos los ensayos moleculares realizados. Concluimos que no hubo evidencia de la presencia de estos virus en los animales estudiados. Nuestro muestreo permite inferir que en el área de estudio estos agentes virales no parecen ser, en este momento, una amenaza para la conservación de estos carnívoros silvestres. Sin embargo, debido al gran número de especies estudiadas, consideramos que la mortalidad no natural por colisiones vehiculares puede ser importante para la perdida de individuos aparentemente sanos. Resaltamos la importancia de aumentar la implementación de medidas de mitigación en las carreteras del estado de São Paulo.

Palabras clave: Atropellamiento. Carnívoros salvajes. Virus ARN. Conservación.

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LIST OF ABBREVIATIONS AND ACRONYMS

ARTESP: Regulatory Agency for Delegated Public Transportation Services of the State of São Paulo.

CCoV: Canine Coronavirus.

CDV: Canine Distemper virus.

CNS: Central nervous system

COVID-19: coronavirus disease 2019.

ELISA: enzyme linked immuno sorbent assay.

FCV: feline calicivirus.

FCoV: feline coronavirus.

FeLV: feline leukemia virus.

FHV1: feline herpesvirus type 1.

FIP: feline infectious peritonitis.

FPV: feline panleukopenia virus.

ICMBio: Instituto Chico Mendes de Conservação da Biodiversidade.

ICTV: International Committee on Taxonomy of Viruses.

IUCN: International Union for Conservation of Nature.

LAPCOM: Laboratory of Wildlife Comparative Pathology.

MERS: Middle East respiratory syndrome.

OCB: operational control base.

PPE: personal protective equipment.

RABV: rabies virus.

RMM: road mortality monitoring.

RT-PCR: reverse transcription polymerase chain reaction.

SARS: severe acute respiratory syndrome.

SARS-COV-2: severe acute respiratory syndrome coronavirus 2.

TGEV: transmissible gastroenteritis virus.

TR: toll road.

WHO: World Health Organization.

WOAH: World Organization for Animal Health.

SUMMARY

1. INTRODUCTION

Brazil is considered one of the 18 most megadiverse countries worldwide, with more than 120,000 invertebrate and 8,930 vertebrate species (IUCN, 2021). (OLIVEIRA, 2006), described 26 species of terrestrial carnivores belonging to five families: Canidae, Felidae, Mephitidae, Mustelidae, and Procyonidae, nine of them are classified as vulnerable to extinction (MACHADO, 2005; IUCN, 2024). Focusing on the state of São Paulo, five out of these nine species inhabit the state: Ocelot (*Leopardus pardalis*), Southern-tiger-cat (*Leopardus guttulus*), Cougar (*Puma concolor*), Maned-wolf (*Chrysocyon brachyurus*) and Hoary-fox (*Lycalopex vetulus*) (BRESSAN, 2009). These carnivores play an important role in natural ecosystems; regulating their pray populations and structuring natural communities, among others (CHIARELLO, 2021).

The impact observed by human activities (e.g., environmental pollution, deforestation, soil erosion, wildlife trafficking, etc.) can influence the transmission of various pathogens that can induce diseases with devastating results (LINDAHL; GRACE, 2015). The importance of the study of viral agents, mainly RNA viruses, is because they are likely to become zoonoses and to be the most frequent cause of spillover because of their predisposition to mutate continuously and to change hosts (ALEXANDE et al., 2018). Four significant examples of viral diseases and respective etiologic agents are: rabies, a zoonotic disease caused by RNA virus of the *Rabdoviridae* family, genus *Lysavirus* (CALISHER, 2012); SARS-CoV-2, formerly known as the novel coronavirus 2019-nCoV, a β-coronavirus related to acute respiratory syndrome outbreak in humans in Wuhan (China) and declared by the World Health Organization (WHO) as a disease of pandemic status and named the COVID-19 (LI, 2020); feline infectious peritonitis (FIP), a disease caused by a feline coronavirus (FCov) belonging to the Coronaviridae family, an immune-mediated, systemic, progressive, and fatal feline disease (FELTEN; HARTMANN, 2019); and, canine distemper virus (CDV; *Morbillivirus* genus, family *Paramyxoviridae*), that can affect a large number of mammals, with a high occurrence rate in dogs, inducing a systemic disease with high morbidity and mortality (SOTO et al., 2018; WILKES, 2022).

Domestic dogs and cats are considered invasive alien species when they inhabit or use natural areas without human intervention (MMA, 2013). Furthermore, dogs have been considered the most abundant carnivores in several rural areas, including the Brazilian Atlantic Forest (GUEDES, ET AL., 2020). Additionally, Companion animals can transmit infectious pathogens causing diseases (e.g., CDV, parvovirus, rabies, leishmaniasis and dirofilariasis). Many studies have reported the flow of pathogens between domestic, wild animals and humans causing devastating consequences such as epizootics or pandemics (BERENTSEN et al., 2013; LESSA et al., 2016; COSTANZI et al., 2021).

According to (HOGUE; BREON, 2022) direct destruction of terrestrial ecosystems and habitat use is one of the greatest threats to wildlife. Direct road mortality is considered one of the meaningful impacts on endangered species, in accordance with the National Action Plans of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) (ICMBio, 2023). The total number of mammals hit by cars per year on 2- and 4-lane paved roads in the state of São Paulo was estimated to be an average of 39,605 (ABRA, ET AL., 2021). The use of roadkill carcasses is a great source of biological samples for different investigations (SCHWARTZ; SHILLING; PERKINS, 2020). For this reason, aiming to increase knowledge about the occurrence of RNA viral pathogens in Brazilian wild carnivores, this study made a molecular screening of the occurrence of four RNA viral pathogens: SARS-CoV-2, FCov, RABV, and CDV in road killed specimens collected on selected highways in the state of São Paulo, Brazil.

2. OBJECTIVES

2.1 GENERAL

Using molecular and anatomopathological methods, the general goal was to survey selected RNA viral pathogens in wild carnivores killed in vehicular collisions on selected highways in Sao Paulo state, Brazil.

2.2 SPECIFICS

- To survey the occurrence of *Coronaviridae*, particularly Sars-Cov2 and FCov in tissue samples from wild road-killed carnivores;
- To survey the occurrence of Rabies virus in brain samples from wild road-killed carnivores;
- To survey the occurrence of CDV in tissue samples from wild road-killed carnivores;
- To identify and describe the anatomopathological findings potentially associated with these viral infection in wild carnivores.

3. LITERATURE REVIEW

3.1 RNA VIRUSES

3.1.1 *Rabies virus*

These viruses are classified within the family *Rhabdoviridae* in the order *mononegavirales* (RNA genome in a segmented, negative sense); inside the genus Lyssavirus there are 16 different viral species and present a bullet-shaped virion morphology with approximate dimensions of B75nm and a length of B180nm depending on the virus; They are formed by 12.000 nucleotides which encode 5 viral proteins with conserved gene order: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and large polymerase protein (L). The N, M, and L proteins retain their structure and length while the P and G proteins vary in length (FOOKS et al., 2021). The Lyssaviruses are responsible for rabies, which is arguably the deadliest encephalitic disease known. RABV is thought to be able to infect all terrestrial mammals (SCOTT; NEL, 2021).

Rabies is one of humanity's oldest described diseases, being mentioned in the writings of the ancient Mesopotamians (2300 BC), by the Chinese in 782 BC, and by Aristotle and Hippocrates around 400 BC. During the Middle Ages, this disease caused several epidemics that generated high mortality in Europe (IBAÑEZ MOLINA; REISSING., 2019). Rabies is present on almost every continent except Antarctica and Oceania, and in some regions of the planet rabies continues to be a public health problem (YAGUANA; DEL ROSARIO LÓPEZ, 2017). Rabies has four cycles: urban, rural, wild, and currently among bats, an airborne cycle. This disease can be transmitted by dogs and cats, so routine vaccination of these animals is an epidemiological strategy to eliminate the urban cycle and prevent the occurrence of human cases (FOOKS; JACKSON, 2020). Diagnosis in the clinical phase is based on demonstrating the presence of the virus in saliva, cerebrospinal fluid, urine, and brain samples by Enzyme

Linked Immuno Sorbent Assay (ELISA) and polymerase chain reaction (PCR) (NEVES, 2006). According to the Brazilian Health Ministry, with the intensification of canine and feline rabies surveillance and the control actions over the last 30 years, Brazil has achieved a significant reduction in human mortality rates due to rabies. Between 2010 and 2021, 40 human cases of rabies were reported. Of these cases, bats were most frequent aggressors (20 cases), followed by dogs (9), non-human primates (4), felines (4), wild canids (2), and in one case it was not possible to identify the aggressor animal (DUARTE et al., 2021).

From 2023 to date, two cases of human rabies have been reported in Brazil; the first was in the municipality of Mantena (Minas Gerais) transmitted by a bovine with the bat variant and the second case was reported in the municipality of Carius (Ceará) in a man attacked by a non-human primate (FARIAS et al., 2024) In carnivores, mainly domestic dogs, a case of rabies was reported on August 31, 2023, diagnosed at the School of Veterinary Medicine and Animal Science at the University of São Paulo of the University of São Paulo, identified with bat variant; the last human case of canine variant was reported more than 25 years ago in Brazil and the last animal case corresponding to this variant was reported in 1998 (SCHNEIDER et al., 2023).

3.1.2 Coronavirus

Members of the family Coronaviridae are a monophyletic group of viruses of the order Nidovirales; they are enveloped positive-sense RNA viruses known to infect four of the seven vertebrate classes: mammals and birds (*Orthocoronaviruses*), amphibians (*Letovirus*), fish (*Pironavirus*) (BUKHARI et al., 2018; MORDECAI; HEWSON, 2019). They are considered the largest RNA viruses identified so far; Orthocoronavirinae subfamily virions are 80 to 160nm spherical enveloped viral particles, with surface projections of spike protein (S), nucleocapsid comprises protein (N), and envelope protein (E). A hemagglutinin esterase (HE) glycoprotein is only present in members of the subgenus *Embecovirus* of the genus *Betacoronavirus*. They infect humans and a variety of animals resulting in diverse clinical manifestations ranging from asymptomatic to severe fatal disease, being highly pathogenic to humans, leading to the severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and coronavirus disease COVID-19 (SARS-CoV-2) pandemics (KSIAZEK et al., 2003; PEIRIS et al., ZAKI et al., 2012; ZHU et al., 2020). In animals, *Alpha coronaviruses* cause diseases characterized by severe gastrointestinal disorders such as Transmissible Gastroenteritis Virus (TGEV), Feline Coronavirus (FCoV), and Canine Coronavirus (CCoV) (CARSTENS, 2009).

3.1.2.1 SARS-CoV-2

Family *Coronaviridae*, subfamily *Orthocoronavirinae*, order *Nidovirales*, genus *Betacoronavirus*; the International Committee on Taxonomy of Viruses (ICTV) divides into 5 subgenera (*Embecovirus, Sarbecovirus, Merbecovirus, Nobecovirus and Hibecovirus*) where SARS-CoV-2 belongs to the subgenus *Sarbecovirus* responsible respectively for the SARS outbreaks of 2002-2003 and the COVID-19 pandemic (BONI et al., 2020). Moreover, they are distributed among mammals and birds, causing respiratory and intestinal infections and, in some cases, neurological diseases and/or hepatitis (ALLUWAIMI et al., 2020).

CoVs have been the causative agent of two large-scale pandemics in the last two decades. SARS-CoV-2, formerly known as the novel coronavirus 2019-nCoV, is a β-coronavirus that caused an outbreak of acute respiratory syndrome in humans in December 2019 in Wuhan, China (LI et al., 2020). In 2020, the WHO declared the disease with a pandemic status and named the disease COVID-19. As of 31st December 2021, deaths that were reported globally reached 5.94 million, however, the estimated number of excess deaths was almost 3.07 times (18.2 million) and the global mortality rate for all ages was 120.3 deaths per 100,000 population (MSEMBURI et al., 2023). In humans, the clinical presentation of the disease includes a wide range of signs and symptoms, such as fever, dry cough, dyspnea, headache, sputum production, hemoptysis, myalgia, fatigue, nausea, vomiting, diarrhea and abdominal pain, loss of smell and taste. Regarding the diagnostic methods, chest X-rays and CT scans can show changes in lung density, but the standard test is reverse transcriptase followed by polymerase chain reaction (RT-PCR) in respiratory samples (nasopharyngeal and oropharyngeal secretions) (OLIVEIRA, 2020).

Concerning domestic and wild animals, SARS-CoV-2 may have implications for animal health, welfare, wildlife conservation, and biomedical research (EKSTRAND et al., 2021). Some studies have shown that animals can be affected naturally or experimentally by SARS-CoV-2; however, acute infection in dogs and cats has been less reported and a few studies indicate that SARS-CoV-2 infection in these animals is detected mainly in individuals living in households with at least one human infected with the virus, suggesting that transmission may be occurring from humans to companion animals (CALVET et al., 2021).

The SARS-CoV-2 infection prevalence, its pathophysiology and whether it jumps intraspecies or interspecies (zoonosis, either between humans or animals) remains unclear in wildlife being a potential topic for further studies. A study conducted between October 2020 and July 2021 in the city of Sao Paulo indicated that the frequency of SARS-CoV-2 infection in domestic animals, both dogs and cats, was very low; however, infected animals were asymptomatic or showed transient signs (AGOPIAN et al. 2022).

Another recent study in northeastern Brazil showed that cats with no clinical signs were considered positive for SARS-CoV-2 due to close contact with owners who were also positive (EPIFANIO, 2021). Finally, SARS-CoV-2 infections were reported in dogs, cats, domestic ferrets, tigers (*Panthera tigris*), lions (*Panthera leo*), cougars (*P. concolor*), snow leopards (*Panthera uncia*) and western lowland gorillas (*Gorilla gorilla*) under human care; in all these cases, transmission from humans to animals has been postulated (MAURIN et al., 2021; AHAW et al., 2023).

3.1.2.2 Feline Coronavirus

Family *Coronaviridae*, subfamily *Orthocoronavirinae*, order *Nidovirales*, genus *Alphacoronavirus 1*, subgenus *Tegacovirus*; they are large, enveloped viruses with a positive-sense single-stranded RNA genome with 29kb in size (DECARO et al., 2015). These are known to cause enteric disease in both domestic and wild felids; there are two serotypes named FCoV type I and Type II and two biotypes according to pathogenesis, FECV biotype (Mild or subclinical enteric disease) and FIPV biotype (Severe enteritis leading to Feline infectious peritonitis) (TEKES; THIEL, 2016).

Feline infectious peritonitis (FIP) was first described in the 1960s and since then cases have been reported in domestic and wild cats worldwide (PEDERSEN, 2009). FIP is an immune-mediated, systemic, progressive, and fatal disease. Its prevalence increases in areas with a high cat population density (FELTEN; HARTMANN, 2019). FIP is more common in young animals between three months and three years old, but cats over the age of 10 can develop the disease. Two forms of the disease are described, the first being called the wet or effusive form, its characterized by effusions in abdomen, chest and/or pericardium; in contrast, the second form is called the dry or non-effusive form, characterized by the presence of multiple granulomas in internal organs (RUBIO; CHAVERA, 2018).

Serology is widely used for diagnosing FCoV infection, measuring antibodies present in the animal’s blood serum; however, a percentage of healthy cats are known to have antibodies to FCoV. RT-PCR analysis of feces provides a sensitive and useful diagnostic method for documenting whether a cat is eliminating FCov enterically. Histopathology and immunohistochemistry are tests that corroborate the occurrence of FIP, as they make it possible to locate the specific areas of the lesions (SHARIF et al. 2010).

In Brazil, few cases of cats exposed to or infected with FCoV are tested, except in some situations in shelters with high sanitary standards or in cases where domestic and pet cats began to show clinical signs. A study of 151 cats in Botucatu, São Paulo, showed that FCoV is widespread in domestic cat populations and that the most relevant epidemiological variants are age group, reproductive status, and access to the outdoors (ALMEIDA, 2019). Several studies in Brazilian wild felids have been conducted to evaluate the presence of the virus (FILONI et al., 2003; FILONI et al., 2006; FILONI et al., 2012; FILONI et al., 2017; FURTADO et al., 2013; FURTADO et al., 2017).

3.1.3 Morbilivirus

The canine distemper virus (CDV) belongs to the Paramyxoviridae family; it is an enveloped, negative-sense single-stranded RNA virus. Mass vaccination of domestic dogs has been proposed as a strategy to protect endangered wildlife in many areas (VIANA et al., 2015). CDV is a systemic disease that in dogs affects an important range of organs, including lymphoid tissues, skin, brain, intestinal, and respiratory tracts, resulting in a variety of respiratory, enteric, and nervous signs and symptoms. CDV spreads primarily through aerosols and contact with respiratory and ocular exudative fluids and other body secretions such as urine or feces; Additionally, transplacental transmission has been documented in domestic dogs (TROGU et al., 2021). The most widely used diagnostic method is RT-PCR, given its high sensitivity, detecting this virus in different types of biological samples and at different stages of the disease (ELIA et al., 2006). Immunohistochemistry helps to show the distribution of the virus in tissues; In addition, the clinical diagnosis can be confirmed by identifying the characteristic viral inclusion, Lentz corpuscle (LC) in epithelial cells and in leukocytes (BARBOSA, 2011).

CDV is a threat to several species of wild carnivores; A prime example is the 1994 outbreak in the Serengeti National Park, when 30% of the lion population died from this disease. A that time, approximately 3,000 unvaccinated domestic dogs cohabitated within the National Park, representing a possible potential source of infection (ROELKE-PARKER et al., 1996). In Brazil, CDV is a common disease in peri-urban and rural dogs due to the lack of vaccination and the large number of feral dogs. In a study published in 2009, the phylogeny of the virus was found to be closely related to the domestic dog variant in the case of a bush dog (*S. venaticus*) (MEGID et al., 2009). Another study, carried out in 2013 by the same author, indicated that a lesser grison (*G. cuja*) with no clinical signs died suddenly after one day in captivity; molecular diagnostic techniques detected CDV and confirmed that retrieved virus was the domestic dog variant (MEGID et al., 2013).

Several studies have been carried out in Brazil for the detection of CDV mainly in Brazilian wild canids (C*. thous, L. vetulus*); where many of these have shown seropositivity indicating that they have been exposed to the virus; likewise, immunohistochemistry, immunolabeling in the cerebellum (CARNIELI et al., 2008;

CORDEIRO et al., 2016; FURTADO et al., 2016; SOUZA et al., 2022; ALVES et al., 2018; WEBER et al., 2020).

3.2. BRAZILIAN TERRESTRIAL CARNIVORES

3.2.1 Canidae

In Brazil, there are five genera of wild canids (i.e., *Atelocynus*, *Cerdocyon*, *Speothos*, *Lycalopex*, and *Chrysocyon*), comprising six different species (i.e., *Atelocynus microtis, Cerdocyon thous, Chrysocyon brachyurus, Lycalopex gymnocercus, Lycalopex vetulus, Speothos venaticus)*. Canids generally have crepuscular habits and can be solitary or gregarious, including bush dogs. Habitat loss or degradation due deforestation, unnatural mortality through conflicts with farmers and/or by motor vehicular collisions (MVC), and disease transmission from domestic dogs represent the major threats to wild canids population (CHIARELLO, 2021). Previous studies have shown the presence of pathogens that affect Brazilian canids. For instance, as indicated by (FIORI et al., 2023), sarcoptic mange is a major problem, and the increasing number of affected individuals, suggests that it is an emerging disease that threatens the maned wolf in Brazil, especially in anthropized areas.

The presence of viral diseases thus CDV has been evidenced in different species of canids in Brazil; in 2009, a young female *C. thous* found in a São Paulo suburban area exhibit generalized myoclonus compatible with the neurologic phase of canine distemper, the presence of the virus was confirmed by PCR (MEGID et al., 2009). In an epidemiological study in the Cerrado of Central Brazil, serum samples were collected from 169 wild carnivores (*C. brachyurus*, *C. thous*, and domestic dogs) and were found to be seropositive for CDV (FURTADO et al., 2016). In a metagenomics analysis, crab-eating fox (*C. thous*) from Brazil were the only animals that tested positive for CDV (WEBER et al., 2020). In Porto Alegre (Brazil), an adult *C*. *thous* that showed clinical signs of CDV was confirmed by immunohistochemistry and molecular techniques (PCR) (ALVES et al., 2018).

Concerning to rabies virus, seropositive *C*. *thous* have been frequently identified in the northeast; these have been implicated in the transmission of RABV to domestic dogs (CARNIELI et al., 2008; CORDEIRO et al., 2016). An experimental study in northeastern Brazil tested serum samples from 52 cases of wild canids (*C. thous* and *L. vetulus*) using fluorescent antibody test (FAT), microagglutination (MIT) and RT-PCR. All samples were positive for all three tests (FAT, MIT, and RT-PCR); antigenic typing suggested a close relation between dog and wild canid biotypes, both belonging to antigenic variant 2 (CARNIELI et al., 2008). Another experimental study in North and Northeast Brazil assessed 100 samples of *C. thous* and domestic dogs positive for RABV by RT-PCR,13 cases were interspecific infections between dogs and *C. thous,* and one dog case presented a lineage associated with the vampire bat *Desmodus rotundus* (DE SOUZA et al., 2017). Viruses such as SARS-CoV 2 and PIF have not yet been reported in Brazilian wild canids.

3.2.2. Felidae

In Brazil, there are four genera (*Herpailurus*, *Leopardus*, *Panthera,* and *Puma*) and ten species. Wild felids have crepuscular and nocturnal habits, are solitary, and are rarely seen in pairs. The main threats include habitat loss and fragmentation, unnatural mortality due to hunting, retaliation for predation by domestic animals, burning and trampling (CHIARELLO, 2021). Infections in wild felids with viruses that are common in domestic carnivores have been reported on several occasions. In a study from different parks in Brazil, blood samples were collected from wild felids in Morro do Diabo State Park (São Paulo) and Ivinhema State Park (Mato Grosso do Sul), resulting in six jaguars and one puma being seropositive for CDV (NAVA et al., 2008).

A study in four biomes in Brazil examined viral agents by serology in wild felids (*Puma concolor, Leopardus tigrinus, Leopardus pardalis),* and found antibody titers to Feline Herpesvirus type-1 (FHV1), Feline Calicivirus (FCV), FCoV, and Feline Panleukopenia Virus (FPV) in 21 serum samples (FILONI et al., 2006). Another retrospective study conducted in the state of São Paulo analyzed blood samples from 97 neotropical felids (*L. tigrinus, H. yagouaroundi, L. geoffroyi, L. wiedii, L. pardalis, P. concolor*) and 51 exotic felids kept in captivity at the São Paulo Zoo Foundation (SPZ). They tested for viral and parasitic agents present in wild felids, including FCoV; 65% of the samples were positive for fluorescent antibody test (FAT), the population of jaguarundi (*H. yagouaroundi*) presented the highest titers being more frequent in captivity. Additionally, two out of 147 samples from zoo-born jaguarundis were positive for Feline leukemia virus (FeLV) p27, and evidence of FHV-1, FCV or FPV infections were found in all neotropical species tested (FILONI et al., 2012).

A more recent study focused on 23 jaguarundis in captivity at the São Paulo Zoo Foundation (FPZSP), used serological and molecular analysis for FeLV, detecting four animals exposed to the virus (FILONI et al., 2017). In contrast, blood analysis from Brazilian wild felid populations showed negative serology for diseases such as FeLV, FCV, FCoV, and CDV infection (FILONI et al., 2003; FURTADO et al., 2013; FURTADO et al., 2017).

3.2.3 Procyonidae

There are four species in Brazil, belonging to the genera *Bassaricyon*, *Nasua*, *Procyon*, and *Potos*. In this family, most species have nocturnal habits, except for the coati, which is diurnal. The main threats include unnatural mortality by hunting due to retaliation, trampling, capture as a pet, and diseases transmitted by domestic dogs (CHIARELLO, 2021).

Aiming to assess the health status of the Procyonidae family, in relation to the presence of disease-causing viral agents in this group, a retrospective and experimental study was realized in Paraná Southern of Brazil, involving 13 cases of ring-tailed coati (*Nasua nasua*) from which samples were taken during necropsy for histopathology and immunohistochemistry (IHC) analysis, showing positive immunoreactivity in bronchiolar epithelium for CDV antigen in three animals; H&E-stained sections of these tissues demonstrated lesions typical for CDV infection (MICHELAZZO et al., 2022). A recent study collected samples from free-living coatis inhabiting the Mangabeiras Municipal Park in Belo Horizonte (Brazil), detecting neutralizing antibodies to SARS-CoV-2 in sera of two coatis (STOFFELLA-DUTRA et al., 2023).

Concerning to rabies virus and according to (FAVORETTO et al., 2013), most cases occurring in terrestrial mammals are the result of infection with variants of RABV circulating in wild reservoirs. For example, A study obtained lineage B isolates from a human bitten by a *P. cancrivorus*; subsequent analysis of *P. cancrivorus* specimens suggests an establishment of a *C. thous* variant in *P. cancrivorus*; this could be attributed to the overlapping niches of these two species, which facilitates their encounter and allows higher prevalence in wild carnivores (FAVORETTO et al., 2006).

3.2.4 Mustelidae

Six species occur in Brazil, belonging to the genera *Eira*, *Galicitis*, *Lontra*, *Mustela*, and *Pteronura*. Mustelids are solitary, but some species form family groups (i.e., giant otters). The main threats are habitat loss, unnatural mortality due to conflicts with farmers, predation by domestic dogs, trampling, and diseases transmitted by domestic dogs (CHIARELLO, A. G., 2021).

Regarding the health status and disease presentation in the family Mustelidae in Brazil, a study conducted in a young free-ranging lesser grison (*Galictis cuja*) found in the city of Votorantim (São Paulo), demonstrated, by the RT-PCR, positivity for a CDV strain compatible with those previously isolated in Brazilian domestic dogs (MEGID et al., 2013). Regarding other diseases, mainly viral, there is not much information available, however, considering the premise that SARS-CoV-2 infection in mink and other animals of the Mustelidae family may occur, it may represent a significant risk to the conservation of this group of animals as to the human health in a public health approach (EFSA et al., 2021).

3.2.5 Mephitidae

In Brazil, there are two species belonging to the genus *Conepatus*, with crepuscular and nocturnal habits. The main threats include habitat loss and fragmentation, unnatural mortality due to trampling, predation by domestic dogs, and pesticide use (CHIARELLO, 2021). Distribution maps indicate that *C. chinga* is present in the states of Paraná, Santa Catarina, and Rio Grande do Sul; while *C. semistriatus* is distributed from southern Mexico to northern Colombia, including Venezuela, Peru, and Brazil. Researchers have proposed that the Brazilian populations of *C. semistriatus* should be recognized as *C. amazonicus*, known as the striped skunk, found mainly in areas of open vegetation, and can occasionally be found in forested areas during the dry season. (MACHADO et al., 2023). The health information on the *Conepatus* genus is very scarce, but the most significant negative impact on its population in Brazil is likely the roadkill (CAVALCANTI, 2010).

3.3. DOMESTIC CARNIVORES IN THE STATE OF SÃO PAULO

In the city of São Paulo, the ratio of men to dogs is 4.34, while the ratio of cats is 19.33. On the other hand, the proportion of dogs with access to the street is 64.4%, significantly higher than cats, estimated at 42.5%. It is important to consider that the number of animals with unrestricted access to the street is very high and leading to home invasions, vehicular collisions, attacks on people and other domestic animals, and transmission of diseases, many of which are zoonotic (CANATTO et al., 2012).

In accordance with IBGE (2015), It was estimated that the population of dogs (52.2 million) and cats (22.1 million) in homes overlap the population of children under 14 in the country. This exponential increase in companion animals is a problem at global and local levels with great impacts on public health due to the possible risk of transmission of infectious diseases to humans and free-living wild animals (FELIPETTO et al., 2022). During the last decades, human activities have broken down many geographical barriers, facilitating the dispersion of numerous non-endemic species into multiple regions. These invasive species interact with native ones through predation, competition, hybridization, and pathogen transmission, causing great impacts on the structure and functioning of ecosystems, ultimately contributing to biodiversity loss and the decline of ecosystem services (ALONSO; CASTRO-DÍEZ, 2015).

Domestic dogs and cats are considered invasive alien species when they use or live-in natural areas without human intervention (without restriction of movement). Domestic dogs (*Canis familiaris*) can be considered a potential threat to the integrity of protected areas in Brazil; the presence of these animals in protected areas or their surroundings can reduce the effectiveness of biodiversity conservation (MMA, 2013). Domestic dogs can be classified into two groups: those that have guardians who provide adequate food and shelter, and stray or free-living dogs that are not under human care and survive opportunistically with the food resources offered by some people; this group represents 75% of the 700 million dogs in the world (HUGHES; MACDONALD, 2013).

Dogs have been considered the most abundant carnivores in several rural areas including the Brazilian Atlantic Forest (PASCHOAL et al., 2012; HUGHES; MACDONALD, 2013). High densities can cause direct impact by injury or death of other predators such as the crab-eating fox (*C. thous*) and jaguarundi (*H. yagouaroundi*), as well as potential transmission of infectious diseases to native wild carnivores. A study shows that *Canis familiaris* was one of the most abundant groups of mammal species captured on camera traps in natural areas of Brazil. For example, a total number of mammal records obtained from 2014 to 2016 by camera trapping in four municipalities in the state of Minas Gerais, southeastern Brazil showed that dogs were the second predominant group, with 92 observations (GUEDES et al., 2020).

In terms of pathogen transmission, dogs function as reservoirs of multiple parasites and pathogens that can affect native animals and human populations; they can also be carriers of multiple diseases (e.g., canine distemper, parvovirus, rabies, leishmaniasis and dirofilariasis). Many studies have reported the flow of pathogens between domestic, wild animals and humans causing devastating consequences such as epizootics or pandemics. Prevention measures and continue research on transmission potential of diseases from domestic dogs, as well as the severity of the impact of diseases on native vertebrates is essential for their conservation (LESSA et al., 2016).

3.4. WILDLIFE ROAD MORTALITY

According to ABRA et al. (2021) direct road mortality is considered one of the most significant impacts on endangered species, in compliance the National Action Plans of the Chico Mendes Institute for Biodiversity Conservation (ICMBio); the total number of mammals hit by cars per year on 2- and 4-lane paved roads in the state of São Paulo was estimated at an average of 39,605, with Rodentia as the most affected order(30,06%), followed by Carnivora (24,50%) and Cingulata (19,30%). According to ARTESP (2019), the number of accidents involving domestic and wild animals in the first 6 months of 2019 was 7,679, being 64% domestic animals and 36%, wild animals all these animals died.

On a global scale, roads are of great importance to society as they enable the transport of people and goods; however, they present some of the greatest threats to biodiversity. Coalitions between animals and vehicles are not only a concern for biological conservation, but also a threat to human safety (ABRA et al., 2019). Therefore, planning measures to reduce vehicle accidents is justified on three grounds: a) animal welfare and the threat to wildlife species; according to (DASOLER; GONÇALVES, 2023) eight million birds and two million mammals are killed on Brazilian roads, which may represent a large-scale loss of wildlife populations; b) the financial costs of vehicular crashes in São Paulo State: the average cost of accidents involving animals was estimated in R$ 21,656.00, and in the case of accidents with injuries and/or deaths it averaged R$ 67,048.00 (ABRA et al., 2019); c) the safety of drivers and passengers: the São Paulo police reported approximately 2,600 animal accidents per year in the state, with nearly18.5% causing human injuries (ABRA et al., 2019).

To minimize impacts, many experts are calling for better planning in the construction of new roads, such as viaducts for wildlife passage, noise barriers, and rafters in the asphalt composition to reduce noise (MARQUES, 2022). In addition, to mitigate negative effects on wildlife, it should be taken into account that movement corridors facilitate gene flow and genetic diversity of populations, therefore it is advisable to consider road segments where the potential for wildlife movement and road mortality are high. According to (CERQUEIRA et al., 2021), a study conducted to monitor wild felids killed by roadkill indicates that the effects of road fragmentation in the Atlantic Forest may be affecting the conservation of these species and therefore requires greater attention to these species by governmental agencies, hence more concessionaires in charge of the different roads increase monitoring and removal of animal corpses and collaborate with research to determine the number of species killed by roadkill per stretch of road in Brazil, especially in the state of São Paulo.

The use of roadkill carcasses provides valuable biological samples for different investigations including, assessing hybridization between endemic and feral species - by physical characteristics analysis, recording geographic distributions, and providing information on the distribution of the species (SCHWARTZ, 2020). The use of roadkill to collect samples of different tissues, internal organs, and parasites (ectoparasites, endoparasites), not only from endemic species that are not at risk but also from species under protection for disease monitoring in different populations, is also an important opportunity to study the health of wildlife populations. In addition, the logistics used to collect samples from roadkill animals, compared to the study of wildlife by live capture, require fewer resources. On the other hand, a major disadvantage of using biological material from roadkill animals is the state of decomposition of the carcasses (SZEKERES et al., 2019).

4. MATERIAL AND METHODS

To access the carcasses of wild carnivores, a partnership was implemented with three toll roads (TR) in the state of São Paulo. Under Brazilian federal and state environmental legislations, every toll company must execute road mortality monitoring (RMM), to propose mitigation strategies (underpasses, overpasses, or fences) (CETESB, 2023). During the RMM and within the framework of the established partnership, traffic inspectors circulate on the road every 3 to 5 hours and, if during the drive a carcass of a wild carnivore was sighted, the traffic inspectors used personal protective equipment (PPE) to collect it in a plastic bag, which then is stored in a freezer (-20C) at the operational control base (OCB); subsequently, the carcasses were transported to the Laboratory of Wildlife Comparative Pathology - LAPCOM at the University of São Paulo, in order to process the diagnostic exams. This flux was implemented by (NAVAS-SUÁREZ et al., 2022). The partnership lasted 19 months (January 2022 to July 2023). The Ethics Committee on Animal Use on Research of the FMVZ/USP approved this study under protocol number: 3436120722, and the System Authorization and Information on Biodiversity (SISBIO) of the Chico Mendes Institute of Biodiversity Conservation (ICMBio) approved this study with the license number: 82263-1. SISGEN (A39AC43)

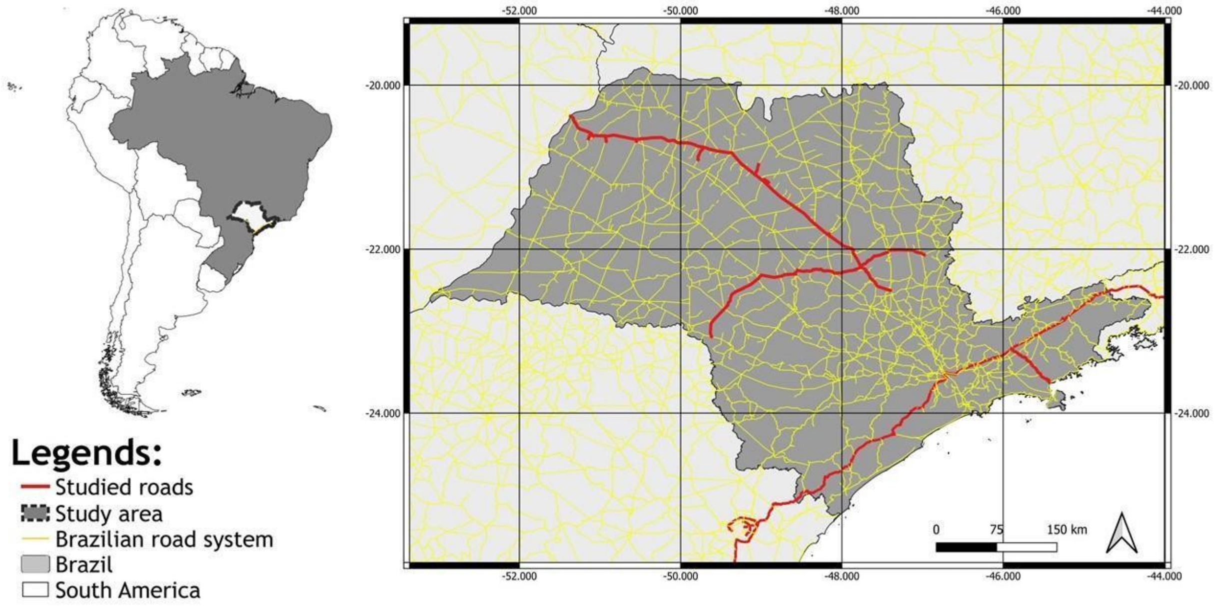
4.1. STUDY AREA

The partnership was made with three roads in the São Paulo State, Brazil (Rodovia Tamoios, Rodovia Regis Bittencourt, and Rodovia Eixo-SP). Details of the selected highways roads are recorded in Table 1 and Figure 1.

Table 1 - General characteristics of the highways selected in this study.

Toll road	Total Length (km)	Roads	Speed limit (km/hr)	Type of surface	Number of lanes
TR-01	82	SP99	60-100	Paved	Mixed (two-lane, single lane)
TR-02	402	BR116	60-80	Paved	Two-lane
TR-03	1273	SP310, SP225	40-110	Paved	Mixed (two-lane, single lane)

Figure 1 – Spatial distribution of the selected highways in this study



Source: ARIAS LUGO (2023). Software: QGIS.

TR-01 (SP99)

SP99 is a mixed road with sections of a single line (mainly in the Serra do Mar National Park) and transects with two-lane with New Jersey separators, connecting the cities of São José dos Campos and Caraguatatuba (Figure 1). The road crosses areas of grasslands, eucalyptus plantations, and urban areas in the Paraíba River valley, and down the Serra do Mar State Park to the coastal lowlands. SP99 begins at BR116

(Rodovia Presidente Dutra) and crosses with SP70 (Rodovia Governador Carvalho Pinto), these two large highways cross the state of São Paulo (SP) in a North-South direction, connecting the city of São Paulo with the state of Rio de Janeiro (RJ). For the RMM, the toll road has an operational vehicle, fully equipped and with professionals trained to carry out this activity. In addition, it has two OCBs positioned at km60 and km82 in which the freezers are located (TAMOIOS, 2021).

TR-02 (BR116)

The BR116 is a highway that connects the cities of Sao Paulo (SP capital) and Curitiba (Paraná (PR) capital) (Figure 1). Around 22,000 vehicles circulate every day on the highway, which runs through 17 municipalities. It is a completely two-lane highway and has a speed limit of 110 km/h for cars and 80 km/h for trucks. It plays an important role in the road network that connects the most important economic poles of the Southeast and South of Brazil, and the latter with the main MERCOSUR countries. There are currently 12 monitored wildlife crossings implemented across this highway. As TR-01, for the RMM, this toll road has an operational vehicle, fully equipped and with professionals trained to carry out this activity (ARTERIS, 2022).

TR-03 (SP310-2P225)

TR-03 is a toll road that manages a total of 1,273 km made up of 12 highways, which interconnect 62 municipalities, linking the central region of the state of São Paulo (municipality of Rio Claro) with the border with the state of Mato Grosso do Sul (MS) (Municipality of Panorama, SP). The SP310 and SP225 roads connect the cities of São Carlos with Bauru (Figure 1). For the RMM, this TR has 32 OCBs positioned along the entire stretch and has 89 operational vehicles with trained staff (EIXO-SP, 2023).

4.2 NECROPSY AND SAMPLE COLLECTION

Necropsy examinations followed a standard protocol (KING, 2014). The epidemiological data were collected for each specific case, comprising sex (male, female), age class (juvenile, adult), and body condition (poor, regular, good, or overweight). For taxonomic identification, we used the Brazilian mammal species identification guide (DOS REIS, M. et al., 2015). Additionally, for each species, specialized literature was reviewed to characterize the type of locomotion, activity pattern, diet, plasticity to land-use changes, and social habits (SCHMIDT, M., 2011). Gross information was collected according to the LAPCOM standardized necropsy form (Annex 1). For data evaluation, descriptive data analysis was performed. Data were reported in absolute (n=number of cases) and relative (%) terms. For each viral agent studied, a standard sample collection was carried out (Table 2). All samples were collected in 1.8ml plastic screw-capped identified with the animal case number and then stored in a −80°C freezer.

Table 2 - Sample collection for each RNA viral molecular protocol.

Agent	Samples
RABV*	Cerebellum, brain, and brainstem
Pan-coronavirus (SARS-CoV-2, FCoV)	Intestine, lung
CDV	Lung, kidney, mesenteric lymph node and CNS

*In the case of cranioencephalic trauma, a sample of the mixed encephalic tissues was collected. RABV: Rabies Virus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, FCoV: Feline Coronavirus; CDV: Canine Distemper Virus; CNS: Central nervous system.

4.3 HISTOPATHOLOGY

Representative samples of each organ were collected during necropsy, fixed in 10% neutral buffered formalin, and embedded in paraffin. Five µm thick sections were cut using a rotating microtome, the sections were stained with hematoxylin and eosin and then mounted in synthetic resin diluted in toluene (Permount TM). Microscopic analysis of the tissues was performed by light microscopy, with magnifications of 4, 10, 40, and 100x (JUNQUEIRA, 2001).

4.4 MOLECULAR ANALYSIS

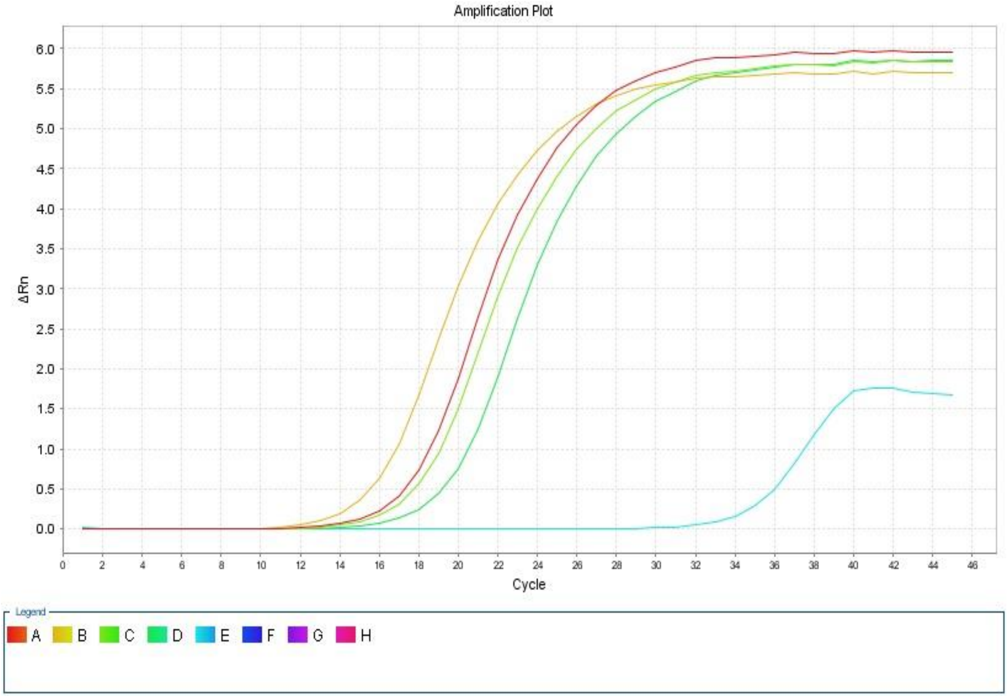
4.4.1 Total RNA extraction

Tissue samples of the central nervous systems (i.e., cerebellum, cerebrum, and brainstem), lungs, kidney, mesenteric lymph node, and intestine were separated in aliquots, macerated, and subjected to total RNA extraction using the RNeasy mini kit, following the manufacturer’s procedures (Qiagen, Valencia, CA, USA). All RNA samples were frozen at -80°C until processing.

4.4.2. Rabies virus screening

Extracted RNA from a pool of central nervous system (i.e., cerebellum, brainstem, and brain) was screened for Lyssavirus rabies using a one-step real time RT-PCR (Reverse Transcription-Polymerase Chain Reaction) adapted from (MACEDO et al., 2006) targeting the terminal portion of the nucleoprotein (N) gene (249pb). An aliquot of 4µL of extracted RNA was included in the RT-PCR mixture containing 10 µL Kapa Master Mix (2X), 50X RT Kapa, Rox high, primers 504 and 304 (Table 3), and RNase-Free water to a final volume of 20 µL. Reactions were performed in StepOnePlus system following a cycle of 5 min at 42°C for RT and a cycle of 3 min at 95°C followed by 40 cycles of 3 seconds at 94ºC, 20 seconds at 55ºC and 10 seconds at 72ºC, and a final cycle of 10 min at 72ºC. A cell culture sample of Lyssavirus rabies provided by the Rabies laboratory (FMVZ/USP) was used as positive control, and DPEC water was used as no template control. For standardization, the positive control was diluted (10-1 to 10-6) to a Cycle Threshold (CT) suitable for the reaction (Figure 2). Suspect samples were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), and confirmed by direct Sanger sequencing in both directions.

Figure 2 – Concentration curve of the positive control with different CT for the standardization protocol of the RT-PCR assay for RABV.



Source: ARIAS LUGO (2023).

Table 3 - Oligonucleotides used in RABV assay.

Primer name	**Sequence**	**Amplicon length**
Sense primer 504	5’TATACTCGAATCATGATGAATGGAGGTCGACT-3’	249 bp
Antisense primer 304	5’-TTGACGAAGATCTTGCTCAT-3’	

4.4.2 *Orthocoronavirinae* screening

RNA extracted from lungs and intestines were tested by a *Orthocoronavirinae* universal semi-nested RT-PCR, this panel evaluates several known coronaviruses and other unrelated human respiratory virus pathogens (Human coronavirus 229E, Human coronavirus NL63, Human coronavirus OC43, Human coronavirus HK, SARS-CoV-2, MERS-CoV, SARS-CoV, SARS-CoV, Canine and feline coronavirus, Porcine respiratory coronavirus, Transmissible gastroenteritis virus, Bovine, coronavirus, Infectious bronchitis virus, Porcine deltacoronavirus, Human adenovirus, Human enterovirus 71, Human parainfluenza virus, Human rhinovirus, Influenza virus A H1N1, Influenza virus A H3N2, Influenza virus B, Influenza virus C, Respiratory syncytial virus A, Respiratory syncytial virus B), targeting RdRP (RNA-directed RNA polymerase) gene (XIU et al., 2020).

For the first round RT-PCR (the external primers) pan-CoV outF and pan-CoV_R were used to amplify from 670- 673bp product; For the second round Semi-nested PCR, an internal primer pan-CoV inF was used with pan-CoV R for amplification of a 599-602bp product (Table 4). In the first round, an aliquot of 4μL of extracted RNA was included in the RT-PCR mixture containing 13.5 μL RNase-Free water, DNTPs 0.5 μL, Qiagen One step mix (5x), One Step enzyme mix and primers pan-CoV outF and pan-CoV R to a final volume of 25 μL for conventional one-step RT-PCR.

The thermal profile includes incubation at 50°C for 30 min for reserve transcription, followed by incubation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 seg, 53°C for 30 seg, and 72°C for 45 seg: a final extension at 72°C for 10 min. The conventional PCR for the second step contained 4μL of first RT-PCR product, 13.5 μL RNase-Free water, 2.5 μL PCR 10X, DNTPs 0.5 μL, 1.25 μL MgCl, 0.25 μL Platinum™ Taq DNA Polymerase and 0.5 μL (pan-CoV_inF and pan-CoV_R). Finally, 599-602 bp RdRp amplicon was obtained by thermocycling using the following parameters: 94°C for 3 min, 36 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s; and final extension of 72°C for 10 min. A previously confirmed sample *of Alphacoronavirus 1* from a domestic cat received by the animal pathology service of the School of Veterinary Medicine and Animal Science (FMVZ) was used as positive control, and DPEC water was used as no template control. PCR products were analyzed by gel electrophoresis (1.5% agarose), and amplicons among 599-602pb were purified with GFX™ PCR DNA and Gel Band Purification Kit (Global life sciences, United Kingdom) and confirmed by direct Sanger sequencing in both directions.

Table 4 - Oligonucleotides used in the *Orthocoronavirinae* assay.

Step	**Primer name**	**Sequence**	**Amplicon length**
First (RT-PCR)	***pan-CoV_outF***	5’-CCAARTTYTAYGGHGGITGG-3’	670-673 bp
	pan-CoV_R	5’-TGTTGIGARCARAAYTCATGIGG-3’	
Second (Semi-nested)	***pan-CoV_inF***	5’-GGTTGGGAYTAYCCHAARTGTGA-3’	599-602 bp

4.4.3 *Morbillivirus* screening

RNA extraction from lungs, kidneys, mesenteric lymph nodes, and central nervous system pool were screened for morbillivirus by a conventional one-step RT-PCR targeting a partial fragment (429pb) of the phosphoprotein gene (BARRETT et al., 1993) (Table 5). An aliquot of 4µL of extracted RNA was included in the RT-PCR mixture containing 13.5 µL RNase-Free water, 5 µL QIAGEN OneStep RT-PCR buffer (5x) ™, DNTP´s 0.5 µL, QIAGEN OneStep enzyme mix™, primers *Sense primer* and *Antisense primer* to a final volume of 25 µL for conventional one-step RT-PCR. Reactions were performed at 30 min at 50°C for RT and a cycle of 15 min at 95°C followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 51°C, 45 seconds at 72°C, and a final cycle of 7 min at 72°C. PCR products were analyzed by gel electrophoresis (1.5% agarose), and amplicons (429pb) were purified with GFX™ PCR DNA and Gel Band Purification Kit (Global life sciences, United Kingdom) and confirmed by direct Sanger sequencing in both directions.

Table 5 - Oligonucleotides used in CDV assay.

Primer name	**Sequence**	**Amplicon length**
Sense primer	5’ATGTTTATGATCACAGCGGT-3’	429bp
Antisense primer	5’-ATTGGGTTGCACCACTTGTC-3’	

5 RESULTS

During the study period, a total of 33 carcasses were collected, representing eleven species encompassing four families: Felidae (36,4%; 12/33), Canidae (27.3%; 9/33), Procyonidae (21.2%; 7/33) and Mustelidae (15.2%; 5/33). Additionally, 4 domestic dogs were collected. Biological and epidemiological data of the carnivore species included in this study are recorded in Tables 6 and 7.

Table 6 – General aspects of wild carnivores included in study.

Species	Age class		Sex		Weight (kg)	Toll road
	Juvenil	Adult	Male	Female		
Crab-eating Fox (*Cerdocyon thous*)	-	100% (3/3)	67% (2/3)	33% (1/3)	5.6	PI-01
Maned Wolf (*Chrysocyon brachyurus*)	-	100% (6/6)	33% (2/6)	67% (4/6)	23.7	All
Jaguarundi (*Herpailurus yagouaroundi*)	-	100% (1/1)	100% (1/1)	-	4.9	PI-01
Ocelot (*Leopardus pardalis*)	33% (2/6)	67% (4/6)	67% (4/6)	33% (2/6)	10.4	PI-01, PI-03
Puma (*Puma concolor*)	33% (1/3)	67% (2/3)	67% (2/3)	33% (1/3)	24.6	PI-02, PI-03
Southern Tiger Cat (*Leopardus guttulus*)	-	100% (2/2)	50% (1/2)	50% (1/2)	2.2	PI-01, PI-03
Crab-eating Raccoon (*Procyon cancrivorus*)	-	100% (2/2)	-	100% (2/2)	7.25	PI-02, PI-03
South American Coati (*Nasua nasua*)	-	100% (5/5)	40% (2/5)	60% (3/5)	5.0	PI-01, PI-03
Lesser Grison (*Galictis cuja*)	-	100% (3/3)	100% (3/3)	-	1.2	PI-01
Neotropical Otter (*Lontra longicaudis*)	-	100% (1/1)	100% (1/1)		7.5	PI-03
Tayra (*Eira barbara*)	-	100% (1/1)	-	100% (1/1)	4.1	PI-02

Table 7 – Biological and ecological characteristics of wild carnivores included in the study.

Species	Locomotion	Activity	Diet	Social Behavior	Conservation trends			Population trend	Habitat loss classification	Total
					IUCN	BRASIL	CITES			
Canidae (n=9)										
Crab-eating Fox (*Cerdocyon thous*)	Terrestrial	Nocturnal	Omnivore	Solitary	LC	LC	II	Stable	Resilient	3
Maned Wolf (*Chrysocyon brachyurus*)	Terrestrial	Nocturnal	Omnivore	Solitary	NT	VU	II	Unknown	Sensitive	6
Felidae (n=12)										
Jaguarundi (*Herpailurus yagouaroundi*)	Terrestrial	Both	Carnivore	Solitary	LC	VU	II	Decreasing	Resilient	1
Ocelot (*Leopardus pardalis*)	Scansorial	Nocturnal	Carnivore	Solitary	LC	LC	I	Decreasing	Sensitive	6
Puma (*Puma concolor*)	Terrestrial	Both	Carnivore	Solitary	LC	VU	I	Decreasing	Resilient	3
Southern Tiger Cat (*Leopardus guttulus*)	Scansorial	Nocturnal	Carnivore	Solitary	VU	VU	I	Decreasing	Resilient	2
Procyonidae (n=7)										
Crab-eating Raccoon (*Procyon cancrivorus*)	Scansorial	Nocturnal	Omnivore	Solitary	LC	LC	-	Decreasing	Resilient	2
South American Coati (*Nasua nasua*)	Terrestrial	Diurnal	Omnivore	Gregary	LC	LC	III	Decreasing	Resilient	5
Mustelidae (n=5)										
Lesser Grison (*Galictis cuja*)	Terrestrial	Both	Carnivore	Small groups	LC	LC	-	Unknown	Resilient	3
Neotropical Otter (*Lontra longicaudis*)	Semiaquatic	Both	Carnivore	Solitary	NT	NT	I	Decreasing	Resilient	1
Tayra (*Eira barbara*)	Scansorial	Nocturnal	Omnivore	Solitary	LC	LC	-	Decreasing	Resilient	1
									Total	**33**

(Michalski et al. 2006), (Macdonald & Courtenay 1996), (Juarez & Marinho-Filho 2002), (Courtenay & Maffei 2004), (Rodden et al. 2004), (Jacomo et al. 2009), (Maia & Gouveia 2002), (Veado 2007), (de Melo et al. 2006), (Trolle et al. 2007), (Silveira et al. 2009), (Sabato et al. 2006), (De Oliveira et al. 2010), (Emmons & Feer 1997), (Murray & Gardner 1997), (de Oliveira 1998) (Currier 1983), (Grigione et al. 2002), (Nascimento & Feijó 2017), (Oliveira & Cassaro 2005), (Gompper & Decker 1998), (Beisiegel & Mantovani 2006), (Yensen & Tarifa 2003), (Lariviere 1999), (Presley 2000).

5.1 MOLECULAR ANALYSIS

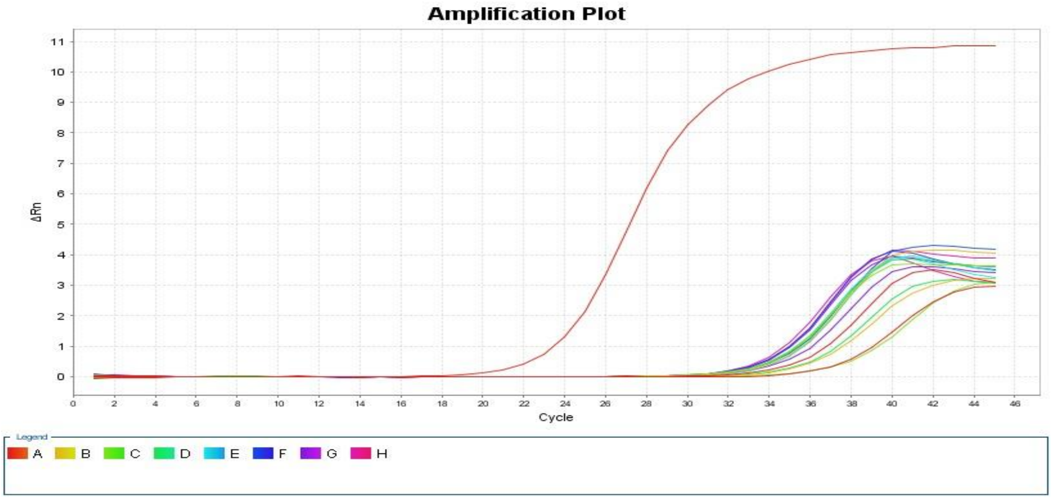
From the 33 wild carnivore carcasses evaluated in this study, 165 tissue samples were collected (33 of each tissue CNS, lung, intestine, kidney, and mesenteric lymph node) and 231 molecular analyses for the selected agents (33 RABV, 132 CDV, 33 PIF, and 33 SARS-CoV-2) were performed. Furthermore, from 4 domestic dogs run over on the roads studied, 20 tissue samples were collected (4 of each tissue CNS, lung, intestine, kidney, and mesenteric lymph node) and 28 molecular analyzes for the selected agent (4 RABV, 16 CDV, 4 *Orthocoronavirinae*) (Table 8). All cases were negative for the tested agents (Figure 3-4).

Table 8 – samples collected for all domestic and wild carnivores using in the molecular screening.

Tissue	**Pathogen**		
	RABV	***Orthocoronavirinae* (SARS-CoV-2, FCoV)**	**CDV**
Wild carnivores			
CNS	33	-	33
Intestine	-	33	
Kidney	-	-	33
Lung	-	33	33
Mesenteric lymph node	-	-	33
Total samples	33	66	132
Total wild carnivores	**231**		
Domestic carnivores			
CNS	4	-	4
Intestine	-	4	-
Kidney	-	-	4
CNS	-	-	4
Intestine	-	4	4
Total samples	4	8	16
Total domestic	**28**		

RABV: Rabies Virus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, FCoV: Feline Coronavirus; CDV: Canine Distemper Virus; CNS: Central nervous system.

Figure 3 - RT-PCR result for rabies virus (RABV) in wild and domestic carnivores



Source: ARIAS LUGO (2023).

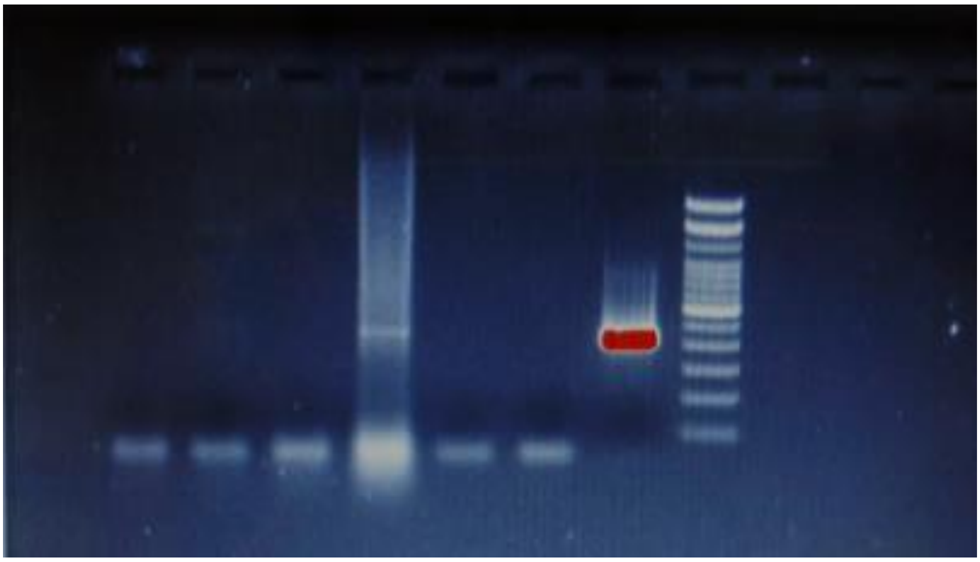
For the molecular study of *Orthocoronavirinae* (FCov, SARS-CoV-2), 33 wild carnivores (66 samples tested) and 4 domestic dogs (8 samples tested), for a total of n= 74 samples tested; all cases were negative (Figure 4). Using conventional PCR, some nonspecific bands, duplicates, and some with an estimated height of 600bp were obtained. However, upon purification and Sanger sequencing, all samples with an estimated size of 600 bp from wild carnivores and domestic dogs were determined to be endogenous genes.

Figure 4 - RT-PCR results for *Orthocoronavirinae* in wild and domestic carnivores. Red bands correspond to positive controls.

Source: ARIAS LUGO (2023).

For the molecular study of CDV, 33 wild carnivores (132 samples tested) and 4 domestic dogs (16 samples tested), for a total of n=148 samples analyzed; all cases were negative. Using conventional PCR, we obtained products with an estimated band size of 429bp (Figure 5). However, upon purification and Sanger sequencing, we determined that these PCR products corresponded to endogenous genes from wild carnivores and domestic dogs.

Figure 5 - PCR results for CDV in wild and domestic carnivores. The red band corresponds to positive control.



Source: ARIAS LUGO (2023).

5.2. MACROSCOPIC FINDINGS

The main gross findings associated with trauma are recorded in Table 9. The most frequent findings were bone fractures (100%; 31/31), rupture of parenchymatous organs (61%; 19/31), hemorrhages (61%; 19/31), hemoperitoneum (42%; 13/31) and hemothorax (45%; 14/31) (Figure 6).

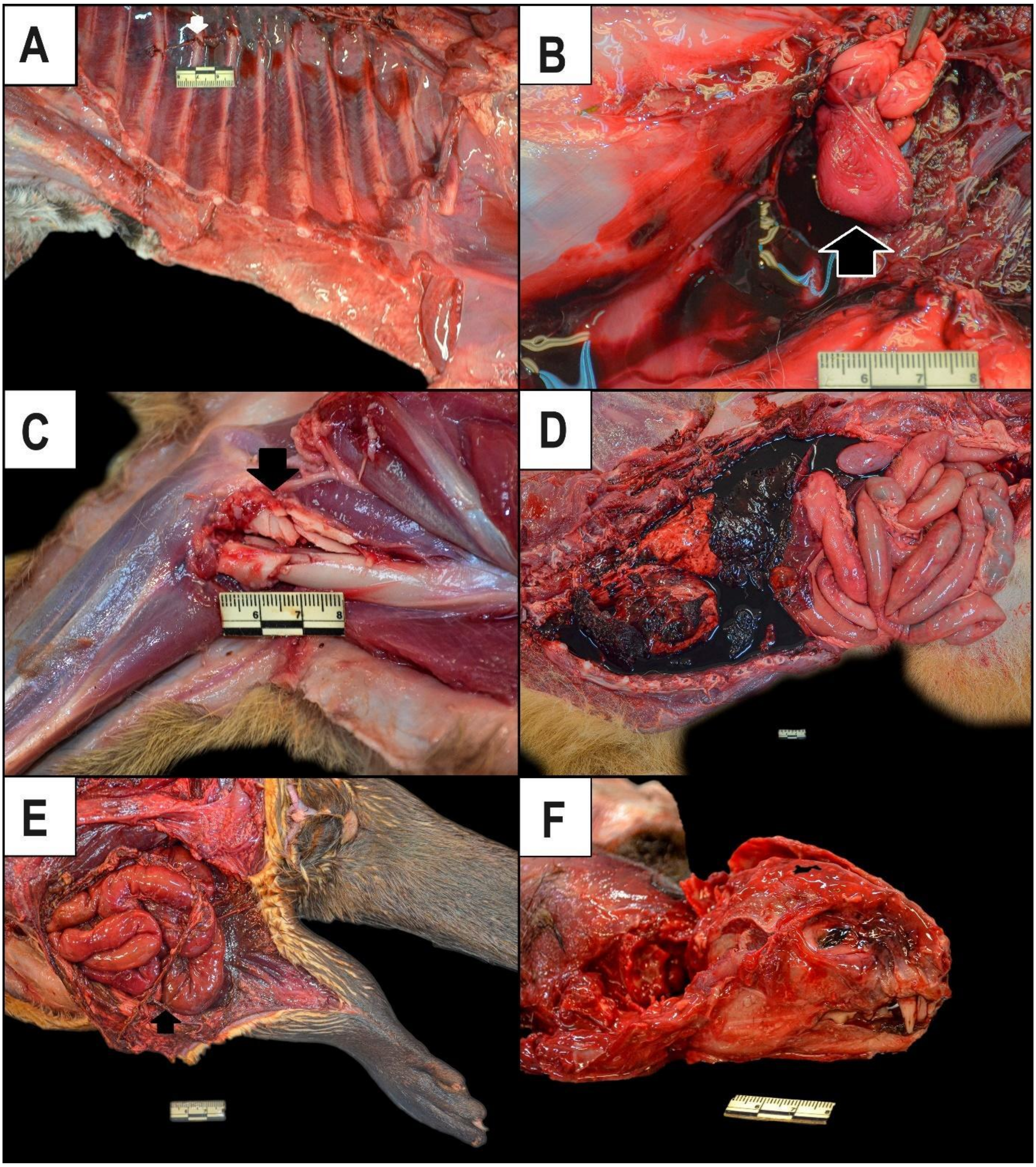
Table 9 - Main gross findings associated with vehicular collisions in wild carnivores (n=31).

Gross findings	T (n=31*)	CA (n=7)	FE (n=12)	MU (n=5)	PR (n=7)
Fracture	31 (100%)	7 (100%)	12 (100%)	5 (100%)	7 (100%)
Skull	20 (65%)	4 (57%)	8 (67%)	3 (60%)	5 (71%)
Claws	6 (19%)	0 (0%)	6 (50%)	0 (0%)	0 (0%)
Rib	14 (45%)	3 (43%)	6 (50%)	3 (60%)	2 (29%)
Jaw	14 (45%)	1 (14%)	6 (50%)	2 (40%)	5 (71%)
Hip	7 (23%)	2 (29%)	2 (17%)	3 (60%)	0 (0%)
Nasal	2 (6%)	0 (0%)	0 (0%)	0 (0%)	2 (29%)
Maxillae	6 (19%)	0 (0%)	2 (17%)	1 (20%)	3 (43%)
Tooth	9 (29%)	2 (29%)	4 (33%)	2 (40%)	1 (14%)
Spine	7 (23%)	3 (43%)	2 (17%)	1 (20%)	1 (14%)
Humerus	6 (19%)	3 (43%)	2 (17%)	0 (0%)	1 (14%)
Femur	3 (10%)	1 (14%)	1 (8%)	1 (20%)	0 (0%)
Zygomatic	1 (3%)	1 (14%)	0 (0%)	0 (0%)	0 (0%)
Rupture	19 (61%)	5 (71%)	7 (58%)	3 (60%)	4 (57%)
Pericardium	6 (19%)	2 (29%)	0 (0%)	1 (20%)	3 (43%)
Liver	14 (45%)	4 (57%)	7 (58%)	2 (40%)	1 (14%)
Kidney	8 (26%)	4 (57%)	3 (25%)	1 (20%)	0 (0%)
Spleen	5 (16%)	1 (14%)	3 (25%)	1 (20%)	0 (0%)
Lungs	12 (39%)	1 (14%)	5 (42%)	3 (60%)	3 (43%)
Heart	6 (19%)	0 (0%)	2 (17%)	1 (20%)	3 (43%)
Stomach	2 (6%)	1 (14%)	0 (0%)	1 (20%)	0 (0%)
Intestine	3 (10%)	0 (0%)	2 (17%)	1 (20%)	0 (0%)
Diaphragm	8 (26%)	1 (14%)	4 (33%)	1 (20%)	2 (29%)
Hemorrhage	19 (61%)	5 (71%)	9 (75%)	1 (20%)	4 (57%)
Oral cavity	10 (32%)	2 (29%)	5 (42%)	0 (0%)	3 (43%)
Muscles	4 (13%)	2 (29%)	2 (17%)	0 (0%)	0 (0%)
Nostrils	12 (39%)	1 (14%)	6 (50%)	1 (20%)	4 (57%)
Hemothorax	14 (45%)	4 (57%)	5 (42%)	2 (40%)	3 (43%)
Hemoperitoneum	13 (42%)	4 (57%)	4 (33%)	3 (60%)	2 (29%)
Eyeball protrusion	7 (23%)	1 (14%)	5 (42%)	0 (0%)	1 (14%)
Evisceration	7 (23%)	2 (29%)	1 (8%)	2 (40%)	2 (29%)

*Two maned wolves were excluded for this study since the necropsy was carried out by staff from another institution.

T=total; CA=Canidae; FE=Felidae; Mu=Mustelidae; PR=Procyonidae

Figure 6 - A. CS001. *Leopardus Pardalis.* Multiple rib fractures (white arrow) and focal hematoma. B. CS002. *Cerdocyon thous.* Urinary bladder evisceration (black arrow) and hematoma. C. CS004. *Cerdocyon thous*. Comminuted fracture of humerus (black arrow). D. CS008. *Chrysocyon brachyurus.* Hemothorax. E. CS010. *Lontra longicaudis.* Small intestine evisceration (black arrow). F. CS014. *Herpailurus yagouaroundi.* Complete skull fracture.



Source: ARIAS LUGO (2023).

DISCUSSION

There are studies indicating that the Carnivora order is highly impacted by un-natural mortality due to vehicle collisions (ABRA et al., 2021; PINTO et al., 2022; NAVAS-SUÁREZ et al., 2022), with rates between 5 to 20 individuals/100km/year (GRILO et al., 2019). Specifically in the Atlantic Forest, the habitat loss and fragmentation of ecosystems due to the high road network could explain the high mortality rates of wild carnivores (CERQUEIRA et al., 2021). In our study, we obtained 33 carcasses of wild carnivores belonging to 11 different species; of which four are categorized as vulnerable (VU) according to the red list of the ICMBio (MMA, 2022). Our study demonstrates the high diversity of wild carnivores' victims of road mortality in the study area.

Some authors postulate that the high frequency of carnivores killed by collisions with vehicles may be related to the fact that the road allows them to reach food sources for more generalist species (e.g., *C*. *thous*) mainly due to higher rates of movement in the dry season. To search for food and water sources, carnivores may have higher rates of movement and, therefore, an increase in road crossings, enhancing the risk of having a vehicle collision (ABRA et al., 2021; SILVEIRA et al., 2009). In this study, we believe that the search for food may be a potential process that explains road mortality since 51.5% (17/33) of the animals had stomachs replete with food. This fact has also been previously reported by NAVAS-SUÁREZ et al., (2022).

It is reported that road mortality is more frequent in adult individuals due to their behavior to find a mate and search for food (MOORE, 2023). In this study, 90.9% (30/33) of the cases correspond to adult individuals, which corroborates the above assumption. Therefore, our data may indicate that these individuals could be in a housing period or searching for food. Studies report that males are more vulnerable to being run over than females (MIOTTO, 2012; GREEN-BARBER; OLD, 2019). This hypothesis may also be supported by our study since 54.5% (18/33) of the individuals were males. It is estimated that the drastic withdrawal of adult males from a population can generate problems in population dynamics, generating the Allee effect (MIOTTO, 2012; GREEN-BARBER; OLD, 2019).

Road mortality studies in megadiverse regions with expanding road networks are still scarce (SILVA; CRANE; SAVINI, 2021). In the state of São Paulo, it is estimated that annually more than 39 thousand mammals are victims of road mortality (ABRA et al., 2021). Although our study is specific to three selected roads and for the Carnivora order, our findings are consistent with ABRA report. In addition, the diversity of the species (11) collected, suggests this anthropogenic loss of biodiversity could pose challenges for the conservation of threatened species, at least at local scale.

The impact observed by human activities from deforestation to the construction of new roads can influence the transmission of various pathogens and diseases with devastating results (LINDAHL; GRACE, 2015). Habitat loss and ecosystem imbalances can lead to changes in the dynamics of some viruses, which can undergo mutations resulting in changes in host diversity as well as morbidity and mortality (BARBIER, 2021). Therefore, the importance of the study of viral agents, mainly RNA viruses, is high, especially because these agents are the most frequent cause of spillover (ALEXANDER et al., 2018). In our study, we performed molecular screening to detect the presence of viral RNA agents causing diseases including, rabies, FIP, canine distemper, and SARS-CoV-2) in the tissues of 33 wild carnivores and 4 domestic dogs as follows.

In relation to the measurement of viral activity in rabies, the detection limit of the N gene-specific RT-PCR is 100.5 LD50/0.03 mL (KANG et al., 2007). In our study, all the individuals analyzed were negative for the N nucleoprotein gene. This indicates that the animals were not presenting infection with the Rabia virus or that the viral load at the time of collection was below the detection level. It has been postulated that neurological diseases could predispose to vehicle collisions (WOBESER, 2013; SÁNCHEZ et al., 2018). However, in our study, as well as in previous ones (NAVAS-SUÁREZ et al., 2022), no macroscopic lesions associated to pre-existing neurologic diseases were observed. In our study, the negative results for rabies by RT-PCR for both wild and domestic carnivores, suggest that the surveyed animals were not acting as host for the virus.

Viral agents have a wide mutational capacity, (e.g., the family *Coronaviridae*) increased incidence of mutations and recombination that lead to the occurrence of new CoVs (CHANG, 2012; YE et al., 2020). In this study, all individuals were negative for the CoV. This negative finding may be explained by multiple factors, including the fact that due to the high mutagenicity of this virus, the degenerate primers used for this reaction were not suitable. Another possibility to explain the results is the fact that the animals could effectively not be infected or that the viral load at the time of collection was very low. In the case of SARS-CoV 2, the high rate of variability and mutation can be attributed to the fact that animals (wild and domestic carnivores) are not adapted to the viral agent as hosts (KLESTOVA, 2023). This may also justify the absence of gross findings and alterations compatible with SARS-CoV 2 and FIP.

CDV may be unique among morbilliviruses, as it is postulated that host species living in low-density populations can sustain the virus (MARIN, 2021). In this study, all individuals were negative and did not present gross pathological lesions suggestive of distemper. This may indicate that the populations studied were not in contact with the virus, since for some of the species studied there are reports of this disease inducing high morbidities (FURTADO et al., 2016; WEBER et al., 2020; ALVES et al., 2018; CARNIELI et al., 2008; CORDEIRO et al., 2016; DE SOUZA et al., 2017). In addition, in previous studies in wild carnivore populations, antibodies to CDV have been identified through serology and, in these individuals, it was postulated that they have been exposed to the virus but already generated an immunological response, reducing the impacts on the individual (TAQUES et al., 2018).

The use of roadkill carcasses is a great source of biological samples for different investigations (SCHWARTZ; SHILLING; PERKINS, 2020). In our study, the gross findings related to vehicular collision were similar to those reported by (NAVAS-SUAREZ et al., 2022). In that study, it is postulated that the most significant injuries affecting the thoracic and abdominal cavities are due to the impact with the vehicle bumper. These injuries are severe enough to cause instant death of the individual. In our study, traumatic injuries were also related to the cause of death of all the individuals studied.

7. CONCLUSIONS

Our results suggest the potential impact of road mortality on carnivores since individuals suitable for reproduction (adults, males, and females) are annually removed from local populations. We consider that monitoring programs and sample collection of road-killed animals should be carried out on an extensive basis, in order to have a better understanding of the potential impacts of this process.

In our study, all samples were negative for the selected pathogens, even using a broad-spectrum RT-PCR in the case of *Coronaviridae*. These results allow us to conclude that there was no evidence of the presence and/or pathogenicity of these viruses in the animals studied, indicating a low prevalence of these infectious agents in this group. These results are important in terms of conservation since they give us information to suggest that the studied viral diseases (RNA), at least on our geographic and temporal scales, do not seem to be a threat to the conservation of wild carnivores in São Paulo state.

On the other hand, it is important to note that the negative results may also be attributed to the inefficiency of detection of the selected diagnostic assay. As RNA viruses have high mutational variability, the primers used, which are mostly degenerate, may not be specific for the agents investigated.

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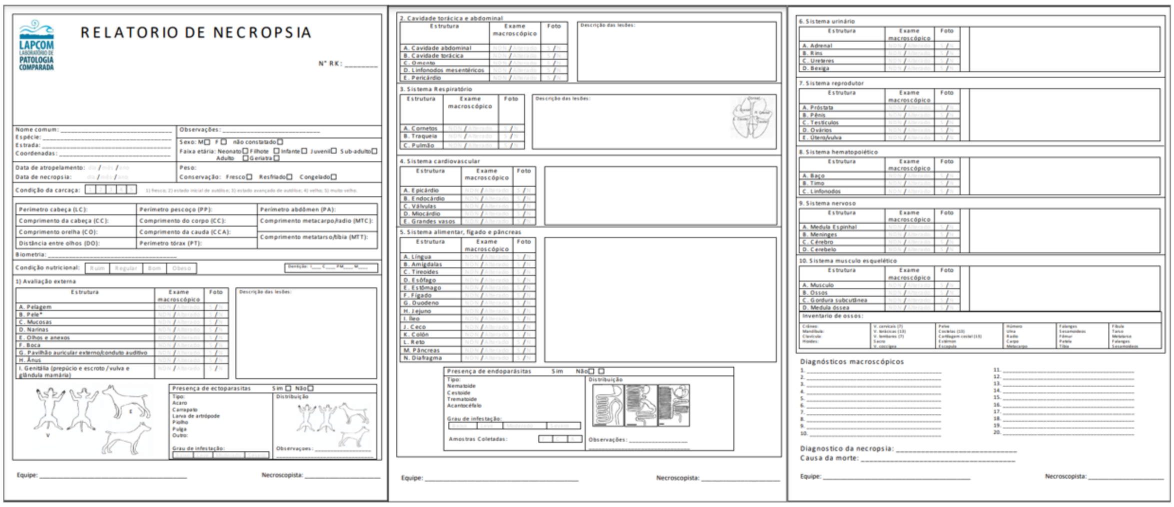
Anexo 1 - Laboratory of Wildlife Comparative Pathology (LAPCOM) Necropsy form LAPCOM (2017)**.**



RELATORIO DE NECROPSIA

Nº RK: ________

Nome comum: ______________________________
Espécie: ______________________________
Estrada: ______________________________
Coordenadas: ______________________________

Observações: ___________________________
Sexo: M☐ F☐ não constatado☐
Faixa etária: Neonato☐ Filhote ☐ Infante☐ Juvenil☐ Sub-adulto☐ Adulto ☐ Geriatra☐

Data de atropelamento: dia / mês / ano
Data de necropsia: dia / mês / ano

Peso:
Conservação: Fresco☐ Resfriado☐ Congelado☐

Condição da carcaça: 1 | 2 | 3 | 4 | 5 — 1) fresco; 2) estado inicial de autólise; 3) estado avançado de autólise; 4) velho; 5) muito velho.

Perímetro cabeça (LC):	Perímetro pescoço (PP):	Perímetro abdômen (PA):
Comprimento da cabeça (CC):	Comprimento do corpo (CC):	Comprimento metacarpo/radio (MTC):
Comprimento orelha (CO):	Comprimento da cauda (CCA):	Comprimento metatarso/tibia (MTT):
Distância entre olhos (DO):	Perímetro tórax (PT):	

Biometria: ______________________________

Condição nutricional: Ruim | Regular | Bom | Obeso

Dentição: I____ C____ PM____ M____

1) Avaliação externa

Estrutura	Exame macroscópico	Foto
A. Pelagem	NDN / Alterado	S / N
B. Pele*	NDN / Alterado	S / N
C. Mucosas	NDN / Alterado	S / N
D. Narinas	NDN / Alterado	S / N
E. Olhos e anexos	NDN / Alterado	S / N
F. Boca	NDN / Alterado	S / N
G. Pavilhão auricular externo/conduto auditivo	NDN / Alterado	S / N
H. Ânus	NDN / Alterado	S / N
I. Genitália (prepúcio e escroto / vulva e glândula mamária)	NDN / Alterado	S / N

Descrição das lesões:

V
E

Presença de ectoparasitas Sim ☐ Não☐
Tipo:
Acaro
Carrapato
Larva de artrópode
Piolho
Pulga
Outro:
Grau de infestação: Baixo | Leve | Moderado | Severo
Distribuição
Observaçoes: ________________

Equipe: ______________________________ Necroscopista: ________________

2. Cavidade torácica e abdominal

Estrutura	Exame macroscópico	Foto
A. Cavidade abdominal	NDN / Alterado	S / N
B. Cavidade torácica	NDN / Alterado	S / N
C. Omento	NDN / Alterado	S / N
D. Linfonodos mesentéricos	NDN / Alterado	S / N
E. Pericárdio	NDN / Alterado	S / N

Descrição das lesões:

3. Sistema Respiratório

Estrutura	Exame macroscópico	Foto
A. Cornetos	NDN / Alterado	S / N
B. Traqueia	NDN / Alterado	S / N
C. Pulmão	NDN / Alterado	S / N

Descrição das lesões:

4. Sistema cardiovascular

Estrutura	Exame macroscópico	Foto
A. Epicárdio	NDN / Alterado	S / N
B. Endocárdio	NDN / Alterado	S / N
C. Válvulas	NDN / Alterado	S / N
D. Miocárdio	NDN / Alterado	S / N
E. Grandes vasos	NDN / Alterado	S / N

5. Sistema alimentar, fígado e pâncreas

Estrutura	Exame macroscópico	Foto
A. Língua	NDN / Alterado	S / N
B. Amígdalas	NDN / Alterado	S / N
C. Tireoides	NDN / Alterado	S / N
D. Esôfago	NDN / Alterado	S / N
E. Estômago	NDN / Alterado	S / N
F. Fígado	NDN / Alterado	S / N
G. Duodeno	NDN / Alterado	S / N
H. Jejuno	NDN / Alterado	S / N
I. Íleo	NDN / Alterado	S / N
J. Ceco	NDN / Alterado	S / N
K. Colón	NDN / Alterado	S / N
L. Reto	NDN / Alterado	S / N
M. Pâncreas	NDN / Alterado	S / N
N. Diafragma	NDN / Alterado	S / N

Presença de endoparásitas Sim Não☐ ☐
Tipo:
Nematoide
Cestoide
Trematoide
Acantocéfalo
Grau de infestação: Baixo | Leve | Moderado | Severo
Amostras Coletadas: F | C | A
Distribuição
Observações: ________________

Equipe: ______________________________ Necroscopista: ________________

6. Sistema urinário

Estrutura	Exame macroscópico	Foto
A. Adrenal	NDN / Alterado	S / N
B. Rins	NDN / Alterado	S / N
C. Ureteres	NDN / Alterado	S / N
D. Bexiga	NDN / Alterado	S / N

7. Sistema reprodutor

Estrutura	Exame macroscópico	Foto
A. Próstata	NDN / Alterado	S / N
B. Pênis	NDN / Alterado	S / N
C. Testículos	NDN / Alterado	S / N
D. Ovários	NDN / Alterado	S / N
E. Útero/vulva	NDN / Alterado	S / N

8. Sistema hematopoiético

Estrutura	Exame macroscópico	Foto
A. Baço	NDN / Alterado	S / N
B. Timo	NDN / Alterado	S / N
C. Linfonodos	NDN / Alterado	S / N

9. Sistema nervoso

Estrutura	Exame macroscópico	Foto
A. Medula Espinhal	NDN / Alterado	S / N
B. Meninges	NDN / Alterado	S / N
C. Cérebro	NDN / Alterado	S / N
D. Cerebelo	NDN / Alterado	S / N

10. Sistema musculo esquelético

Estrutura	Exame macroscópico	Foto
A. Musculo	NDN / Alterado	S / N
B. Ossos	NDN / Alterado	S / N
C. Gordura subcutânea	NDN / Alterado	S / N
D. Medula óssea	NDN / Alterado	S / N

Inventario de ossos:

Crâneo: Mandíbula: Clavícula: Hioides:	V. cervicais (7) V. torácicas (13) V. lombares (7) Sacro V. coccígea	Pelve Costelas (13) Cartilagem costal (13) Estérnon Escápula	Úmero Ulna Radio Carpo Metacarpo	Falanges Sesamoideos Fêmur Patela Tíbia	Fíbula Tarso Metatarso Falanges Sesamoideos

Diagnósticos macroscópicos

1. ________________________________
2. ________________________________
3. ________________________________
4. ________________________________
5. ________________________________
6. ________________________________
7. ________________________________
8. ________________________________
9. ________________________________
10. ________________________________
11. ________________________________
12. ________________________________
13. ________________________________
14. ________________________________
15. ________________________________
16. ________________________________
17. ________________________________
18. ________________________________
19. ________________________________
20. ________________________________

Diagnostico da necropsia: ____________________________
Causa da morte: ____________________________

Equipe: ______________________________ Necroscopista: ________________

Source: LAPCOM (2017).