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DETECTION OF BLOOD PATHOGENS IN NON-HUMAN PRIMATES OF THE ECUADORIAN AMAZON USING NON-INVASIVE TECHNIQUES

DÉTECTION DE PATHOGÈNES SANGUINS CHEZ LES PRIMATES NON HUMAINS DE L'AMAZONIE ÉQUATORIENNE À L'AIDE DE TECHNIQUES NON INVASIVES

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Acronyms

ARCSA	National Agency for Regulation, Control and Phytosanitary
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BS	Blood smears
CatL-like	Cathepsin L-like
CDC	Center for Disease Control and Prevention
CF	Complement fixation test
cm	Centimeter
COA	Organic environenmental Code
DAT	Direct Agglutination Test
dDNTp	Dideoxyribonucleotides
DFA	Direct Immunofluorescence Assay
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FF	Flooded forest
g	Grams
HA	Hemagglutination test
HC	Hemoculture
HI	Hemagglutination inhibition test
IFA	Immunofluorescence Assay
IFAT	Indirect Fluorescent Antibody Technique
IHC	Immunohistochemistry
IIF	Indirect Immunofluorescence Assay
INSPI	National Institute of Research on Public Health
IPCC	Intergovernmental Panel on Climate Change
ITS	Internal transcribed spacers
IUCN	International Union for Conservation of Nature
km^2	Square kilometer
M	Monogamous
MAATE	Ministry of the Environment, Water and Ecological Transition
MAT	Modified agglutination test
ml	Milliliter
ML	The Maximum-Likelihood Phylogeny
mM	Milimolar
MM	Multi-males
MM-MF	Multi male- multi female
MNT	Mouse neutralization test
MYA	Million years ago
N.A.	Not available
NATb	Neutralizing antibody survey
NCBI	National Center for Biotechnology Information
NGOs	Non-governmental organizations
NHPs	Non-Human Primates
NT	Neutralization test
NWs	New World Monkeys
OIE (WAHO)	Office international des Epizooties (World Organization for Animal Health)
OWs	Old World
PA	Polyandry

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Polygynandry
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PRN	Plaque-reduction neutralizing
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real Time Polymerase chain reaction
seg	Seconds
Sp	Species
TEM	Transmission electron microscopy
TFF	Terra firme forest
UICN	International Union for Conservation of Nature
UV	Ultraviolet
WHO	The World health organization
WMUs	Wildlife Management Units
XD	Hemoculture Xenodiagnosis
%	Percentage
°C	Degree Celsius
°	Degree
`	Minutes
``	Seconds

Glossary

Biosecurity: “the management of risks to the economy, the environment and the community, of pests and diseases entering, emerging, establishing or spreading” (COAG, 2012). Biosecurity can also be defined as the set of precautions (management and physical features) taken to minimise the risk of introducing a pest or infectious disease into (from and within) an animal (or human) population, or to a group or individual (WOAH 2023).

Biosafety: it refers to the safe handling practices in a laboratory environment, where it aims at the prevention of unintentional exposure to biological materials (WOAH, 2023).

Bloodborne pathogens: infectious organisms in human or animal blood (or other blody fluids) that cause or can cause disease.

CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora. This convention is a resolution to protect and regulate endangered species from wildlife trade.

Cryptic species: a species which is difficult to see in his normal environment due to its ecology (Carrillo-Bilbao et al., 2005).

Disease: any disturbance of the normal structural or physiological state of a living organism such as animals or humans.

Emerging diseases: “diseases that appear in a population for the first time, or that may have existed previously but are rapidly increasing in incidence or geographic range.” (WHO, 2023a). However, it can also be attributed to a change of pathogen to a new area or species (WOAH, 2021).

Endemic: a) of an animal: native of restricted to a certain place (Paim *et al*., 2019; Raghunathan *et al*., 2020) b) of a disease: a disease or parasite that occurs within a defined location (Magalhães *et al*., 2023; Mas-Coma *et al*., 2023; Zein *et al*., 2023).

Hotspot (Biodiversity): a hotspot is a region with high biodiversity but which is directly or indirectly threatened by human activities (Paige *et al*., 2015) such as population growth, human development and deforestation (Jha *et al*., 2006).

Infection: refers to the presence of a pathogen (or infectious disease agent) within an individual’s tissues, cells. Not all infections result in disease.

IUCN protection categories: Animals, plants and fungi are listed under a category of protection according to their endemism and area of distribution, population status, ecology and treats. There are nine categorie: Not Evaluated, Data Deficient, Least Concern, Near Threatened, Vulnerable, Endangered, Critically Endangered, Extinct in the Wild and Extinct.

Neotropical zoogeographic region: A.R. Wallace in 1876 delimited the world in zoogeographical regions. The neotropical region extends from the south part of Mexico into South America and to the subantarctic zone.

Parasite: an organism that, for all or some part of its life, lives in or on a living organism of another species and can have a cost on that host at several levels of its life cycle (Claar *et al*., 2021).

Pathogen: any infectious agent capable of causing disease in a host e.g. viruses, bacteria, fungi, protozoa, internal parasites such as worms and external parasites such as lice and mites (WHA, 2018). A pathogen is defined as "microorganisms (including bacteria, viruses, parasites, fungi) and other agents such as prions, which can cause disease in humans or animals" (WOAH, 2023).

Pathogenicity: the ability of a pathogen to cause a disease in its host and the severity of the disease caused (Cann, 2016).

Vector (pathogens): living organisms or non-living carrier that transports or transmits an infectious agent from one individual, food, or environment to another place, or susceptible host (WHO, 2020).

Wild reservoir host: an animal inside of which a pathogen survives (and mantains in the population) and can coevolve with it, the animal may or may not be ill. For example, an animal that can provide an environmental place for a pathogen, where it doesn't show any sign of disease, but it can infect another animal (Hallmaier-Wacker *et al*., 2017; Roberts *et al*., 2020).

Virus: "A virus is an infectious microbe consisting of a segment of nucleic acid (either DNA or RNA) surrounded by a protein coat. A virus cannot replicate alone; instead, it must infect cells and use components of the host cell to make copies of itself. Often, a virus ends up killing the host cell in the process, causing damage to the host organism" (NIH, 2023).

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Abstract
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Abstract

Background: Non-human primates (NHPs) act as a wild reservoir for bloodborne pathogens, and the study of this will allow us to better understand the ecology of diseases and the role of NHPs in the emergence of human diseases in Ecuador, a small country in South America, that lacks information on most of these pathogens. Most of those pathogens are of zoonotic importance and should be the focus of attention to understand the socioecology of zoonotic diseases and their actors.

Methodology: This study was divided into 4 parts: the first part is to provide a baseline of consolidated scientific information on bloodborne pathogens recorded in non-human primates in the Neotropical region, a region where some countries have gaps in this type of information as evidenced in the **study 1** "**Zoonotic BloodBorne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review"**, information that was strengthened with the collaboration reflected in the publication: "**Arboviruses in Mammals in the Neotropics: A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine".** The second part of the study aimed to perform PCR analyses to molecularly identify the following blood pathogens in collected faecal samples: *Leishmania, Babesia, Plasmodium, Trypanosoma* and *Anaplasma*. One of the main aspects was to improve the protocols for sample collection, storage and transport as mentioned in **study 2** "**Molecular Identification of *Plasmodium falciparum* from Captive Non-Human Primates in the Western Amazon Ecuador"**. The third part consisted of the analysis of samples collected as a result of the improvements in the initial protocols in search of the blood pathogens *Babesia* and *Trypanosoma* as highlighted in study 3. **"First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon"**. Finally, the fourth part of this study **"*Perception of biosecurity and risks in Ecuadorian Wildlife Management Units*"** sought to provide a baseline assessment of biosecurity perceptions and practices in wildlife management units to contribute to biosecurity programmes in these wildlife management units. To this end, we developed two instruments (for owners, visitors, and volunteers) which were validated by experts to determine the perception of biosecurity in wildlife management units.

Results: In the first part of the study, we determined that, at the neotropical level, a total of 56 bloodborne pathogens were identified in a total of 127 publications, and 25 genera (132 species) of NHPs. The total number of arboviruses in neotropical mammals we found allowed us to quantify the richness of arboviruses. In the second part of our study using a pan-Plasmodium PCR, we detected *Plasmodium falciparum* in one sample which was obtained from an adult female of *Leontocebus lagonotus*. And in the third part of our study, we identified Trypanosoma in *Leontocebus lagonotus* and in *Cebus albifrons*. In the fourth part of our study, we collected a total of 150 complete answered surveys.

Owners and visitors had a higher index in Education than the other two domains (Infrastructure and Practices) while volunteers had a higher score in Infrastructure than Education and Practices.

Conclusion: We managed to consolidate the bibliography and the respective reports of bloodborne pathogens in non-human primates in the Neotropical region. This information did not exist or was not published in some countries, such as Ecuador. However, these gaps on bloodborne pathogens were strengthened by the information and the analysis of reports of arboviruses present at the neotropical level in the mammalian class, a taxonomic group to which non-human primates belong and which also has geographical regions with insufficient information. We detected for the first time in Ecuador *Plasmodium falciparum* in a faecal sample from *Leontocebus lagonotus* contributing to the parasite ecology of NW NHP and we also made the first record of *Trypanosoma* for the NHP species *Leontocebus lagonotus* and the first report for Ecuador of *Trypanosoma* in *Cebus albifrons*. The fourth study allowed us to provide a local and regional tool to identify the risk pathways of Wildlife Management Units. This will provide an evaluation of the human actions involved in biosecurity with the aim of reducing the risk of transmission of diseases and their pathogens between wildlife and humans.

Resumen

Antecedentes: Los primates no humanos (NHP) actúan como reservorio silvestre de patógenos transmitidos por la sangre, y su estudio nos permitirá comprender mejor la ecología de las enfermedades y el papel de los NHP en la aparición de enfermedades humanas en Ecuador, un pequeño país de Sudamérica, que carece de información sobre la mayoría de estos patógenos. La mayoría de esos patógenos son de importancia zoonótica y deberían ser el foco de atención para entender la socioecología de las enfermedades zoonóticas y sus actores

Metodología: Este estudio se dividió en 4 partes: la primera parte es proporcionar una línea base de información científica consolidada sobre patógenos de transmisión sanguínea registrados en primates no humanos en la región Neotropical, región donde algunos países presentan vacíos en este tipo de información como se evidenció en el estudio 1 "**Zoonotic BloodBorne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review**", información que se fortaleció con la colaboración reflejada en la publicación: "**Arboviruses in Mammals in the Neotropics: A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine**". La segunda parte del estudio tenía como objetivo realizar análisis PCR para identificar molecularmente los siguientes patógenos sanguíneos en las muestras fecales recogidas: *Leishmania*, *Babesia*, *Plasmodium*, *Trypanosoma* y *Anaplasma*. Uno de los aspectos principales fue mejorar los protocolos de recogida, almacenamiento y transporte de muestras, como se menciona en el estudio 2 "**Molecular Identification of *Plasmodium falciparum* from Captive Non-Human Primates in the Western Amazon Ecuador**". La tercera parte consistió en el análisis de las muestras recogidas como resultado de las mejoras en los protocolos iniciales en busca de los patógenos sanguíneos *Babesia* y *Trypanosoma* como se destaca en el estudio 3. "**First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon**". Finalmente, la cuarta parte de este estudio buscó proporcionar una evaluación de base de las percepciones y prácticas de bioseguridad en las unidades de manejo de fauna silvestre para contribuir a los programas de bioseguridad en estas unidades de manejo de fauna silvestre. Con este fin, desarrollamos dos instrumentos (para propietarios, visitantes y voluntarios) que fueron validados por expertos para determinar la percepción de la bioseguridad en las unidades de manejo de fauna silvestre.

Resultados: En la primera parte del estudio determinamos que, a nivel neotropical, se identificaron un total de 56 patógenos de transmisión hemática en un total de 127 publicaciones, y 25 géneros (132 especies) de NHP. El número total de arbovirus en mamíferos neotropicales que encontramos nos permitió cuantificar la riqueza de arbovirus. En la segunda parte de nuestro estudio, utilizando una PCR pan-Plasmodium, detectamos *Plasmodium falciparum* en una muestra obtenida de una hembra adulta de *Leontocebus lagonotus*. Y en la tercera parte de nuestro estudio identificamos

Trypanosoma en *Leontocebus lagonotus* y en *Cebus albifrons*. En la cuarta parte de nuestro estudio, pudimos recoger un total de 150 encuestas completas contestadas. Los propietarios y visitantes tuvieron un índice más alto en Educación que en los otros dos dominios (Infraestructura y Prácticas), mientras que los voluntarios tuvieron una puntuación más alta en Infraestructura que en Educación y Prácticas.

Conclusiones: Se logró consolidar la bibliografía y los respectivos reportes de patógenos de transmisión sanguínea en primates no humanos en la región Neotropical. Esta información no existía o no estaba publicada en algunos países, como Ecuador. Sin embargo, estos vacíos de información sobre patógenos de transmisión sanguínea fueron reforzados con la información y análisis de los reportes de arbovirus presentes a nivel neotropical en la clase mamíferos, grupo taxonómico al que pertenecen los primates no humanos y que también tiene regiones geográficas con información insuficiente. Detectamos por primera vez en Ecuador *Plasmodium falciparum* en una muestra fecal de *Leontocebus lagonotus* contribuyendo a la ecología parasitaria de NHPs del NW y también realizamos el primer registro de *Trypanosoma* para la especie de NHP *Leontocebus lagonotus* y el primer reporte para Ecuador de *Trypanosoma* en *Cebus albifrons*. El cuarto estudio nos permitió proporcionar una herramienta local y regional para identificar las vías de riesgo de las Unidades de Manejo de Fauna Silvestre y evaluar las acciones humanas involucradas en la bioseguridad con el objetivo de reducir el riesgo de transmisión de enfermedades y sus patógenos entre la fauna silvestre y el hombre.

Résumé

Contexte: Les primates non humains (NHP) constituent un réservoir sauvage d'agents pathogènes transmissibles par le sang. Leur étude nous permettra de mieux comprendre l'écologie des maladies et le rôle des NHP dans l'émergence de maladies humaines en Équateur, un petit pays d'Amérique du Sud qui manque d'informations sur la plupart de ces agents pathogènes. La plupart de ces pathogènes ont une importance zoonotique et devraient faire l'objet d'une attention particulière pour comprendre la socio-écologie des maladies zoonotiques et leurs acteurs.

Méthodologie : Cette étude a été divisée en 4 parties : la première partie est de fournir une base d'informations scientifiques consolidées sur les pathogènes à diffusion hématogène enregistrés chez les primates non humains dans la région néotropicale, une région où certains pays ont des lacunes dans ce type d'informations comme le montre l'étude 1 "**Zoonotic BloodBorne Pathogens in Non-Human Primates in the Neotropical Region : A Systematic Review**", information qui a été renforcée par la collaboration reflétée dans la publication : "**Arbovirus in Mammals in the Neotropics : A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine**". La deuxième partie de l'étude vise à effectuer des analyses PCR pour identifier moléculairement les pathogènes sanguins suivants, dans les échantillons fécaux collectés: *Leishmania*, *Babesia*, *Plasmodium*, *Trypanosoma* et *Anaplasma*. L'un des principaux aspects consistait à améliorer les protocoles de collecte, de stockage et de transport des échantillons, comme indiqué dans l'étude 2, "**Molecular Identification of *Plasmodium falciparum* from Captive Non-Human Primates in the Western Amazon Ecuador**". La troisième partie consiste en une analyse des échantillons collectés à la suite des améliorations apportées aux protocoles initiaux, à la recherche des pathogènes sanguins *Babesia* et *Trypanosoma*, comme indiqué dans l'étude 3, "**First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon** ". Enfin, la quatrième partie de cette étude *" **Perception of biosecurity and risks in Ecuadorian Wildlife Management Units",*** vise à fournir une référence de base des perceptions et des pratiques en matière de biosécurité dans les unités de gestion de la faune sauvage, afin de contribuer aux programmes de biosécurité dans ces unités. À cette fin, nous avons mis au point deux instruments (pour les propriétaires, les visiteurs et les bénévoles) qui ont été validés par des experts, afin de déterminer la perception de la biosécurité dans les unités de gestion de la faune sauvage.

Résultats : Dans la première partie de l'étude, nous avons déterminé qu'au niveau néotropical, nous conservons un total de 127 publications et 25 genres, 132 espèces de NHP, un total de 56 agents pathogènes transmissibles par le sang. Le nombre total d'arbovirus chez les mammifères néotropicaux nous a permis de quantifier la richesse des arbovirus. Dans la deuxième partie de notre étude, nous avons

détecté le *Plasmodium falciparum* dans un échantillon provenant d'une femelle adulte de *Leontocebus lagonotus*, en utilisant une PCR pan-plasmodium. Dans la troisième partie de notre étude, nous avons identifié des Trypanosoma pour les espèces de NHP *Leontocebus lagonotus* et *Cebus albifrons*. Dans la quatrième partie de notre étude, nous avons pu recueillir un total de 150 questionnaires complets. Les propriétaires et les visiteurs ont obtenu un indice plus élevé dans le domaine de l'éducation que dans les deux autres domaines (infrastructure et pratiques), tandis que les bénévoles ont obtenu un score plus élevé dans le domaine de l'infrastructure que dans celui de l'éducation et des pratiques.

Conclusion : Nous avons réussi à consolider la bibliographie et les rapports respectifs sur les pathogènes à diffusion hématogène chez les primates non humains dans la région néotropicale. Ces informations n'existaient pas ou n'ont pas été publiées dans certains pays, comme l'Équateur. Cependant, ces lacunes sur les agents pathogènes transmis par le sang ont été renforcées par les informations et l'analyse des rapports sur les arbovirus présents au niveau néotropical dans la classe des mammifères ; un groupe taxonomique auquel appartiennent les primates non humains et qui comprend également des régions géographiques pour lesquelles les informations sont insuffisantes. Nous avons détecté pour la première fois en Équateur du *Plasmodium falciparum* dans un échantillon fécal de *Leontocebus lagonotus*, contribuant ainsi à l'écologie parasitaire des NHP du NW. Nous avons également réalisé le premier enregistrement de *Trypanosoma* dans l'espèce *Leontocebus lagonotus* de NHP et le premier registre en Équateur de *Trypanosoma* chez le *Cebus albifrons*. La quatrième étude nous a permis de fournir un outil local et régional qui permet d'identifier les voies des risques dans les unités de gestion de la faune, et d'évaluer les actions humaines impliquées dans la biosécurité, afin de réduire le risque de transmission de maladies et de leurs agents pathogènes entre la faune et les humains.

General preamble

Wild animals have a direct relationship with emerging diseases as it has been recognized that they are responsible for more than 71% of all emerging diseases and of these more than 60% are considered zoonotic (Jones *et al*., 2008; Kuiken *et al*., 2005). It is therefore extremely important to protect the health of wildlife (OIE, 2021), not least primates are considered a major source of human infectious diseases because of our strong kinship relationships due to a genetic homology of 75 to 98.5% (Jiang *et al*., 2023; Wolfe *et al*., 2007). Non-human primates are reservoirs of diseases such as yellow fever (Aliaga-Samanez *et al*., 2022; de Azevedo Fernandes *et al*., 2021; Goes de Jesus *et a*l., 2020), and other arboviruses such as dengue and zika (Valentine *et al*., 2019). Malaria, for example, is tought to be originated in non-human primates (Liu *et al*., 2010a). The low representativeness of NHPs over the total number of vertebrate species (0.5% of vertebrate species), have contributed to 20% of infectious diseases among humans (Harper *et al*., 2013).

Primates have a worldwide distribution with a complex system of species classification that is poorly understood in different areas, as is the case in Ecuador *(Carrillo-Bilbao et al., 2021)*. Non-human primate species maintain an important role in forest dynamics as indicators of good forest quality as well as being susceptible to emerging diseases (Holzmann *et al*., 2010). Habitat destruction (Onderdonk *et al*., 2000), hunting and live capture are the main threats to their conservation status (Alves *et al*., 2016; Hofner, 2017). NHPs are potential reservoirs of many bloodborne pathogens. In Ecuador, research activities should be focused on bacteria and viruses, where there is a gap of information for neotropical NHPs, to implement surveillance programs with regular and effective monitoring protocols adapted to NHPs.

All advances in the protection of wildlife, including non-human primates, are about developing approaches that allow us to better understand diseases and their pathogens using non-invasive techniques to generate conservation tools as well as to contribute directly to human and non-human primate health and their role as potential zoonotic reservoirs. Environmental tools that are developed studying NHPs pathogens and using non-invasive samples are as follows: environmental guidelines, education guidelines, management plans of protected areas and captive settings, policies for disease prevention, and red lists (Bicca-Marques *et al*., 2010). It is also important to identify the origin of zoonotic reservoirs in wildlife and their transmission in captive NHP and should be considered a public health concern.

Chapter 1 - Introduction

1. General introduction

Wild animals are the cause of almost 70% of all emerging diseases (Kuiken *et al*., 2005), and more than 60% of these diseases are zoonotic (Jones *et al*., 2008) (Fig. 1). It is a public health and conservation problem (Daszak *et al*., 2000; Gillespie *et al*., 2008). Non-human primates (NHPs) are infected not only by gastrointestinal parasites (Martin-Solano *et al*., 2017), but also by ectoparasites, blood pathogens, bacteria, viruses and some arthropods that affect lungs. Until recently (Liu *et al*., 2010a; Victoria *et al*., 2009), just a few studies identified blood pathogens from faecal samples due to the presence of DNA (deoxyribonucleic acid) inhibitors in faecal samples. Thereafter, just a few studies have identified blood pathogens such as *Plasmodium* sp. (de Assis *et al*., 2016; Siregar *et al*., 2015) and *Trypanosoma brucei* (Jirků *et al*., 2015), and viruses such as adenovirus (Sukmak *et a*l., 2017), and astrovirus (Wang *et al*., 2016) from NHPs faecal samples. Most NHPs species are under a category of conservation (IUCN, 2020; Schwitzer *et al*., 2019) and identification of blood pathogens and viruses thanks to molecular identification in faecal samples is going to be of great advantage to monitor NHPs population that can be a potential zoonotic reservoir for humans.

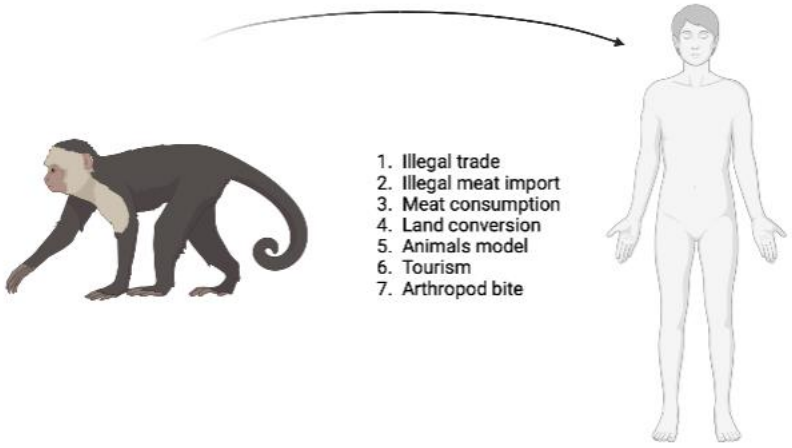

Figure 1. Drivers of interspecies pathogens from NHP to humans (Created with BioRender.com).

Gastrointestinal pathogens have been monitored in neotropical primates (Helenbrook *et al*., 2015; Kowalewski, 2012; Martin-Solano *et al*., 2017; Montesinos-López *et al*., 2014; Parr *et al*., 2013; Perea-Rodriguez *et al*., 2010; Phillips *et a*l., 2004; Valdes Sanchez *et al*., 2009), however restricted to some countries such as Mexico (Aguilar Cucurachi *et al*., 2007; Cristóbal-Azkarate *et al*., 2010; Estrada, 2006; Gonzalez Hernández, 2004; González-Hernández *et al*., 2014; Solórzano-García et al., 2017; Stoner *et al*., 2005; Trejo-Macías *et al*., 2011; Valdespino *et al*., 2010; Venturini et al., 2003; Villanueva-García *et al*., 2017; Vitazkova *et al*., 2006) and Brazil (da Silva Barbosa *et al*., 2015; David *et al*., 2014; dos Santos Sales *et al*., 2010; Fernandes *et al*., 2011; Mati *et al*., 2013; Monteiro et al., 2003; Pinto *et*

al., 2013; Souza de *et al.*, 2010; Tenorio Mati *et al*., 2014; Vicente *et al*., 1992; Volotão *et al*., 2008). However, the study of blood pathogens and arbovirus in neotropical primates is restricted just to a few studies in Brazil (Brasil *et al*., 2017; Figueiredo *et al*., 2017; Guimaraes *et al*., 2012; Leite *et al*., 2008), Venezuela (Muñoz *et al.*, 2012; Navarro *et al.*, 2016) and French Guyana (Muñoz *et al*., 2012), and Ecuador has no published data on them (Pappas *et al*., 2009), even if most of those blood pathogens are present in Ecuador (Anez *et al*., 2012; Browne *et al*., 2017; CDC, 2018; Rückert *et al*., 2017; WHO, 2018). Finally, we wish to focus on blood pathogens because they are the cause of millions of infections and thousands of deaths per year in humans (Anez *et al*., 2012; Gubler *et al*., 1999; WHO, 2013; WHO., 2016). Understanding whether primates act as a wild reservoir for blood pathogens and viruses will allow us to better understand the ecology of diseases (Hudson *et al*., 2002) and the role of NHPs in the emergence of human diseases (Wolfe *et al*., 2007) as well as the way to implement control programs (Drosten, 2010; Joseph *et a*l., 2013) for endemic (da Silva-Nunes *et al*., 2012) and incoming pathogens (Maljkovic Berry *et al*., 2020) and for NHPs conservation/management plans (Tirira *et al*., 2018b).

Some NHPs can become infected with blood pathogens species of protozoans. For example, wildlife harbours *Toxoplasma gondii*, which has a worldwide distribution and affects a wide range of hosts from humans (Iddawela *et al*., 2017), and domestics animals (Gazzonis *et al*., 2018) to wildlife (Chadwick *et al.*, 2013; Conrad *et al*., 2005; Daszak *et al*., 2000; Wendte *et al*., 2011), including NHPs, Old World Monkyes (OWs) and New World Monkeys (NWs) (Leite *et al*., 2008; McConnell *et al*., 1973; Pena *et al*., 2011; Poirotte *et al*., 2016; Spencer *et al*., 2004). In Ecuador, however, just the islands of Galapagos were screened for *Toxoplasma* (Deem *et al*., 2010; Deem *et a*l., 2012; Verant *et al*., 2014). Another bloodborne pathogen, *Leishmania* sp., also occurs in a wide range of hosts (Gómez-Hernández *et al*., 2017; Hashiguchi *et a*l., 2018; Lainson *et al*., 1989; Leite *et a*l., 2008; Malta *et al*., 2010; Otranto *et al.*, 2010; Quinnell *et al*., 2009; Trüeb *et al*., 2017). However, studies in the neotropics are limited to Brazil and countries such as Ecuador are under surveyed even though the parasite is widely distributed (Hashiguchi *et al*., 2017). In NW monkeys, it is very common to find several species of trypanosomes such as *Trypanosoma (megatrypanum) minasense* (Sato *et al*., 2008), and also zoonotics trypanosomes such as *Trypanosoma rangeli* and *T. cruzi* (Aysanoa *et al*., 2017). In Ecuador, despite surveillance and control campaigns, trypanosomiasis is still present (Dumonteil *et al*., 2016), however there are just a few studies of trypanosomiasis on wildlife (Bernal *et al*., 2016; Cottontail *et al*., 2014; Ocaña-Mayorga *et al*., 2015; Pinto *et al*., 2015), and unfortunately there aren't any studies on primates.

Viral infections also pose a threat to NHPs's health. Several type of viruses may affect NHPs: deoxyribonucleic acid (DNA) viruses, and ribonucleic acid (RNA) viruses (Wachtman *et al.*, 2012). Among the latter, Arboviruses (arthropod-borne viruses) are a diverse range of viruses from 6 families: Togaviridae (*Alphavirus*), Flaviviridae (*Flavivirus*), Bunyaviridae (*Bunyaviruses, Nairoviruses, and Phleboviruses*), Reoviridae (*Orbovirus*), Rhabdoviridae (*Vesiculoviruses*) and Orthomyxoviridae

(*Thogotoviruses*). Arboviruses are of public health concern, because of the threat to humans and animals (Jonduo *et al*., 2012; Young *et al*., 2014). The host type of arboviruses can vary from a specific taxonomic group to several hosts. The range of vectors can also vary in the same way. For some arbovirus the zoonotic origin is linked to primates because of their close genetic distance while others are linked to other vertebrates or the vector itself (Hanley *et a*l., 2008). In the neotropics, NHPs have been identified as host for the following diseases: yellow fever (Fernandes *et al*., 2017; Holzmann *et al*., 2010; Moreno *et a*l., 2015), mayaro (Muñoz *et al*., 2012) zika, chikungunya (Moreira-Soto *et al*., 2018), hepatitis A (Svoboda *et al*., 2016), Cacipacoré virus (Batista et al., 2013), St. Louis encephalitis virus *(*SLEV) and Oropouche virus (OROV) (Barreto Almeida *et al*., 2016; Muñoz *et al*., 2012).

2. Hotspots, Biodiversity and the Emerging Zoonotic Diseases

A biodiversity hotspot is a region with high biodiversity but which is directly or indirectly threatened by human activities (Paige *et al*., 2015) such as population growth, human development and deforestation (Jha *et al*., 2006). There are a total of 36 biodiversity hotspots around the world (Fig. 2) (Koenig 2022). Ecuador is located in two biodiversity hotspots (Myers *et al*., 2000), the first one is called Andes Tropicales and the second one Tumbes – Chocó – Magdalena. Ecuador is also one of the few countries with a high number of endemism. Ecuador belongs to one of the only two regions worldwide considered as a hotspot for mammals (Orme *et al*., 2005). Parasites are part of the biodiversity and the number of species is underestimaded (Luque *et al*., 2007). One recommendation in these wildlife hotspots is to vaccinate people that live in those areas or close to (Leendertz *et al*., 2021), since they can pose a threat to biodiversity. For example, with the recent discovery of the Dengue vaccin and the Malaria vaccin, the WHO recommends to vaccinate people in endemic zones (Freedman, 2023; WHO, 2023a).

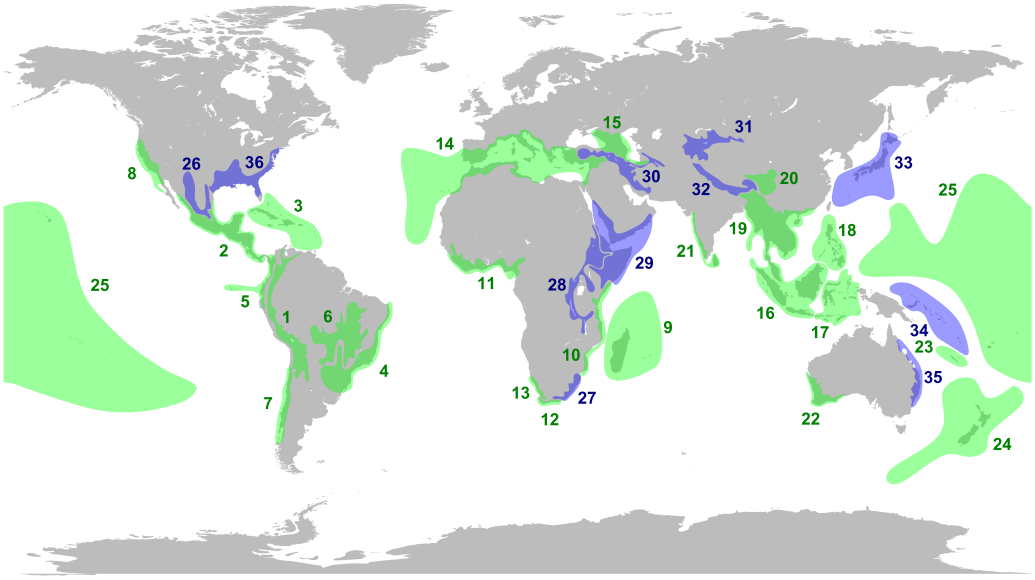

Figure 2. Map of World Biodiversity Hotspots (Koenig, 2022; Ninjatacoshell, 2010). Note: 1. The Tropical Andes 2. Mesoamerica 3. The Caribbean Islands 4. The Atlantic Forest 5. Tumbes-Chocó-Magdalena 6. The Cerrado 7. Chilean Winter Rainfall-Valdivian Forests 8. The California Floristic Province 9. Madagascar and the Indian Ocean Islands 10. The Coastal Forests of Eastern Africa 11. The Guinean Forests of West Africa 12. The Cape Floristic Region 13. The Succulent Karoo 14. The Mediterranean Basin 15. The Caucasus 16. Sundaland 17. Wallacea 18. The Philippines 19. Indo-Burma 20. The Mountains of Southwest China 21. Western Ghats and Sri Lanka 22. Southwest Australia 23. New Caledonia 24. New Zealand 25. Polynesia and Micronesia 26. The Madrean Pine-Oak Woodlands 27. Maputaland-Pondoland-Albany 28. The Eastern Afromontane 29. The Horn of Africa 30. The Irano-Anatolian 31. The Mountains of Central Asia 32. Eastern Himalaya 33. Japan 34. East Melanesian Islands 35. The Forests of East Australia 36. North American Coastal Plain.

In 1876, A.R. Wallace, published a distribution of animals (mainly vertebrates) defined according to zoogeographical regions (Wallace, 1876). He provided this first map divided in six zoogeographical regions. However, there was an update of this map published by Procheş *et al*. (2012) (Fig. 3) using cross-taxon analysis with a total of 14 zoogeographic regions.

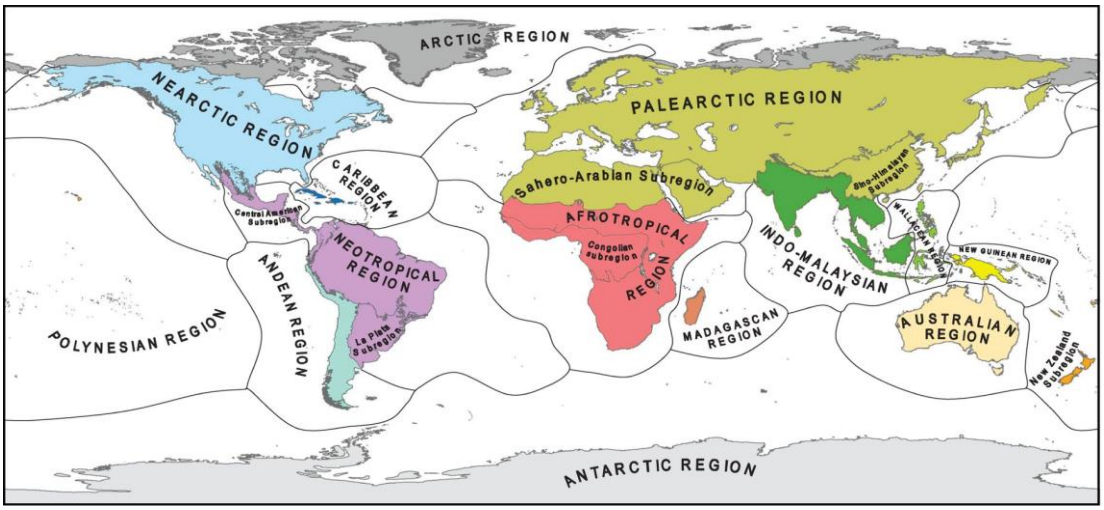

Figure 3. Zoogeographic regions and subregions of the world (Procheş *et al*. 2012)**.**

3. Current Situation of non-human primates:

3.1. World Taxonomy and classification

The Order Primates, it is one of the largest within the vertebrates, where we find a total of two suborders: Strepsirrhini with three infraorders Lemuriformes, Chiromyiformes and Lorisiformes; while the suborder Haplorrhini has two infraorders which are Tarsiiformes and Simiiformes; the latter with two Parvorder; Platyrrhini and Catarrhini (Fig. 4) (IUCN, 2023).

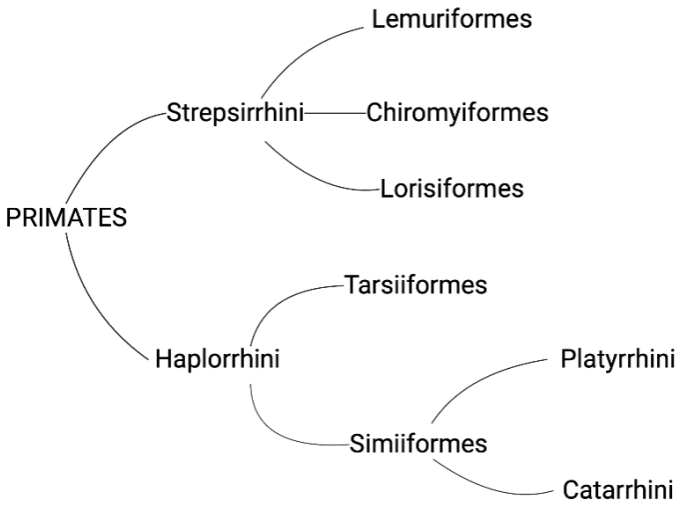

Figure 4. Taxonomy of non-human primates according to IUCN 2023 (Created with BioRender.com).

3.2. Worldwide Primate Richness

Globally, there is a total of 17 families, 83 genera and 532 species (Table 1) of non-human primates' species, of which 60% are endangered and 75% of the world's populations are in decline (Estrada *et al*., 2017; IUCN, 2023).

Table 1. Number of primates species per continent according to (IUCN, 2023).

Region	Families	Genera	Number of Species
Africa	4	26	107
Asia	5	19	131
Madagascar	5	15	108
Neotropics	5	23	186
Total	**17**	**83**	**532**

The Neotropical region is the region with the largest number of primates species recorded worldwide with 186 species, 23 genera grouped in 5 families, representing 25% of species worldwide. Of which 24 % are critically endangered and endangered and 42 % are threatened. The Amazon, records 134 species, in 19 genera (IUCN, 2023), which represents 72 % of the total number of species in the Neotropical region and 25 % of species worldwide.

3.3. Ecuadorian NHP Richness

Ecuador registers a total of 21 species and 22 taxa; grouped in 5 families, of which 18 are registered in the Amazon**,** size, weight, group structure, social system, and varied habitat preferences (Table 2).

Table 2. Species of non-human primates from Ecuador (Carrillo-Bilbao *et al*., 2021)

Family	Primate species	Size (cm)	Weight (g)	Group Size (# of individuals)	Social System[1]	Habitat[2]
Aotidae	*Aotus lemurinus*	50	1300	Small (1 to 5)	M	TFF
	Aotus vociferans	50	698	Small (2–5)	M	TFF, FF, and crops
Atelidae	*Alouatta seniculus*	43–60	3600–9000	Small (3–7) or Large (15)	MM–MF or a group of bachelor males	FF
	Alouatta palliata aequatorialis	48–67	3100–9800	Small and Medium (2–12)	MM–MF	All types of forests
	Ateles belzebuth	40–60	6000–10,500	Large (≈ 20)	MM–MF	TFF
	Ateles fusciceps fusciceps	39–58	8800	Large (20–30)	MM–MF	TFF
	Lagothrix lagotricha lagotricha	40–58	3600–10,000	Large (6–60)	PG/MM–MF/MM	TFF
	Lagothrix lagotricha poeppigii	40–58	3600–10,000	Large (2–25)	PG/MM–MF/MM	TFF
Cebidae	*Cebus albifrons*	81–84	1900–3900	Medium (5–30)	PG/MM–MF	All types of forests
	Cebus aequatorialis	35–46	1200–3600	Medium (5–20)		
	Sapajus macrocephalus	35–50	1700–4500	Medium (5–20)		
	Cebus capucinus capucinus	35–45	2900–3900	Medium (2–42)		
	Cebus yuracus	81–84	2900–3900	Medium and Large (10 and 35–54)		
	Saimiri cassiquiarensis macrodon	25–32	600–1400	Large >10 and up to 100		
Callitrichidae	*Cebuella pygmaea*	12–15	100–140	Small and Medium (2–9)	M/PA	TFF, FF
	Leontocebus nigricollis graellsi	15–28	350–470	Small and Medium (2–30)	M/MM–MF	TFF and FF
	Leontocebus lagonotus	17–27	330–430	Medium (2–10)	All kinds of social structures	All kind of forest
	Leontocebus tripartitus	21–24	218–240	Small and Medium-sized (4–10)	PG	TFF and FF
Pithecidae	*Plecturocebus discolor*	28–36	900–1400	Small (2–5)	M	FF-lianas forest–forest gaps

Cheracebus lucifer	30–38	800–1500	Small (2–5)	TFF
Pithecia aequatorialis	39–44	2000–2500	Small (1–4)	TFF
Pithecia milleri	37–48	2100–2600	Small (2–6)	TFF and FF
Pithecia napensis	37–48	2100–2600	Small and Medium (4–8)	TFF

[1]Social system: M = Monogamous: A mating system in which only one male copulates with only one female; MM–MF = Multimale–Multifemale groups: A social group consisting of multiple adult males and multiple adult females; MM = Multi-males: Strong social relationships among males, often deriving from close kinship among those males as a result of male philopatry; PA = Polyandry: A mating system in which one female copulates with multiple males; PG = Polygynandry: A mating system in which members of both sexes copulate with multiple members of the opposite sex (Swedell, 2012); [2]Habitat: TFF—Terra firme forest: forest with soils composed of clay or loam (Tuomisto *et al*., 2003); FF—Flooded forests: Forests characterized by trees waterlogged or submerged during a large part of the year (Parolin *et al*., 2004). Data on NHPs were obtained from the following references: (Tirira *et al*., 2018a),(Smuts *et* al., 1987).

The reasons why primate conservation efforts should be made is because they play a crucial ecological role in the ecosystems where they are found. All primate species are part of food chains acting as prey or predators and are also seed dispersers (Carrillo Bilbao *et al*., 2010). They comprise the majority of the total biomass of neotropical frugivores (Chapman *et al.*, 2001). They also have low growth rates, reproduction with long birth intervals and a long development time (Cowlishaw *et al*., 2000). They are our closest biological relatives and provide the closest living biological information to us. They offer critical insight into the evolution, biology and behaviour of humans and the threat of emerging and zoonotic diseases (Estrada *et al*., 2017).

Primates populations are declining (Fig. 5). Among the main threats that directly affect primates are anthropogenic activities, including habitat loss, expansion of industrial agriculture, large-scale cattle ranching, logging, oil and gas extraction, mining, dam construction, hunting and illegal trade in primates, climate change and anthroponotic diseases (Estrada *et al*., 2017).

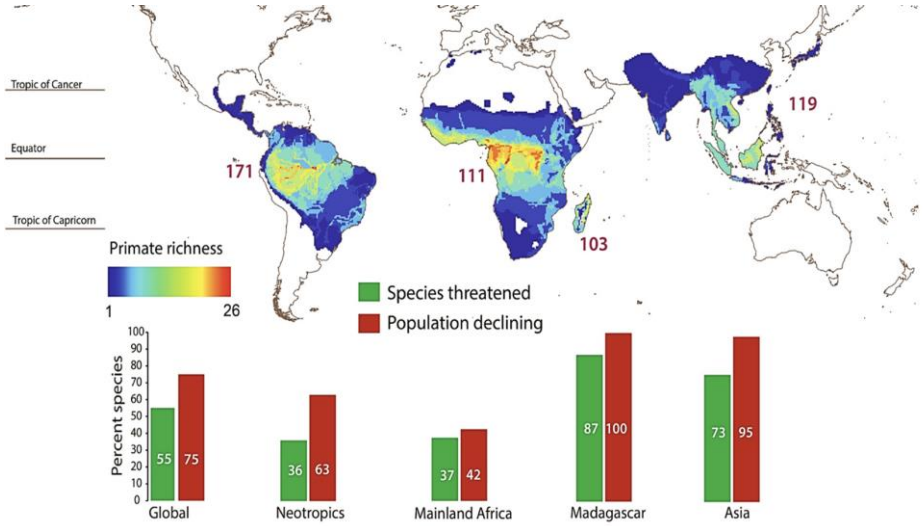

Figure 5. Global primate species richness, distributions, and the percentage of species threatened and with declining populations (Estrada *et al*., 2017)**.**

In relation to emerging and zoonotic diseases, we must highlight the following: non-human primates are social, and as social animals they are at risk of infectious or parasitic diseases (Rushmore *et al*., 2017). Factors such as group size, movement between groups, and sexual selection (number of mating partners), are among the variables of host-parasite interactions in NHPs that are considered as a driver of parasite transmission (Altizer *et al*., 2003).

3.4. Current situation of pathogens in Ecuadorian NHPs

No reports on pathogens in Ecuadorian NHPs were found, only on gastrointestinal parasites in the Amazon region and southern Ecuador (Helenbrook *et al*., 2015; Martin-Solano *et al*., 2017a). In addition, there are a few undergraduate theses that we have supervised on Giardia (Chávez *et al*., 2019), bacterial resistance (Haro León *et al*., 2020) and the use of non-invasive sampling in pathogens (Pérez *et al*., 2018). Therefore, the low number of studies on pathogens in NHPs in Ecuador highlights the importance of developing this line of research in NHPs.

4. Methods of detection of pathogens

Within the study of wildlife, the collection of biological samples is very important, among which two types of samples are considered: invasive and non-invasive. The former are those that obligatorily involve the capture and handling of the animal. This handling entails a risk both for the personnel taking the sample and for the species. These risks are greater when it comes to the collection of disease-related samples and the use of chemicals to obtain them (Muñoz García *et al*., 2016).

4.1. Invasives samples

Traditional genetic methods to collect samples in wildlife species are considered invasive. The most common sample is blood (Cosme *et al*., 2013; Kirmaier *et al.*, 2009). In NHPs it is recommended to obtain the blood from the femoral vein (Diehl *et al*., 2001; Jeneby, 2011). Other tissues are also considered such as skin, saliva and semen (Davoust *et a*l., 2018). When collecting tissues, the external ear is recommended (Teixeira *et al*., 2022). When NHPs are dead, brain tissue can also be collected (Morales *et al*., 2006). However, when you work with invasive samples in NHPs, there are some restrictions according to CITES.

4.2. Non-invasive samples

Wildlife studies as well as the conservation of species in protection categories are of great importance, so the samples that allow us to extract genetic information has become a challenge, highlighting that many species are rare, cryptic and have complex and poorly understood socio-ecological characteristics (Carrillo-Bilbao *et al*., 2018; Costa *et al*., 2016; Di Fiore *et al*., 2006; Fernandez-Duque *et al*., 2008; Martin-Solano *et al*., 2017; Yasuda *et al*., 2015; Zinner *et al*., 2013). These are some of the main reasons to focus conservation efforts on the collection and preservation of non-invasive samples (Costa *et a*l., 2016; Maturrano H. *et al*., 2012). Non-invasive methodologies have been applied to wild populations for genetic studies to avoid disturbance in wildlife (Taberlet *et al*., 1999a).

All contributions made to promote non-invasive sampling facilitate wildlife management and even minimise human contact and its impacts on animals, communities, and their ecosystems. They also promote the protection of a wide range of plant and animal genotypes and facilitate the perpetuation of species and their evolutionary processes. Finally, non-invasive sampling provides crucial information on species and microbial communities (Gardipee, 2007; Maturrano *et al*., 2012; Su *et al*., 2018; United States Department of the Interior, 2006; Yildirim *et al*., 2010).

4.3. Types of non-invasive samples

DNA has been extracted from hairs (Fig. 6), feces (Fig. 7), urine (Fig. 8), feathers, snake skins, egg shells, and even skulls found in owl pellets (Morin *et al*., 1996; Taberlet *et al*., 1996). Fecal DNA samples can provide individual identification, kinship estimates, pedigree construction, sex identification, census and effective population size estimates, mark recapture data, and determine genetic variation within and among populations (Frantz *et al*., 2003; Luikart, 2008; Taberlet *et al*., 1999b).

Figure 6. Hair of a wild mammal in a hair trap (NIBIO, 2015).

Figure 7. Fecal sample of non-human primate.

Figure 8. A sample of urine (Vogel, 2014)**.**

Indeed, it has been suggested that all conservation genetics studies should use non-invasive samples (Desalle *et al*., 2004) since it is the only way to obtain genetic data of cryptic or endangered species without disturbing the animals by reducing the risk of injury and disturbing socio-ecological aspects of the groups under study (Taberlet *et al*., 1999b; Waits, 2009). Almost any evidence left by the taxa can be used for DNA extraction (Beja-Pereira e*t al*., 2009; Waits, 2009). Faeces are one of the most common sources and allow to obtain not only genetic data but also other information such as

physiological hormones, diet, parasites and even blood (Arregui *et al*., 2015; De Nys *et al*., 2015; De Nys *et al*., 2014; Kaisin *et al*., 2023; Liu *et a*l., 2010a; Luikart, 2008; Martin-Solano *et al*., 2013).

4.4. Importance of fecal samples for wildlife studies

In the stool sample, not only genetic information from the host, but also from other taxa such as parasites and diet have been identified. It should be noted that host DNA present in stool samples is the most common, mainly because it is mostly derived from the epithelial cells of the digestive tract. However, the amount of extracted DNA is low and PCR inhibitors are present (Taberlet *et al*., 1996; Taberlet *et al*., 1999a). Another disadvantage for this type of samples is the time of exposure to environmental conditions, since the success of extraction generally is governed by the temperature and the humidity, variables that in the field is very difficult to have them under control. Several authors mentioned that the success of the investigation would depend on: the time and the type of collection of samples, the season of sampling, the diet of the host, the methods of extraction and the interaction between all those factors (Beja-Pereira *et al*., 2009; Frantzen *et al*., 1998; Maudet *et al*., 2004; Piggott *et* al., 2003; Soto-Calderón *et al*., 2009).

Comparative studies indicate that the diet and the environmental conditions are the two main factors affecting the quality and quantity of DNA extracts. Differences in the digestive tract of different species also influence an impact on extraction success rates (Costa *et al*., 2016). Non-invasive samples are sensitive and often do not provide reliable results when standard methodologies are applied (Costa *et al*., 2016). It is essentially widely accepted that non-invasive sampling has three main problems: DNA degradation, DNA quantity, and DNA quality. It is not possible to overcome degradation of pre-existing DNA, however, problems of DNA quantity and quality can be solved by prioritizing DNA performance and minimizing the effects of inhibitors (Costa *et al*., 2016). Factors affecting fecal samples are generally the presence of microorganisms, digested and undigested food, mucus, soluble and non-soluble products of the intestinal tract, enzymes released by the cells, and these samples can be degraded by environmental effects, , and the presence of variable concentrations of hemoglobin, bilirubin, bile salts, pectins, and phenols (Fuentes B. *et al*., 2009; Iacovacci *et al*., 2003; Menghi *et al*., 2006; Sandoval Arias, 2014). Therefore, improving the protocols in all phases will directly affect the quality of the final product as well as faster and more costly times. The choice of method will depend on the type of research, which should give priority to the collection, preservation and transportation of samples (Vianna *et al*., 2009; Wang *et al*., 2018).

Knowledge of the ecology and diet of the species under study, whether it is a host or a guest (for example parasites), is indispensable for studies that seek to identify genetic aspects based on feces, for example in relation to diet. Animals with a diet richer in easily digestible food have a faster and less abrasive digestion process for the intestinal epithelium and therefore there is a lower number of epithelial

cells found in the feces. Depending on the type of diet, herbivores, omnivores, among others, have a direct impact on the quantity and quality of DNA. For example, if sampling is made in the dry season and the diet is primarily herbivorous, it will be drier and this increases the content of less digestible fiber due to an increase in cellulose and lignin, and digestion will be more abrasive and provide more epithelial cells (Costa *et al*., 2016; Maudet *et al.*, 2004).

Another ecological characteristic to consider is whether the sample was exposed to environmental conditions such as high amounts of UV light. For example, sampling at high altitude sites or in the desert affects the quality of the extracted DNA. It is extremely important to seriously consider collection and storage methods that do not result in a reduction in the quantity and quality of the DNA (Frantzen *et al.*, 1998; Liu *et al*., 2010b; Piggott *et al*., 2003; Soto-Calderón *et al.*, 2009).

Seasonal differences in the environment are evident in the diet of taxa, for example in Ungulates, rates of extracted DNA were much lower, and genotype error rates were much higher in seasons where forage quality is quite high, such as spring or summer. In addition, non-invasive faecal DNA samples can produce high error rates due to allele desertion and false alleles, so it is necessary to estimate the number of replicated genotypes needed to confidently assign the correct genotypes (Maudet *et al*., 2004; Taberlet *et al*., 1999a).

Feces in human and non-human primates are generally composed of water, proteins, polysaccharides, including pathogens, blood, undigested food residues, undigested fat, biomass, ash, physiological hormones, which would allow us to obtain not only genetic information about the host but also data related to the elements that make up the faecal sample (Arregui *et al.*, 2015; De Nys *et al*., 2015; De Nys *et al*., 2014; Liu *et al*., 2010a; Luikart, 2008; Martin-Solano *et al.*, 2013; Martin-Solano *et al*., 2017; Snyder *et al*., 1975). Although we can consider them very useful, it is important to obtain quality genetic information in the right quantity, for which it is necessary: to know environmental ecological aspects, the diets of the species, the presence of microorganisms that are not typical of the digestive tract (Costa *et al*., 2016; Fuentes B. *et al*., 2009; Maudet *et al.*, 2004; Menghi *et al.*, 2006; Sandoval Arias, 2014). Other aspects must also be taken into account, such as the optimisation of protocols in all their phases; the collection, transfer and processing of samples, which must be adapted to the type of research. The verification of these aspects will have a direct impact on the quantity and quality of the final product, which will even be reflected in better time and lower costs (Vianna *et a*l., 2009; Wang *et al*., 2018). This type of non-invasive sampling allows us to extract genetic information and others, needed in rare, cryptic species with complex and poorly understood socio-ecological characteristics, thus promoting species conservation (Carrillo-Bilbao *et al*., 2018; Costa *et al*., 2016; Di Fiore, 2004a, 2004b; Fernandez-Duque *et al*., 2008; Martin-Solano *et al*., 2017; Yasuda *et al*., 2015; Zinner *et al*., 2013).

4.5. Why study bloodborne pathogens in NHPs

Non-human primates (NHPs) and human share many bloodborne pathogens. Protozoa pathogens such as *Plasmodium* sp. and *Trypanosoma* sp. are two of the most important diseases in the neotropics and around the world (WHO, 2023b). Wild animals have a direct relationship with emerging diseases as it has been recognized that they are responsible for more than 71% of all emerging diseases and of these more than 60% are considered zoonotic. Long term studies offer insights into various fields: biology, ecology, behaviour, psychology, conservation and health. This study belongs to a long-term research. Bloodborne pathogens can cause NHPs population decline which is why the knowledge of bloodborne pathogens in NHPs aims directly to their conservation. Finally, bloodborne pathogens in New World (NWs) NHPs are poorly documented and there are no reports from Ecuador.

4.6. The PCR technique and its use in non-invasive fecal samples

The chain reaction technique (PCR) has become a successful tool for the evaluation of the genetic composition of social groups, genealogies, populations, biogeography, parasites, phylogeny (microbial DNA-RNA), hormones, diet and diseases of non-human primate societies microbial DNA. Fecal samples are an important source of information about parasites (viruses, prokaryotes or eukaryotes) that infect wild primate species. Molecular analysis of faecal samples has already been used to decipher the origins of the main pathogens of human and non-human primates (Acharya *et al*., 2017; Al-Areeqi *et al*., 2017; Arregui *et al*., 2015; Bairami *et al*., 2018; Bezjian *et al.*, 2008; Boom *et al*., 1990; Carozzi *et al*., 2013; Cerda-Molina *et al*., 2006; Chinchilla *et al.*, 2007; Conga *et al*., 2014; De Nys *et al*., 2015; De Nys *et al.*, 2014; Jirků *et al*., 2012; Mathay *et al*., 2015; Strier *et al*., 1999; Yasuda *et al*., 2015; Ziegler *et al*., 1997; Zinner *et al*., 2013).

Molecular genetic analysis has had its limitations due to the access to blood or tissue samples for their extraction from wild populations (Chaves *et al*., 2006) so it has been opted for worldwide non-invasive sampling whose adversity is mainly due to the low amount of genetic material obtained (Chaves *et al.*, 2006). Boom *et al*. (1990) presented the first study that succeeded in isolating DNA from epithelial cells that were mixed with feces. Since then, with improvements included in their protocols, studies in conservation genetics have used faecal samples for DNA extraction prioritizing species that are in some category of protection or threat (Creel *et al.*, 2003; Taberlet *et al.*, 1999a).

The analysis of molecular stool samples offers a non-invasive option that becomes a valid alternative to traditional sampling methods, one of its difficulties being the quality of the sample collected for which at least these suggestions must be maintained (Carozzi *et al.*, 2013):

1. Sample collection should maximize the recovery of DNA from the samples.

2. Preserves DNA in feces by preventing DNA degradation and ensuring DNA integrity.

3. Limits the action of inhibiting factors present in faeces that could inhibit the efficiency of PCR.

With regard to the limitations that have been recorded in molecular analysis of DNA collection is the contamination of faecal DNA by inhibitors in all groups of taxa studied which have forced to counteract the PCR inhibitors with different adaptations to protocols generally used which allows standardization and optimization operationally analysis (Abu Al-Soud *et al*., 2000; Chaves *et al*., 2006; Crellen *et al*., 2016; Farrell *et al*., 2017; Kohn *et al*., 1997; Palomares et al., 2002).

The adaptations mentioned above seek to maximize DNA recovery and preserve DNA in the stool in such a way that it does not degrade during sample handling, so manual extraction is laborious and it is recommended to add DNA stabilizers (RNAlater, FTA Whatman, 70% alcohol, 2.5% potassium dichromate) and freeze the sample immediately after collection and strictly maintain the cold chain, recommended from -20 °C to -80 °C, to prevent degradation immediately after defecation and thus improve testing. This allows to improve and achieve an optimization of the fecal sample and total yield of extracted DNA and its purity (Mathay *et al*., 2015).

Extraction methods generally use organic solvents to separate proteins and lipids from DNA to isolate it by precipitation with ethanol and performed by traditional and commercial methods (Tarqui-Terrones *et al*., 2019). The latter are faster and generally based on silica membrane promoting the absorption of the DNA molecule to the membrane positively charged in adequate quality and quantity. They are free of contaminants and inhibitors, which is reflected in a guaranteed diagnosis or molecular identification by reducing errors and eliminating PCR inhibition. Constituent elements of stool samples such as: proteins, haemoglobin, bile salts, immunoglobulins, lactoferrin are considered PCR inhibitors (Acharya *et al.*, 2017; Schrader *et al.*, 2012; Tarqui-Terrones *et al.*, 2019). Several authors suggest the use of commercial column kits, such as QIAGEN DNA extraction kit, which has a silica membrane as a solid material that retains or binds molecules such as DNA and allows other molecules to pass through, to facilitate purification and to eliminate derivatives of the extraction process and the presence of chemical inhibitors and thus increase amplification rates (Greenspoon *et al.*, 1998; Molina *et al.*, 2006; Schrader *et al.*, 2012).

5. Bloodborne pathogens

Based on international concepts and guidelines as well as on the terminology used and identified in Carrillo-Bilbao *et al.* (2021), we determined that bloodborne pathogens are viruses, bacteria, protozoa and fungi; which are found in the blood and can cause disease in the host (Fotedar, 2019; Méthot *et al.*, 2014). A parasite is defined as a single-celled or multiple cell organism that lives on or in another

species, known as the host, where it obtains nutrients and may or may not reproduce (CDC, 2022). Sometimes but not always it can be detrimental to the host (Bessette *et al*., 2022). However the definition of parasite is still controversial and has changed many times (Kaishian *et al.*, 2022). A pathogen is defined as "microorganisms (including bacteria, viruses, parasites, fungi) and other agents such as prions, which can cause disease in humans or animals" (WOAH, 2023).

Pathogenicity is the ability of an organism to infect another organism in this case the host, which differs from parasitism which is an interaction between two species where one of them is benefited. Pathogens may include bacteria, protozoa, fungi and viruses. Disease is an abnormal condition in any part of the body or in the whole animal with clinical signs (Méthot *et al.*, 2014).

The transmission of bloodborne pathogens is not well understood, noting that the pathogens have many ways of transmission and dissemination, but it is known that it can be transmitted by blood or other contaminated materials or fluids such as: blood, blood serum, vaginal secretions, semen, cerebrospinal fluid, pleural, peritoneal, and other joint capsule or organ fluids, amniotic fluid (García-Romero *et al.*, 2023).

5.1. Viruses

Viruses are small, intracellular, infectious units (Fig. 9), consisting of RNA and DNA, surrounded by a protein coat, ranging from 16 nm to 300 nm, which have evolved and have allowed them to adapt to specific organisms and their cells (Gelderblom, 1996; Modrow *et al*., 2014). A virus "cannot replicate alone; instead, it must infect cells and use components of the host cell to make copies of itself. Often, a virus ends up killing the host cell in the process, causing damage to the host organism" (NIH, 2023).

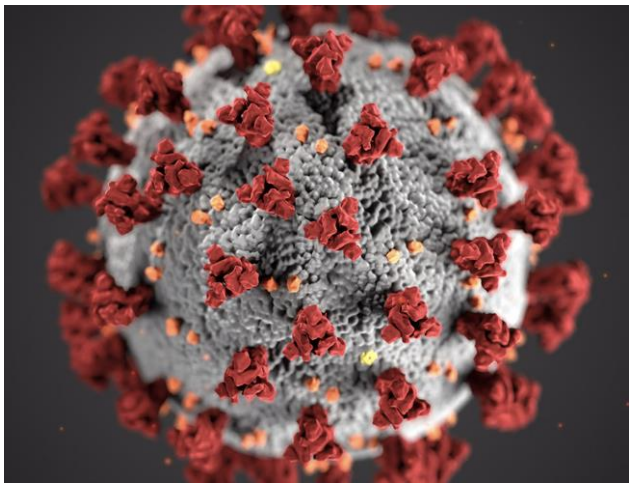

Figure. 9. Image of ultrastructural morphology of the Novel Coronavirus (2019-nCoV)
(Eckert *et al*., 2020)

The classification of viruses is given by families, and it is propitiated by the international commission of viruses, and it is being adapted based on the advances and discoveries; this classification is based basically on the size, shape, chemical composition and structure of its genome and its replication model, being its chemical classification and mode of replication in DNA and RNA viruses (Table 3).

Table 3. Virus taxonomy according to (Modrow *et al*., 2014).

Virus family	Subfamily/genus	Some examples
Picornaviridae	Enterovirus	Poliovirus, coxsackievirus, human enteroviruses
	Cardiovirus	Encephalomyocarditis virus, mengovirus
	Aphthovirus	Foot-and-mouth disease virus
	Parechovirus	Human parechovirus
	Hepatovirus	Hepatitis A virus
	Erbovirus	Equine rhinitis B virus
	Kobuvirus	Aichi virus
	Teschovirus	Porcine teschoviruses
Astroviridae	Mamastrovirus	Human, bovine and feline astroviruses
	Avastrovirus	Avian astroviruses
Caliciviridae	Norovirus	Norwalk virus
	Sapovirus	Sapporo virus
	Vesivirus	Feline calicivirus
	Lagovirus	Rabbit haemorrhagic disease virus
	Nebovirus	Newbury-1 virus
Hepeviridae	Hepevirus	Hepatitis E virus
Flaviviridae	Flavivirus	Yellow fever virus, dengue virus, West Nile virus, tick-borne encephalitis virus
	Pestivirus	Classical swine fever virus, bovine viral diarrhoea virus
	Hepacivirus	Hepatitis C virus
Togaviridae	Alphavirus	Sindbis virus, Semliki Forest virus, equine encephalitis viruses
	Rubivirus	Rubella virus
Arteriviridae	Arterivirus	Equine arteritis virus, porcine reproductive and respiratory syndrome virus
Coronaviridae	Coronavirinae/ Alphacoronavirus	Human coronaviruses 229E and NL63, feline coronavirus, porcine transmissible gastroenteritis virus

	Coronavirinae/ Betacoronavirus	SARS-associated coronavirus, mouse hepatitis virus, bat corinaviruses HKU5 and HKU9
	Coronavirinae/ Gammacoronavirus	Avian infectious bronchitis virus
	Torovirinae/Torovirus	Bovine and equine toroviruses
Rhabdoviridae	Vesiculovirus	Vesicular stomatitis virus
	Lyssavirus	Rabies virus
	Ephemerovirus	Bovine ephemeral fever virus
	Novirhabdovirus	Infectious haematopoietic necrosis virus
Bornaviridae	Bornavirus	Borna disease virus
Paramyxoviridae	Respirovirus	Parainfluenza virus
	Rubulavirus	Mumps virus
	Avulavirus	Newcastle disease virus
	Morbillivirus	Measles virus, canine distemper virus
	Henipavirus	Hendra virus, Nipah virus
	Pneumovirus	Respiratory syncytial virus
	Metapneumovirus	Human metapneumovirus
Filoviridae	Marburgvirus	Marburg marburgvirus
	Ebolavirus	Zaire ebolavirus, Reston ebolavirus
Arenaviridae	Arenavirus	Lymphocytic choriomeningitis virus
Bunyaviridae	Orthobunyavirus	California encephalitis virus
	Phlebovirus	Rift Valley fever virus, sandfly fever virus
	Nairovirus	Crimean-Congo fever virus
	Hantavirus	Hantaan virus, Puumala virus, Sin Nombre virus
	Topsovirus	Tomato spotted wilt virus
Orthomyxoviridae	Influenza A virus	Influenza A virus
	Influenza B virus	Influenza B virus
	Influenza C virus	Influenza C virus
	Thogotovirus	Thogoto virus, Dhori virus
	Isavirus	Infectious salmon anaemia virus
Birnaviridae	Avibirnavirus	Gumboro virus
	Aquabirnavirus	Infectious pancreatic necrosis virus
	Entomobirnavirus	Drosophila X virus
Reoviridae	Orthoreovirus	Reoviruses
	Orbivirus	Bluetongue virus, African horse sickness virus
	Rotavirus	Rotaviruses
	Coltivirus	Colorado tick fever virus

	Aquareovirus	Golden shiner virus
Retroviridae	Alpharetrovirus	Rous sarcoma virus
	Betaretrovirus	Mouse mammary tumour virus
		Jaagsiekte sheep retrovirus
	Gammaretrovirus	Feline leukaemia virus, murine leukaemia virus
	Deltaretrovirus	Human T-lymphotropic viruses 1 and 2
	Epsilonretrovirus	Diverse fish retroviruses
	Lentivirus	Human immunodeficiency viruses
	Spumavirus	Simian foamy virus
Hepadnaviridae	Orthohepadnavirus	Hepatitis B virus
	Avihepadnavirus	Duck hepatitis B virus
	Deltavirus (virusoid); infection along with hepatitis B virus as helper virus	Hepatitis D virus
Polyomaviridae	Polyomavirus	BK polyomavirus, simian virus 40
Papillomaviridae	Alphapapillomavirus	Human papillomaviruses 6, 10, 16, 18 and 32 (mucosa, oral/genital)
	Betapapillomavirus	Human papillomaviruses, 5, 9 and 49 (dermal)
	Gammapapillomavirus	Human papillomaviruses 4, 48 and 50 (dermal)
	Deltaapillomavirus	Ruminant papillomaviruses (cattle, sheep, deer)
	Lambdapapillomavirus	Canine and feline papillomaviruses
Adenoviridae	Mastadenovirus	Human and canine adenoviruses
	Aviadenovirus	Avian adenoviruses
	Siadenovirus	Turkey haemorrhagic enteritis virus
	Atadenovirus	Chicken egg drop syndrome virus
Herpesviridae	Alphaherpesvirinae	Herpes simplex viruses, bovine, equine, porcine, canine, feline and gallid herpesviruses
	Betaherpesvirinae	Cytomegalovirus, human herpesvirus 6
	Gammaherpesvirnae	Epstein-Barr virus, human herpesvirus 8, alcelaphine herpesvirus 1
Poxviridae	Orthopoxvirus	Variola viruses, vaccinia virus, bovine and simian variola viruses
	Parapoxvirus	Orf virus
	Avipoxvirus	Canarypox virus
	Molluscipoxvirus	Molluscum contagiosum virus
	Suipoxvirus	Swinepox virus

	Yatapoxvirus	Tanapox virus, Yaba monkey tumour virus
Asfarviridae	Asfivirus	African swine fever virus
Parvoviridae	Parvovirus	Feline panleucopenia virus, canine parvovirus
	Erythrovirus	Parvovirus B19
	Bocavirus	Human bocavirus, bovine bocavirus
	Amdovirus	Aleutian mink disease virus
	Dependovirus	Adeno-associated viruses
Circoviridae	Gyrovirus	Chicken anaemia virus
	Circovirus	Porcine circovirus, beak and feather disease virus
Anelloviridae	Alphatorquevirus	Torque teno virus
	Betatorquevirus	Torque teno mini virus
	Gammatorquevirus	Torque teno midi virus

5.2. Arboviruses

The description of arboviruses was promulgated by the WHO in 1976, where it mentions that they are viruses that are generally present in nature through transmission between vertebrate hosts by arthropod vectors (Arredondo-García *et al.*, 2016). There are approximately 250 spp. of arboviruses, other authors mention that there could be around 500 spp. of which between 80 and 150 spp. can cause disease in humans (Navarrete-Espinosa *et al.*, 2006; Stuchin *et al.*, 2016; WHO, 2022).

Arboviruses are not a taxonomic category but are grouped into different families that are transmitted by arthropods, mainly mosquitoes. Of the arboviruses that have been recognized as groups that affect the human population, we can highlight the Zika, Dengue, yellow fever and Chikungunya viruses (Fig. 10), which are found in tropical and subtropical regions where about 3.9 billion people live (WHO, 2022). Within the arbovirus group there are several families as follows (Yuill, 2023):

- *Arenaviridae*
- *Bunyaviridae (bunyavirus, flebovirus, nairovirus y hantavirus)*
- *Togaviridae*
- *Flaviviridae (flavivirus)*
- *Filoviridae*
- *Orthomyxoviridae (thogotovirus)*
- *Reoviridae (coltivirus y orbivirus)*
- *Togaviridae (alfavirus)*

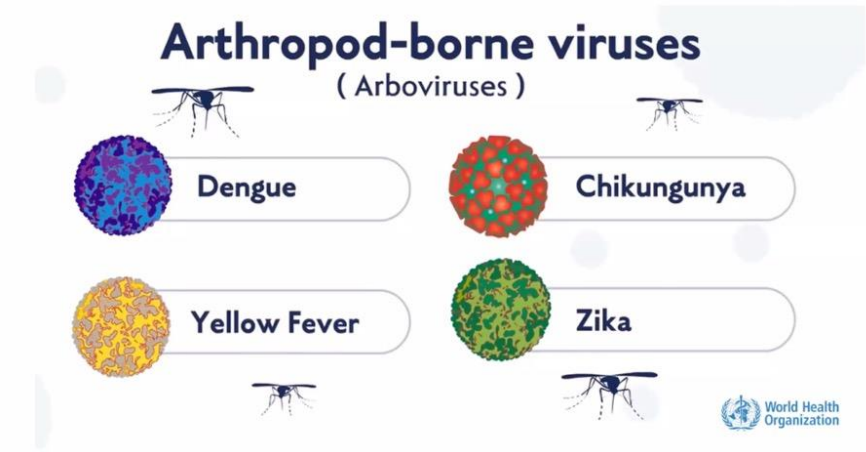

Figure 10. Common arboviruses of the neotropical region from (Sanofi/Pasteur, 2020)**.**

5.3. Bacteria

Bacteria are a broad group of organisms related to prokaryotes, lacking a nucleus. They have a wide range of distribution, they can be found in soil (Wang *et al.*, 2023), water, in our bodies and in other living species as well as in extreme environments (Connelly *et al.*, 2023; Vreeland *et al.*, 2000), some of them even infect blood (Fig. 11). To date it has been possible to identify within this phylum approximately 45 555 species (Parks *et al.*, 2022) and are estimated to a total of $5x10^{30}$ cells, and are therefore a fundamental part of the biomass on Earth (Whitman *et al.*, 1998).

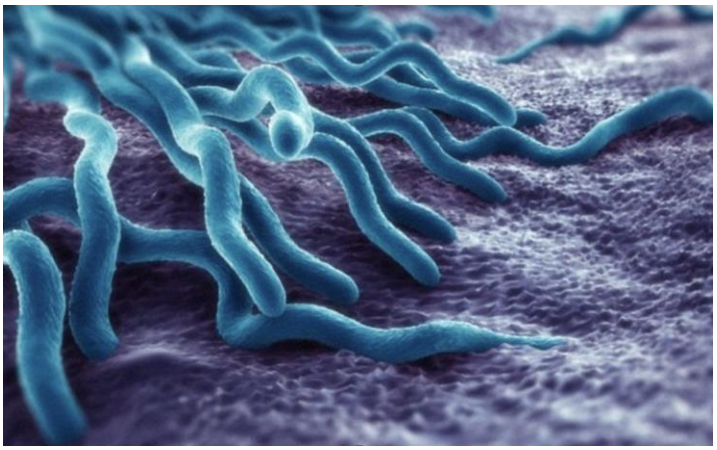

Figure 11. *Anaplasma* sp. taken from (Elsheika, 2016).

The classification of this group is complex. It is based on apropos to the diversity of the domains of Archaea and Bactaeria, where the *Archaea* differ from *Bacteria* in many ways (Boone *et al.*, 2001). Archaea are divided into two phyla based **Crenarchaeota** and the **Euryarchaeota**. The two phyla are divided into eight classes and 12 orders. The bacteria are an extraordinarily diverse assemblage of prokaryotes that have been divided into 23 phyla (DeLong *et al.*, 2001).

Phyla present in the domain Bacteria: Proteobacteria, Acidobacteria, Aquificae, Chlorobi, Bacteroidetes, Chlamydiae/Verrucomicrobia, Planctomycetes, Spirochaetes, Actinobacteria, Chloroflexi, Cyanobacteria, Firmicutes, Tenericutes, Fusobacteria, Synergistetes, Thermotogae, and Deinococcus/Thermus (Boone *et al.*, 2001).

Bacteria are traditionally classified into three major groups based on classification and pathogenicity:

1. Gram Positive and Gram Negative (Fig.12).

2. Acid-fast bacilli.

3. Spirochaetes.

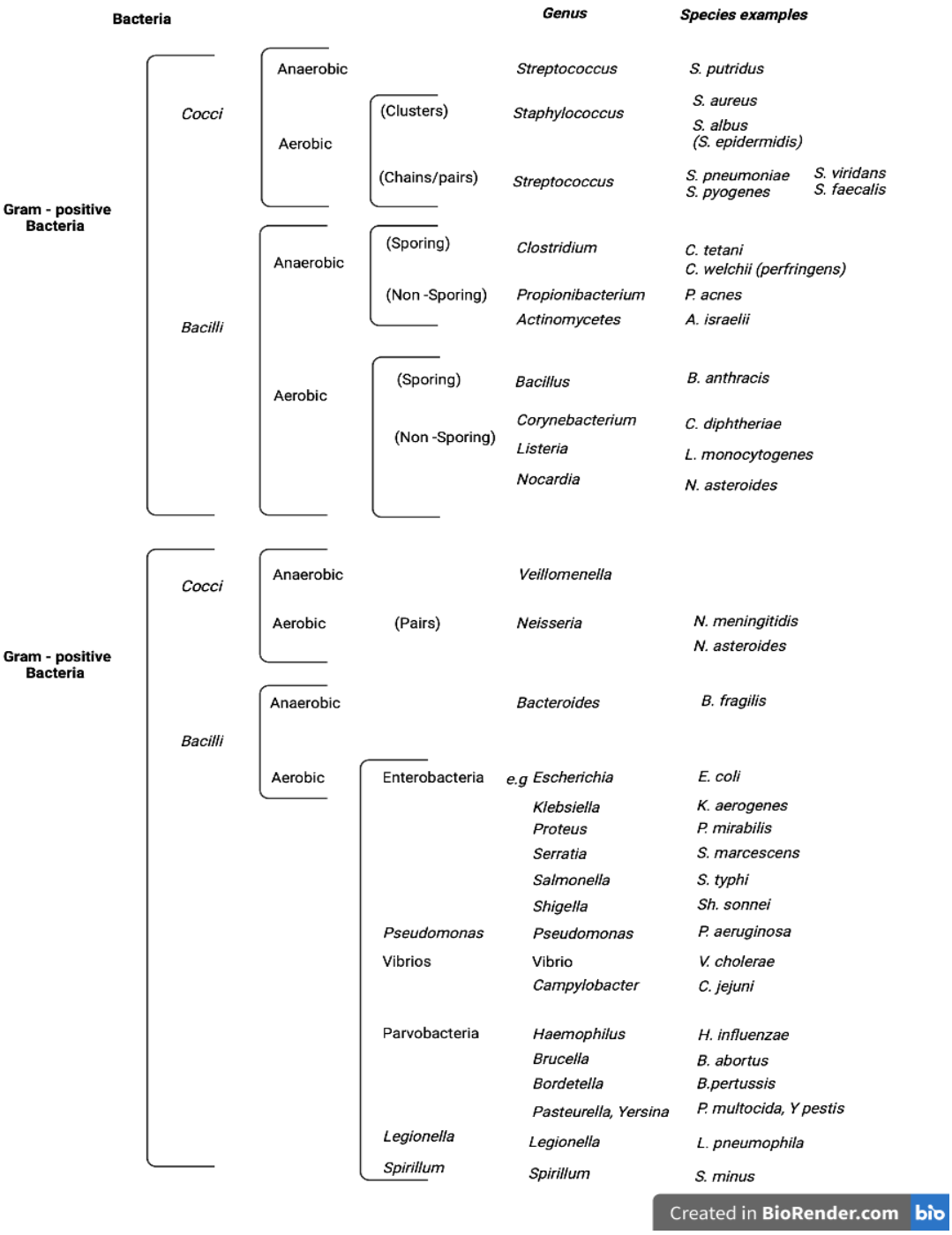

Figure 12. Classification of Gram- pathogens bacteria. Based on (Shanson, 1989)**.**

Currently, there is no official classification for procaryotes (Oren, 2023), however, based on recent genomic studies (Hug *et al*., 2016; Puigbò *et al*., 2019), classification of Bacteria is changing according to the molecular identification where some lineages can be isolated due to the lack of information (Fig. 13).

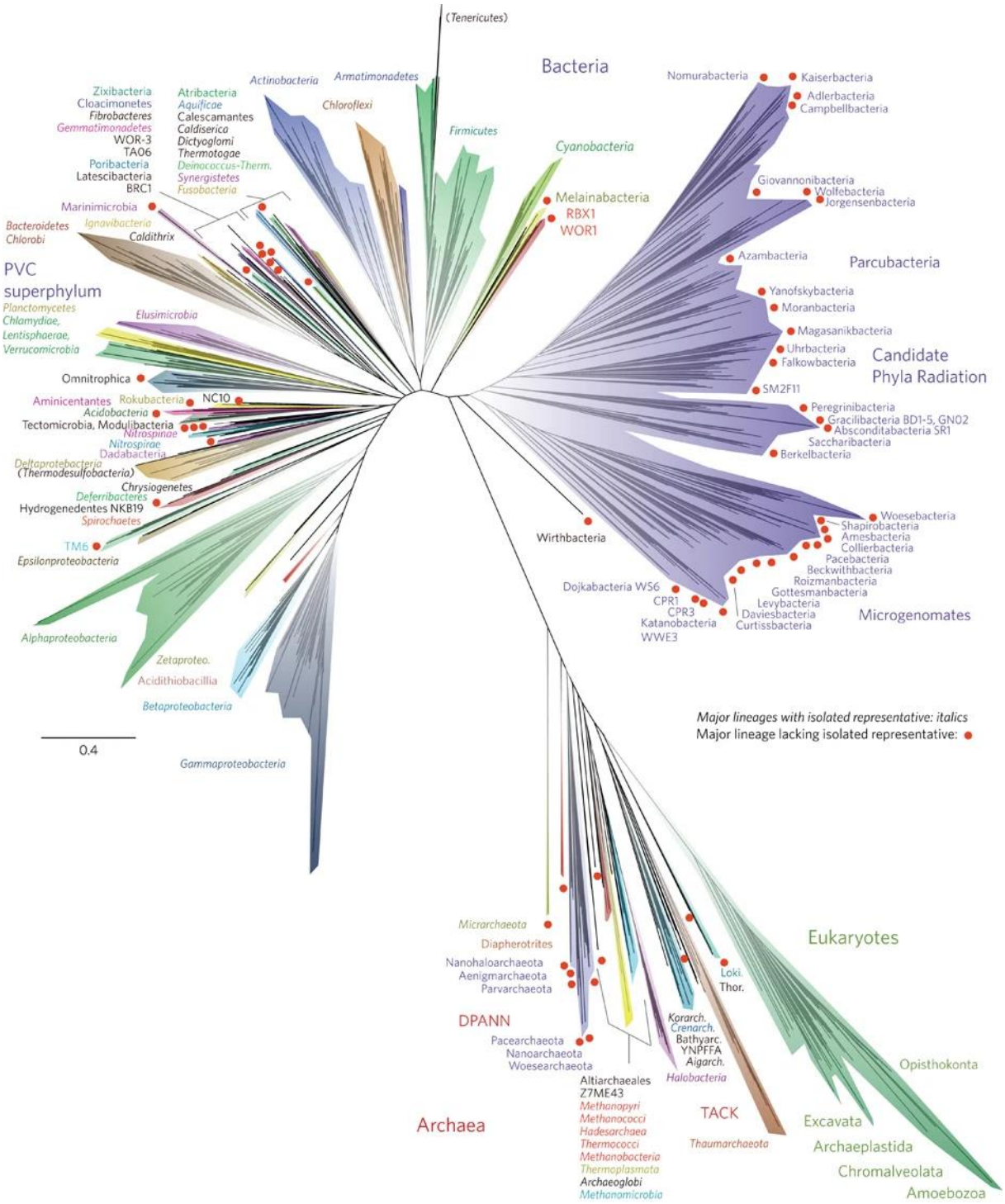

Figure 13. Classification of Major Bacteria Lineages by sequenced genomes (Hug, *et al.* 2016).

5.4. Protozoa

Pathogenic protozoa are generally microscopic, unicellular eukaryotes. They can be free-living, mutagenic, commensal, or parasitic. Some may be solitary and others colonial and may be intestinal,

urogenital, bloodborne, with all types of symbionts present, lacking germinal lamellae, consisting of DNA (Fig. 14) (Yaeger, 1996).

Figure 14. ***Trypanosoma cruzi*** (Dr Microbe, 2019)**.**

Among the Protozoa are the groups of ciliates, amoebae, apicomplexans and flagellates (Kreier *et al*., 1987). They have complex life cycles, as they go through several stages. They may involve a sexual phase, either by conjugation or syngamy, and asexual phase, the latter by fission, budding or cysts. They have no organs or tissues but have specialised organelles. Locomotion in some cases is by propulsion or other types of movements produced by pseudopods, flagella, and cilia (Sleigh, 2001; Yaeger, 1996)**.**

The number of described species worldwide is about 64 000 species, of which about 10 000 are symbiotic with animals and plants. They are widely distributed but many have limits within their environmental ranges (Mayen-Estrada *et al*., 2016; Yaeger, 1996).

In relation to the classification of the Phylum Protozoa, the Society of Protozoologists distributed them into four subphyla, being Microspora, Ciliophora and the phyla of Sarcomastigophora and Apicomplexa, which group together the pathogens causing diseases in humans (Fig. 15) (Yaeger, 1996):

SUBPHYLLUM: SARCOMASTIGOPHORA.

Superclass: Mastigophora

Class: Phytomastigophora Ex: *Euglena.*

Class: Zoomastigophora Ex: *Leishmania, Trypanosoma, Trichomonas, Trichonympha*

Superclass: Opalinata Ex: *Opalina.*

Superclass: Sarcodina:

Class: Rhizopodea Ex: *Amoeba, Entamoeba*

Class: Piroplasmea Ex: *Babesia*

Class: Actinopodea Ex: *Collozoum, Actinophrys, Acanthometra*

SUBPHYLUM: SPOROZOA

Class: Telosporea Ex: *Plasmodium*

Class: Toxoplasmea Ex: *Toxoplasma*

Class: Haplosporea Ex: *Haplosporidium, Ichthyosporidium*

SUBPHYLUM: CNIDOSPORA

Class: Myxosporidea Ex: *Myxobolus*

Class: Microsporidea Ex: *Nosema bombycis*

SUBPHYLUM IV: CILIOPHORA:

Class: Ciliatea Ex: *Paramoecium, Vorticella, Balatidium*

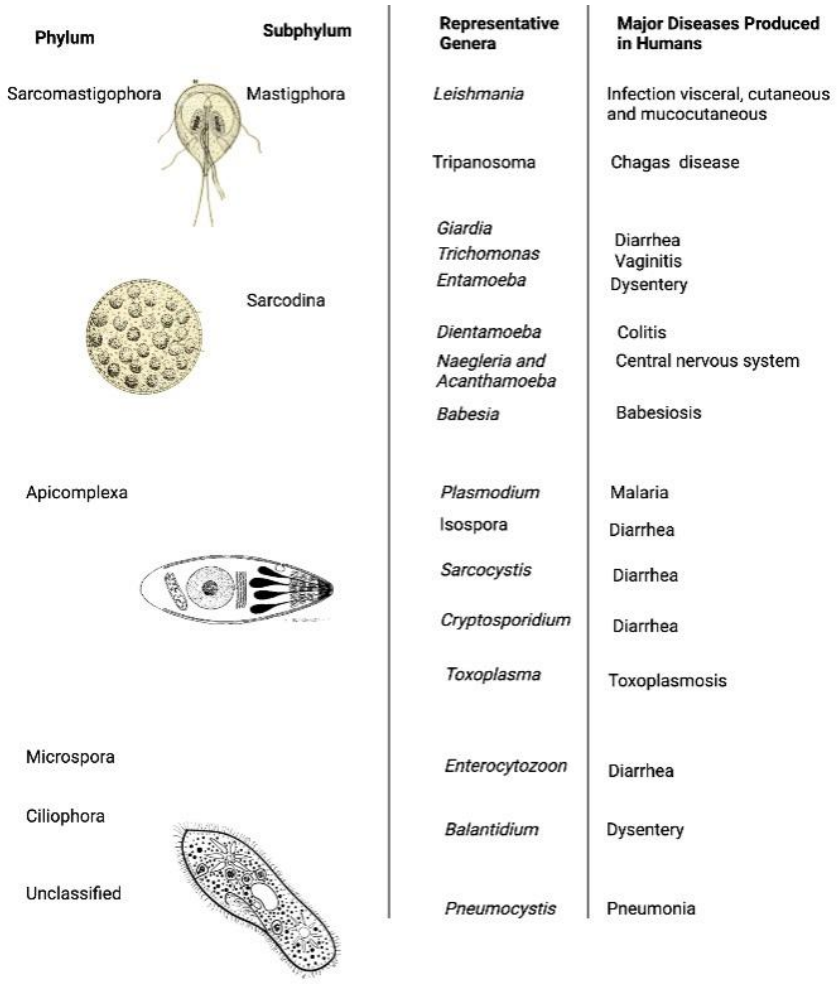

Figure 15. Classification of Pathogens Protozoa. Adapted from (Yaeger, 1996), (Created with BioRender.com)

5.5. Fungi

Fungi (Fig. 16) are eukaryotic microorganisms and constitute one of the most numerous groups with varied characteristics including ecological contributions and functions such as acting in some cases as mutualists or pathogens (McLaughlin *et al*., 2014; Tedersoo *et al*., 2018).

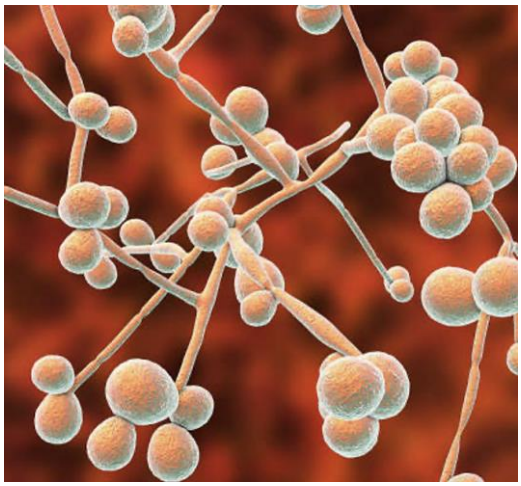

Figure 16. *Candida* sp. from (Dr Microbe, 2018)

Approximately 12 000 species have been recorded in the fungi kingdom and it is estimated that there could be around 1.5 million species. It should be noted that the classification of the group is still being refined with respect to species, genera and higher taxonomic levels (Hawksworth, 2001; Kirk *et al*., 2008; Tedersoo *et al*., 2018), which have a total of three described phyla (Webster et al., 2007).

Phylum Chytridiomycota:

They generally live in aquatic environments, with unicellular and few multicellular forms. They reproduce sexually and asexually, and some species have been considered pathogenic for animal and plant species. Approximately 900 species are grouped into 5 orders, among which the most important are the cryptids that affect populations of living beings located in water bodies where they are recorded (Kirk *et al*., 2008; Webster *et al*., 2007).

Phylum Zygomycota:

They are saprophytic and in many cases pathogens of plants and animals. An example of this phylum is the genus *Rhizopus*, with approximately 1 100 species, some of which affect plant and animal species (Kirk *et al*., 2008; Liu *et al*., 2023; Webster *et al*., 2007).

Phylum Ascomycota:

It is one of the groups with the largest number of species, estimating that there could be more than 32 000 species grouped in 3 400 genera (Kirk *et al*., 2008). Their common name is sac fungi and they are usually terrestrial. Some are saprophytes, neocrotrophs of both plant and animal species. These include the genera *Saccharomyces*, *Penicillum* and *Aspergillus* (Schubert *et al*., 2018; Webster *et al*., 2007).

Phylum Basidomycota:

It is one of the largest phyla in the kingdom, with a more than 30 000 species. They have a worldwide distribution and live on a wide variety of organisms. Some species of the phylum have been considered toxic to humans (Garcia *et al*., 2015; Webster *et al*., 2007).

6. Arthropod vectors of diseases in NHPs

6.1. Ticks

Ticks are arachnids of the order Ixodida and divided into three families (Nava *et a*l., 2017). In the neotropical zoogeographic region we found the description of 137 species of ticks among the following two families: Ixodidae and Argasidae which correspond to hard and soft ticks, respectively (Guglielmone *et al*., 2021). In Ecuador, there were until now 41 tick species found (Enríquez *et* al., 2023; Enríquez *et al*., 2020; Maya-Delgado *et al*., 2020; Pesquera *et a*l., 2015) among following genera: *Ixodes*, *Amblyoma*, *Haemaphysalis*, *Dermacentor*, and *Rhipicephalus* ., which are all hard ticks. Ticks are important vectors of pathogens of humans and wild and domestic animals (Rodriguez-Morales *et al*., 2018), such as bacteria (Epelboin *et al*., 2023; Martins *et al*., 2020), viruses (de Figueiredo *et al*., 2017) and protozoa (Coutinho *et al*., 2005; Dantas-Torres *et al.*, 2010; Marotta *et al*., 2018) and can be coinfected with these pathogens (Diuk-Wasser *et al*., 2016; Ferreira *et al.*, 2022) which makes them relevant vectors for the study of human and animal health. Ticks hard and soft, were identified in several families of neotropical NHP (Lima *et al*., 2023; Martins *et al*., 2021). However, currently, no studies of ticks in NHPs have been done in Ecuador.

6.2. Mosquitos

Mosquitos are insects of the phylum Arthropoda, where females, which are known to feed on blood, can transmit diseases in humans (Fontes *et a*l., 2020) and animals (Santiago-Alarcon *et al*., 2012), including NHPs. They are known to transmit a diverse range of pathogens, from viruses (Morales *et al*., 2017; Tranquilin *et al*., 2013) to protozoa (Rondón *et al*., 2019) and filaria (Famakinde, 2018). Feeding habits (preferred host and time of feeding) of mosquitos determine the ecology of vector-host and play a key role in epidemiology. For example, an application of vector epidemiology is vector management

(Rojo-Ospina *et al*., 2023) such as to targeted control of vectors (Pescod *et al*., 2023) and, disease and intensity of transmission modelling (Katzelnick *et al*., 2023; Zeng *et al*., 2023).

Unfortunately, in South America the number of studies of feeding behaviour of mosquitos is lower than in other continents (Loaiza *et al*., 2023; Melgarejo-Colmenares *et al*., 2022). In Ecuador, there are just two studies on feeding behaviour, one in mainland Ecuador (north coast) (Ligña Cachago, 2018), and the other one in Galápagos (Asigau *et al*., 2019). They identified the following genera: *Aedes*, *Anopheles*, *Culex*, *Psorophora*, and *Wyeomyia*. Most species of mosquitos have a generalist feeding behaviour (Bakker *et al*., 2020; Melgarejo-Colmenares *et al*., 2022), which allows host shift when the preferred host is not available (Kilpatrick *et al*., 2006; Rucci *et al*., 2023). In the Neotropics, from the 260 mosquito species, we know the feeding behaviour of only 38 species, of which 13 are opportunistic (generalist). Indeed, mosquitos can feed on several hosts. In the neotropics, neotropical NHPs (Kumm *et al*., 1938) are feeding hosts of several species of mosquitos such as *Haemagogus leucocelaenus* and *Sabethes* (Marassá *et al*., 2009), *Aedes fluvus*, and *Psorophora albipes* (Hoyos Loaiza, 2018), *Ae. scapularis Ps. albigenu* (Mucci *et al*., 2015). The time of feeding also influences the type of host; for example, most Anopheline (Moreno *et al*., 2007) and *Culex* species feed at night while other species of the genus *Sabethes* and *Haemagogus* feed during the day (Pinto *et al.*, 2009). Some species of the genera *Aedes*, *Haemagogus*, and *Sabethes* can transmit yellow fever to neotropical non-human primates (Abreu *et al*., 2019; Cunha *et al*., 2020).

7. Surveillance networks

In Ecuador, at the national level, the Ministry of Environment and Ecological Transition, together with Agrocalidad, oversee the control and monitoring of wildlife diseases and their impact on domestic animals. At regional level, in coordination with national authorities, the World Organisation for Animal Health (OIE) provides guidance to member countries on animal diseases. Recently, in 2022, the World Organisation for Animal Health, the World Health organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the United Nations Environment Program (UNEP) formed a quadripartite group to strengthen One Health collaborative capacity worldwide (FAO, 2022).

8. Biosecurity Guidelines

Biosecurity in wildlife is important at two levels: human and animal welfare. In the field, biosecurity is important when sampling animals (including NHPs) to minimize the risk of cross-contamination in samples and to have validated data. In captive settings, biosecurity guidelines can limit the spread of pathogens to other areas, regions, or other populations of NHPs. When working with wildlife and following biosecurity guidelines (Table 4), there are several considerations to be addressed:

Table 4. Biosecurity guidelines when working with captive and free-ranging populations of NHPs

To reduce the risks with captive populations	**To avoid cross-contamination in samples**	**To reduce diseases risks associated with wildlife translocations (reintroduction)**
Record keeping and animal (sample) identification		
Quarantine / Isolation and Treatment of sick animals.	All types of equipment (including vehicles to transport samples) need to be clean and if necessary disinfected prior to use (in captive and in the field).	The sending and receiving facility must have appropriate quarantine procedures. Animals that are in a process of a reintroduction need to be quarantined before being moved to another area.
Test of principal diseases such as *Mycobacterium tuberculosis*	Managing hygiene in the field such as hand hygiene, use of masks and goggles.	Animals must have a routine screening and health diagnostic.
Check for aerosol minimisation.	Maintenance of the cold chain of samples when handling and managing samples	Identify and mitigate the risks from animal imports, exports and movements.
Consider possible sacrifice of positive animals (depending on the results).	Clean-up of personnel and equipment at the end of each procedure.	Consider a pre-release treatment according to diagnostic results.
Control of pest animals.	Management of samples, live animals, carcasses and waste at the end of the procedure.	Appropriate animal training to handle a range of taxa and adapt transportation systems for each taxa.
Work and Hygiene procedures for staff and visitors: - Equipment: use of heat, pressure, radiation or chemical disinfection or sterilisation,		Vehicles to transport animals need to be clean and if necessary disinfected before and after use.

- At the facility: Hand Hygiene (prior to commencement of the work, as well as between handling each animal), appropriate management of laundry, food management - Safe food practices (e.g.: identification of food inappropriately stored, check for water quality and supply).		
Selection and use of personal protective equipment (PPE): e.g., the use of disposable gloves.		Emergency biosecurity response plans should be elaborated before any translocation.
Check for appropriately constructed and maintained facilities (fences, security barriers), and develop biosecurity plans to improve facilities according to the plans.		A veterinarian may be required to accompany in animal. transfers, to assist in management of biosecurity and health concerns.
Control of drainage and waste disposal (safe use and disposal of sharps such as needles and scalpel blades, as well as knives for food preparation).		
Ensure that food, water, equipment, or work practices do not introduce or spread pests or diseases.		

Control of carcasses and biological samples (cleaning spills of blood and body substances).		

Chapter 2 - Objectives

General objective

To determine the prevalence of blood pathogens in captive and free-ranging non-human primates in the Provinces of Pastaza, Morona Santiago and Napo Ecuadorian Amazon.

Specific objectives

The first specific objective was to unify the concepts to be used based on existing international guidelines and related information on zoonotic bloodborne pathogens in non-human primates and mammals at the neotropical level, based on the number of terms identified throughout our research: Zoonotic BloodBorne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review **(Study 1)**. In addition, we collaborated directly with another review on the evidence of arborviruses in mammals in the neotropics: Arboviruses in Mammals in the Neotropics: A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine**.** With this objective we aimed to provide a baseline of consolidated scientific information on bloodborne pathogens in non-human primates.

The second objective allowed us to conduct a preliminary study to improve the protocols for the determination of *Leishmania, Babesia, Plasmodium, Trypanosoma* and *Anaplasma* in faecal samples in which we identified some limitations that we found during the study "Molecular identification of *Plasmodium falciparum* from captive non-human primates in the Western Amazon Ecuador" **(Study 2)**.

The third objective, with validated collection, storage and transport protocols for faecal samples, was to screen faecal samples of non-human primates for the following blood pathogens: *Trypanosoma* sp. and *Babesia* sp., for which we published the following manuscript: First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon **(Study 3).**

Finally, the fourth objective aimed to determine the perception of biosecurity knowledge and its application in wildlife management centers and provide a baseline assessment to create an input to promote the correct application of biosecurity measures in these centers, which is entitled: Perception of biosecurity and risks in Ecuadorian Wildlife Management Units **(Study 4).**

Chapter 3 - Experimental section

Experimental section

Study 1:

Zoonotic BloodBorne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review

Pathogens 10(8):1009-33

Carrillo-Bilbao Gabriel, Martin-Solano Sarah, Saegerman Claude

Preamble

Non-human primates can act as reservoirs for bloodborne pathogens, however information on this topic is scarce for countries like Ecuador. Therefore, this study, Zoonotic Bloodborne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review, allows us to better understand the role of NHPs in human emerging diseases in the Neotropical region with an emphasis on Ecuadorian NHPs.

We identified 56 bloodborne pathogens in the neotropical region, of which the Protozoa are the most recorded pathogens and the *Alouatta* genus is the most studied non-human primate in the Neotropical region. Finally, we identified the different socioecological factors associated with the transmission of this type of pathogens, as a fundamental part of the important elements for understanding the transmission process and the different interactions between the pathogen, its host, and its ecosystems.

With the results presented in this study, it is of utmost importance that the governmental units responsible for the countries with a high risk of transmission of this type of diseases and their pathogens prioritize the implementation of control and prevention measures with their respective protocols.

pathogens

MDPI

Review

Zoonotic Blood-Borne Pathogens in Non-Human Primates in the Neotropical Region: A Systematic Review

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Abstract: Background: Understanding which non-human primates (NHPs) act as a wild reservoir for blood-borne pathogens will allow us to better understand the ecology of diseases and the role of NHPs in the emergence of human diseases in Ecuador, a small country in South America that lacks information on most of these pathogens. **Methods and principal findings**: A systematic review was carried out using PRISMA guidelines from 1927 until 2019 about blood-borne pathogens present in NHPs of the Neotropical region (i.e., South America and Middle America). **Results**: A total of 127 publications were found in several databases. We found in 25 genera (132 species) of NHPs a total of 56 blood-borne pathogens in 197 records where Protozoa has the highest number of records in neotropical NHPs (n = 128) compared to bacteria (n = 12) and viruses (n = 57). *Plasmodium brasilianum* and *Trypanosoma cruzi* are the most recorded protozoa in NHP. The neotropical primate genus with the highest number of blood-borne pathogens recorded is *Alouatta* sp. (n = 32). The use of non-invasive samples for neotropical NHPs remains poor in a group where several species are endangered or threatened. A combination of serological and molecular techniques is common when detecting blood-borne pathogens. Socioecological and ecological risk factors facilitate the transmission of these parasites. Finally, a large number of countries remain unsurveyed, such as Ecuador, which can be of public health importance. **Conclusions and significance**: NHPs are potential reservoirs of a large number of blood-borne pathogens. In Ecuador, research activities should be focused on bacteria and viruses, where there is a gap of information for neotropical NHPs, in order to implement surveillance programs with regular and effective monitoring protocols adapted to NHPs.

Keywords: Ecuador; non-human primates; *Alouatta*; blood-borne pathogen; protozoa; *Plasmodium*; *Trypanosoma*; yellow fever

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1. Introduction

Wild animals are the cause of almost 70% of all emerging diseases [1], and more than 60% of these diseases are zoonotic [2]. This is a public health concern and a conservation problem [3,4]. Non-human primates (NHPs) are infected not only by gastrointestinal parasites [5], but also by ectoparasites, hemoparasites, bacteria, viruses and some arthropods that affect the lungs. Until recently [6,7], just a few studies identified blood pathogens from fecal samples due to the presence of DNA (deoxyribonucleic acid) inhibitors in fecal samples. Thereafter, just a few studies have identified hemoparasites such as *Plasmodium* sp. [8,9] and *Trypanosoma brucei* [10] and viruses such as adenovirus [11] and astrovirus [12]

from NHPs' fecal samples. Most NHP species are listed under a category of conservation [13,14]. Molecular identification in fecal samples of blood-borne pathogens will be of great advantage to monitor NHP populations that can be a potential zoonotic reservoir for humans.

Gastrointestinal parasites have been monitored in neotropical primates [5,15–21]; however, they are restricted to some countries such as Mexico [22–33] and Brazil [34–44]. Regarding the study of hemoparasites and arboviruses in neotropical primates, this one is restricted just to a few studies in Brazil [45–48], Venezuela [49,50] and French Guiana [49], and Ecuador has no data on them [51], even if most of those hemoparasites and arboviruses are present in Ecuador [52–56]. Finally, we wish to focus on hemoparasites and arboviruses because they are the cause of millions of infections and thousands of deaths per year in humans [56–59]. Understanding whether primates act as a wild reservoir for hemoparasites and viruses in the neotropical region will allow us to better understand the ecology of diseases [60] and the role of NHPs in the emergence of human diseases [61], as well as the way to implement control programs [62,63] for endemic [64] and incoming pathogens [65] and NHP conservation/management plans in Ecuador [66].

Some NHPs can become infected with hemoparasite species of protozoans. For example, wildlife harbors several species of *Plasmodium* [67–69], especially NHPs. However, in the neotropics, just recently, there is evidence of natural infection in humans with *Plasmodium brasilianum* in Venezuela [70] and *Plasmodium simium* in Brazil [45]. Therefore, in order to identify potential zoonotic reservoirs in wildlife, it is essential to monitor *Plasmodium* sp. in the Amazon region of Ecuador. Another example, *Toxoplasma* sp., has a worldwide distribution and affects a wide range of hosts from humans [71] and domestic animals [72] to wildlife [3,73], including marine mammals [74], freshwater mammals [75] and NHPs, Old World (OW) and New World (NW) monkeys [48,76–79]. In Ecuador, however, screening to detect *Toxoplasma* was only carried out in the islands of Galapagos. Indeed, some studies found *Toxoplasma* in birds [80,81], domestic animals [82], as well as in environmental waters. *Leishmania* sp. occurs in a wide range of hosts [83–88], including human [89] and non-human primates [48]. In NHPs, experimental [90] and natural infections [91] have been registered. However, studies in the neotropics are restrained to Brazil, and countries such as Ecuador are under-surveyed even though the parasite is widely distributed [92]. In Ecuador, despite surveillance and control campaigns, trypanosomiasis is still present [93]. However, there are just a few studies of trypanosomiasis in wildlife: bats [94,95], marsupials and rodents [96], and frogs [97], and unfortunately there are none on primates. In addition, in NW monkeys, it is very common to find several species of trypanosomes such as *Trypanosoma (megatrypanum) minasense* [98], and also zoonotic trypanosomes: *Trypanosoma rangeli* and *T. cruzi* [99].

Viral infections also pose a threat to NHPs' health. Four types of viruses may affect NHPs: enveloped DNA (deoxyribonucleic acid) viruses, non-enveloped DNA viruses, enveloped RNA (ribonucleic acid) viruses and non-enveloped RNA viruses [100]. Among the latter, arboviruses (arthropod-borne viruses) are a diverse range of viruses from eight families: *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivirus*), *Peribunyaviridae* (example: genus *Orthobunyavirus*), *Nairoviridae* (example: genus *Orthonairovirus*), *Phenuiviridae* (example: genus *Phlebovirus*), *Reoviridae* (genus *Orbivirus*), *Rhabdoviridae* (genus *Vesiculovirus*) and *Orthomyxoviridae* (genus *Thogotovirus*).

Arboviruses are a public health concern due to the threat to both humans and animals [101,102]. Arbovirus hosts can vary from a specific taxonomic group to several hosts. The range of vectors can also vary in the same way. For some arboviruses, the zoonotic origin is linked to primates because of their close genetic distance, while others are linked to other vertebrates or the vector itself [103]. In the neotropics, NHPs have been identified as hosts for the following diseases: yellow fever [104–106], Mayaro virus [49], Zika virus, Chikungunya virus [107], hepatitis A [108], Cacipacoré virus [109], St. Louis encephalitis virus (SLEV) and Oropouche virus (OROV) [49,110].

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2. Results

2.1. Current Situation of Non-Human Primates

Non-Human Primate Biodiversity

Primates from all over the world are divided into two groups: Old World Monkeys (Catarrhinni) and New World Monkeys (Platyrrhini). Around the world, we reported 504 species, including 171 species in the Neotropical region (i.e., South America and Middle America). The Neotropical region is the zoogeographical region with the highest number of species, and Ecuador registers 21 species (Table 1). All groups are mainly arboreal and they play an important role in cultures [111], in religions [112], in human livelihoods [113], and in the threat of emerging diseases [105]. They are also a good indicator of the quality of the environment [114], and at this time the destruction of their habitats, hunting and the capture of live specimens for export and local use are the greatest threats to their conservation [115–119].

Table 1. Species of non-human primates from Ecuador.

Family	Primate Species	Size (cm)	Weight (g)	Group Size (# of Individuals)	Social System [1]	Habitat [2]
Aotidae	*Aotus lemurinus*	50	1300	Small (1 to 5)	M	TFF
	Aotus vociferans	50	698	Small (2–5)	M	TFF, FF, and crops
Atelidae	*Alouatta seniculus*	43–60	3600–9000	Small (3–7) or Large (15)	MM–MF or a group of bachelor males	FF
	Alouatta palliata aequatorialis	48–67	3100–9800	Small and Medium (2–12)	MM–MF	All types of forests
	Ateles belzebuth	40–60	6000–10,500	Large (≈20)	MM–MF	TFF
	Ateles fusciceps fusciceps	39–58	8800	Large (20–30)	MM–MF	TFF
	Lagothrix lagotricha lagotricha	40–58	3600–10,000	Large (6–60)	PG/MM–MF/MM	TFF
	Lagothrix lagotricha poeppigii	40–58	3600–10,000	Large (2–25)	PG/MM–MF/MM	TFF
Cebidae	*Cebus albifrons*	81–84	1900–3900	Medium (5–30)	PG/MM–MF	All types of forests
	Cebus aequatorialis	35–46	1200–3600	Medium (5–20)		
	Sapajus macrocephalus	35–50	1700–4500	Medium (5–20)		
	Cebus capucinus capucinus	35–45	2900–3900	Medium (2–42)		
	Cebus yuracus	81–84	2900–3900	Medium and Large (10 and 35–54)		
	Saimiri cassiquiarensis macrodon	25–32	600–1400	Large >10 and up to 100		
Callitrichidae	*Cebuella pygmaea*	12–15	100–140	Small and Medium (2–9)	M/PA	TFF, FF
	Leontocebus nigricollis graellsi	15–28	350–470	Small and Medium (2–30)	M/MM–MF	TFF and FF
	Leontocebus lagonotus	17–27	330–430	Medium (2–10)	All kinds of social structures	All kind of forest
	Leontocebus tripartitus	21–24	218–240	Small and Medium-sized (4–10)	PG	TFF and FF

Table 1. *Cont.*

Family	Primate Species	Size (cm)	Weight (g)	Group Size (# of Individuals)	Social System [1]	Habitat [2]
Pithecidae	*Plecturocebus discolor*	28–36	900–1400	Small (2–5)	M	FF-lianas forest–forest gaps
	Cheracebus lucifer	30–38	800–1500	Small (2–5)		TFF
	Pithecia aequatorialis	39–44	2000–2500	Small (1–4)		TFF
	Pithecia milleri	37–48	2100–2600	Small (2–6)		TFF and FF
	Pithecia napensis	37–48	2100–2600	Small and Medium (4–8)		TFF

[1] Social system: M = Monogamous: A mating system in which only one male copulates with only one female; MM–MF = Multimale–Multifemale groups: A social group consisting of multiple adult males and multiple adult females; MM = Multi-males: Strong social relationships among males, often deriving from close kinship among those males as a result of male philopatry; PA = Polyandry: A mating system in which one female copulates with multiple males; PG = Polygynandry: A mating system in which members of both sexes copulate with multiple members of the opposite sex [120]; [2] Habitat: TFF—Terra firme forest: forest with soils composed of clay or loam [121]; FF—Flooded forests: Forests characterized by trees waterlogged or submerged during a large part of the year [122]. Data on NHPs were obtained from the following references: [123,124].

2.2. Terminology

2.2.1. Key Concepts

Blood pathogens can infect NHPs. However, a lot of terms have been identified across studies. This is why we propose the following concepts based on international guidelines. A disease is considered to be an abnormal condition in one part of the body or in the entire animal with clinical signs [125]. An infectious disease is caused by an agent that infects a host and can be transmitted to other hosts [126]. Blood-borne pathogens are viruses, bacteria and parasites found in the blood that can cause a disease.

2.2.2. Non-Invasive Samples and Detection Methods

The source of DNA in NHPs can be hairs [127,128], feces [129,130], buccal cells from swabs [131–133] or food wadges [134], urine [135] and blood [98,136]. Non-invasive genetic sampling was defined by Taberlet, et al. [137] as "*the source of the DNA left behind by the animal and that can be collected without having to catch or disturb the animal*". Non-invasive samples have been used in several studies of a wide range of vertebrates, such as birds [138–140], marine mammals [141–143], wolves [144,145], amphibians [146,147], reptiles [148], fish [149,150] and non-human primates. Non-invasive samples are known to have low quality and low quantity of DNA [151–153]. Samples such as pure blood have better results, but their collection is considered to be invasive. There are even cases where wild animals have died when trapped or manipulated for sampling. Therefore, the use of non-invasive samples can minimize disturbance to animals when collected correctly. However, sometimes non-invasive samples can disturb the ecology of animals. For example, in animals where their feces is used to mark their territory [154], collecting the whole feces can disturb the territory of the animal. In conservation biology, the use of non-invasive samples is of the utmost importance when it comes to threatened or endangered species such as gorillas [155], and in some cases is legally mandated.

Bacteria (n = 3), protozoa (n = 29) and viruses (n = 24) have been reported to infect the blood of neotropical NHPs (Tables 2–4). Studies in NHPs use invasive samples to detect blood pathogens. Most detection methods on protozoa focus on a combination between microscopy, polymerase chain reaction (PCR) and serological methods such as enzyme-linked immunosorbent assay (ELISA) (Table 3). However, for viruses, they focus primarily on a hemagglutination test (Table 4), which is considered a test for the presence of a humoral immune response of NHPs to an infectious agent such as viruses.

Table 2. Bacteria found in neotropical non-human primates.

Bacteria	Host	Location	Sampling (Invasive Non-invasive)	Detection Methods [1]	References
Borrelia burgdorferi	*Leontopithecus chrysomelas*	Brazil	Invasive	Nested PCR	[156]
Leptospira spp.	*Ateles fusciceps* *Ateles geoffroyi vellerosus* *Ateles geoffroyi yucatanensis* *Ateles hybridus* *Callithrix jacchus* *Callithrix pennicilata* *Cebus* sp. *Cebus albifrons* *Cebus capucinus* *Leontopithecus* sp. *Leontopithecus chrysomelas* *Saguinus leucopus* *Saimiri sciureus* *Sapajus apella*	Brazil Colombia French Guiana Mexico	Invasive	PCR MAT Serology	[157] [158] [159] [160] [161] [162] [163] [164] [165]
Mycoplasma spp.	*Alouatta* sp. *Alouatta caraya* *Saimiri sciureus* *Sapajus apella* *Sapajus flavius* *Sapajus nigritus* *Saguinus midas niger*	Brazil French Guiana	Invasive	TEM PCR	[166] [167] [168] [169] [170] [171] [172]

[1] TEM = Transmission electron microscopy; MAT = Modified agglutination test.

Table 3. Protozoa found in neotropical non-human primates.

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
Babesia sp.	*Alouatta seniculus* *Ateles paniscus* *Callithrix* sp. *Pithecia pithecia* *Saimiri sciureus*	French Guiana	Invasive	BS	[173] [68]
Leishmania sp. *Leishmania (Viannia)*	*Alouatta guariba* Atelidae (unknown species) *Aoutus azarai azarai* *Aotus nigriceps* *Callicebus nigrifrons* *Callithrix jacchus* *Callithrix penicillata* *Cebus macrocephalus* *Lagothrix cana* *Leontopithecus crysomelas* *Pithecia* sp. *Pithecia irrorata* *Saguinus imperator* *Saimiri ustus madeirae* *Sapajus apella* *Sapajus xanthosternos*	Argentina Brazil	Invasive	ELISA PCR IIF DAT PCR-RFLP	[174] [175] [91] [176] [177]
Leishmania amazonensis	*Ateles paniscus*	Brazil	Invasive	DNA PCR and RFLP	[178]

Table 3. *Cont.*

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
Leishmania braziliensis	*Saguinus geoffroyi*	Panama	Invasive	NA [1]	[179]
Leishmania chagasi *Leishmania infantum*	*Callicebus nigrifrons* *Callithrix jacchus*	Brazil	Invasive	PCR IHC DAT	[91] [180]
Leishmania mexicana	*Alouatta palliata* *Alouatta pigra*	Mexico	Invasive	ELISA IIF Western Blot	[181]
Leishmania (Viannia) shawi	*Chiropotes satanus* *Sapajus apella*	Brazil	Invasive	Monoclonal antibodies	[85]
Plasmodium sp.	*Alouatta seniculus*	Brazil	Invasive	Conventional microscopy (GIEMSA) PCR	[182]
Plasmodium vivax	*Alouatta caraya* *Alouatta guariba clamitans* *Alouatta seniculus macconnelli* *Sapajus apella*	Brazil French Guiana	Invasive	Microscopy IFA ELISA PCR Real-time PCR	[183] [184] [185] [186] [187]
Plasmodium brasilianum	*Alouatta* sp. *Alouatta seniculus* *Alouatta seniculus straminea* *Alouatta caraya* *Alouatta guariba clamitans* *Alouatta guariba guariba* *Aotus nigriceps* *Ateles* sp. *Ateles belzebuth* *Ateles chamek* *Ateles paniscus* *Aotus nigriceps* *Brachytheles arachnoides* *Cacajao calvus* *Cacajao rubicundus* *Callicebus bruneus* *Callicebus dubuis* *Callicebus moloch* *Callicebus personatus* *Callicebus torquatus* *Callithrix geoffroyi* *Cebus* sp. *Chiropotes albinasus* *Chiropotes chiropotes* *Chiropotus* sp. *Chiropotes satanas* *Lagothrix cana cana* *Lagothrix lagotricha lagotricha* *Lagothrix lagotricha poeppigii* *Leontopithecus chrysomelas* *Leontopithecus rosalia* *Mico humeralifer* *Pithecia monachus*	French Guiana Brazil Venezuela	Invasive	BS Conventional microscopy (GIEMSA) PCR ELISA	[188] [68] [189] [182] [190] [191] [192]

Table 3. *Cont.*

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
	Pithecia irrorata *Pithecia pithecia* *Saguinus martinsi martinsi* *Saguinus martinsi ochraceous* *Saguinus midas niger* *Saguinus midas* *Saimiri* sp. *Saimiri sciureus* *Saimiri sciureus sciureus* *Saimiri sciureus boliviensis* *Saimiri ustus* *Sapajus apella apella* *Sapajus apella macrocephalus* *Sapajus robustus* *Sapajus xanthosternos*				
Plasmodium simium	*Alouatta guariba clamitans* *Callicebus nigrifrons* *Cebus* sp. *Sapajus robustus* *Sapajus xanthosternos*	Brazil	Invasive Non-Invasive	BS PCR PCR from fecal samples Nested PCR	[193] [174] [183] [190] [9]
Plasmodium falciparum	*Alouatta caraya* *Alouatta guariba* *Alouatta puruensis* *Alouatta seniculus macconnelli* *Ateles chamek* *Callicebus bruneus* *Lagothrix cana cana* *Sapajus apella*	Brazil French Guiana	Invasive	ELISA IFA PCR	[188] [185]
Toxoplasma sp.	*Alouatta seniculus*		Invasive	DAT	[9]
Toxoplasma gondii	*Alouatta* sp. *Alouatta belzebul* *Alouatta caraya* *Alouatta seniculus* *Alouatta seniculus straminea* *Ateles* sp. *Ateles geoffroyi* *Ateles paniscus paniscus* *Aotus* sp. *Aotus nigriceps* *Brachyteles arachnoides* *Cebus* spp. *Cebus albifrons* *Cebus capucinus* *Callithrix* sp. *Callithrix penicillata* *Chiropotes satanas* *Erythrocebus* sp. *Leontopithecus* sp. *Leontopithecus chrysomelas* *Leontopithecus chrysopygus* *Leontopithecus rosalia* *Leontopithecus rosalia rosalia*	Argentina Brazil Colombia French Guiana Mexico	Invasive Necropsy	DAT IFAT MAT PCR Latex agglutination kit Indirect hemagglutination kit ELISA IHC PCR-RFLP Histology	[194] [195] [196] [197] [198] [199] [200] [201] [202] [203] [204] [205] [206] [207] [48] [208] [209] [210] [211] [212] [213] [214] [215] [76]

Table 3. *Cont.*

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
	Lagothrix lagotricha *Saguinus imperator* *Saguinus labiatus* *Saguinus oedipus* *Saimiri* sp. *Saimiri boliviensis* *Saimiri sciureus* *Saimiri sciureus sciureus* *Sapajus* spp. *Sapajus apella* *Sapajus libidinosus* *Sapajus flavius* *Pithecia pithecia pithecia*				[216] [217] [218]
Trypanosoma sp.	*Alouatta seniculus* *Ateles paniscus* *Pithecia pithecia* *Saguinus leucopus* *Saimiri sciureus*	Colombia French Guiana Panama	Invasive	BS	[68] [219] [220]
Trypanosoma cruzi	*Alouatta palliata* *Alouatta pigra* *Alouatta caraya* *Alouatta seniculus* *Ateles belzebuth* *Ateles geoffroyi* *Ateles fusciceps* *Aotus* sp. *Aotus azarai* *Aotus nigriceps* *Cacajao calvus* *Callicebus personatus* *Callicebus nigrifrons* *Callithrix geoffroyi* *Callithrix jacchus* *Callithrix penicillata* *Cebuella pygmaea* *Cebus albifrons* *Cebus capucinus* *Cheracebus torquatus* *Chiropotes satanas* *Leontopithecus chrysopygus* *Leontopithecus chrysomelas* *Leontopithecus rosalia* *Leontocebus fuscicollis* *Leontocebus fuscicollis weddelli* *Leontocebus nigricollis* *Mico chrysoleucus* *Mico argentatus* *Mico emiliae* *Pithecia irrorata* *Plecturocebus brunneus* *Saguinus niger* *Saguinus geoffroyi* *Saguinus bicolor bicolor* *Saguinus imperator imperator*	Argentina Brazil Mexico French Guiana Panama Peru	Invasive	ELISA IIF PCR BS IFA HC XD	[181] [221] [203] [222] [223] [179] [224] [225] [226] [227] [228] [229] [230] [231]

Table 3. *Cont.*

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
	Saguinus labiatus *Saguinus leucopus* *Saguinus midas* *Saguinus mystax* *Saguinus ustus* *Saimiri boliviensis* *Saimiri sciureus* *Saimiri ustus* *Sapajus libidinosus* *Sapajus robustus* *Sapajus xanthosternos*				
Trypanosoma devei	*Cebuella pygmaea* *Callimico goeldii* *Leontocebus fuscicollis weddelli* *Leontocebus tamarin tamarin* *Saguinus imperator imperator*	Brazil	Invasive	HC	[231] [232] [233]
Trypanosoma diasi	*Sapajus apella apella*	Brazil	Invasive	HC	[232]
Trypanosoma forestali	*Alouatta guariba* *Alouatta caraya*	Argentina Brazil	NA [1]	NA [1]	[223]
Trypanosoma hippicum	*Alouatta guariba* *Alouatta seniculus*	NA	NA [1]	NA [1]	[219]
Trypanosoma lambrechti	*Alouatta seniculus* *Cebus albifrons* *Cheracebus torquatus* *Chiropotes satanas* *Pithecia pithecia* *Sapajus apella*	Brazil	NA [1]	NA [1]	[219] [179] [223]
Trypanosoma lesourdi	*Ateles paniscus*	French Guiana	NA [1]	NA [1]	[223]
Trypanosoma mycetae	*Alouatta belzebul* *Alouatta belzebul belzebul* *Alouatta palliata* *Alouatta caraya* *Alouatta seniculus* *Chiropotes satanas*	Brazil Guatemala Panama French Guiana	Invasive	XD Direct observation of blood	[229] [233] [232] [223] [224] [219]
Trypanosoma minasense	*Alouatta belzebul* *Alouatta caraya* *Alouatta guariba* *Alouatta seniculus* *Aotus trivirgatus* *Ateles fusciceps* *Ateles geoffroyi griscescens* *Callithrix jacchus* *Callithrix penicillata* *Cebus albifrons* *Cebus capucinus* *Leontocebus weddelli* *Leontocebus fuscicollis weddelli* *Plecturocebus ornatus*	Argentina Brazil Colombia French Guiana Panama Peru	Invasive	PCR Stained films of peripheral blood	[232] [179] [203] [229] [234] [223] [98] [224] [231] [230] [235]

Table 3. *Cont.*

Protozoa	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods	References
	Saguinus geoffroyi *Saguinus imperator imperator* *Saguinus midas* *Saimiri sciureus* *Saimiri sciureus macrodon* *Saimiri ustus* *Sapajus apella*				
Trypanosoma rangeli (like)	*Alouatta seniculus* *Cebuella pygmaea* *Cebus albifrons unicolor* *Cebus capucinus* *Callimico goeldii* *Leontocebus fuscicollis weddelli* *Pithecia pithecia* *Saguinus bicolor* *Saimiri boliviensis* *Saimiri ustus* *Saimiri sciureus* *Saguinus geoffroyi* *Saguinus imperator imperator* *Saguinus midas* *Saimiri boliviensis* *Sapajus apella*	Brazil Colombia French Guiana Panama Peru	Invasive	BS	[236] [203] [237] [224] [230] [231] [229]
Trypanosoma saimiri	*Saimiri sciureus sciureus*	Brazil	Invasive	HC	[233]
Trypanosoma venezuelensis	*Alouatta guariba* *Alouatta seniculus*	NA [2]	NA [2]	NA [2]	[219]

[1] BS = Blood smears; ELISA = Enzyme-Linked Immunosorbent Assay; IIF = Indirect Immunofluorescence Assay; DAT = Direct Agglutination Test; MAT = Modified Agglutination Test; IFAT = Indirect Fluorescent Antibody Technique; IFA = Immunofluorescence Assay; IHC = Immunohistochemistry; XD = Hemoculture Xenodiagnosis; HC = Hemoculture. [2] N.A.: Not available.

Table 4. Viruses found in neotropical non-human primates.

Virus	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods [1]	References
Arbovirus (not specified)	*Alouatta caraya* *Sapajus* sp. *Sapajus apella*	Brazil	Invasive	HA	[238] [109]
Eastern equine encephalitis virus	*Ateles paniscus chamek* *Sapajus libidinosus*	Bolivia Brazil	Invasive	Antibody titers (IgG and IgM antibodies) HI	[239] [240]
Western equine encephalitis virus	*Cebus libidinosus*	Brazil	Invasive	HI	[240]
Alphavirus (not specified)	*Sapajus apella*	Brazil	Invasive	HA	[238]

Table 4. *Cont.*

Virus	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods [1]	References
Mayaro virus	*Alouatta villosa* *Alouatta seniculus* *Callithrix argentata* *Pithecia pithecia* *Saguinus midas* *Sapajus apella* *Sapajus libidinosus*	Brazil Panama French Guiana	Invasive	HA Serologic (PRN antibodies) HI	[238] [203] [241] [240] [242] [243]
Una virus	*Alouatta caraya*	Argentina Paraguay	Invasive	NTAb survey	[244]
Venezuelan equine encephalitis virus	*Sapajus apella*	Colombia	Invasive	NTAb survey	[245]
Mucambo virus	*Sapajus libidinosus*	Brazil	Invasive	HI	[240]
Flavivirus (not specified)	*Leontopithecus chrysomelas* *Sapajus apella* *Sapajus xanthosternos*	Brazil	Invasive	HA HI	[238] [246]
West Nile virus	*Alouatta caraya*	Argentina	Invasive	RT-nested PCR	[247]
Yellow fever virus	*Alouatta* sp. *Alouatta caraya* *Alouatta clamitans* *Alouatta guariba clamitans* *Alouatta fusca* *Alouatta macconnellii* *Alouatta seniculus* *Ateles paniscus chamek* *Callicebus* sp. *Callithrix* sp. *Cebus* sp. *Leontopithecus* sp. *Leontopithecus chrysomelas* *Pithecia pithecia* *Saguinus midas* *Sapajus* sp. *Sapajus libidinosus*	Argentina Bolivia Brazil Colombia French Guiana Panama Trinidad Venezuela	Invasive Necropsy	Immunoperoxidase staining method HI ELISA IgM / inoculated in mice (Isolation by RT-PCR) IHC RT-qPCR RT-PCR Serologic (PRN antibodies) Antibody titers (IgG and IgM antibodies) Isolation in cell cultures (*Aedes albopictus* C3/36), followed by DFA, RT-PCR generic for Flavivirus, and genome sequencing IFAT IIF using monoclonal antibodies	[248] [249] [250] [251] [246] [252] [203] [105] [239] [240] [253] [254] [255] [256] [257] [258] [242] [259] [260]
Ilheus virus	*Alouatta caraya* *Callithrix jacchus* *Callithrix penicillata* *Leontopithecus chrysomelas* *Sapajus libidinosus*	Argentina Brazil	Invasive	HI NT RT-nested PCR	[261] [246] [240] [247] [262]

Table 4. *Cont.*

Virus	Host	Location	Sampling (Invasive Non-Invasive)	Detection Methods [1]	References
Saint Louis encephalitis virus	*Alouatta caraya* *Alouatta seniculus* *Ateles paniscus chamek* *Leontopithecus chrysomelas* *Pithecia pithecia* *Saguinus midas* *Sapajus nigritus* *Sapajus cay* *Sapajus libidinosus*	Brazil Argentina French Guiana	Invasive	HI NT MNT RT-nested PCR	[261] [246] [240] [239] [203] [247] [263]
Rocio virus	*Leontopithecus chrysomelas* *Sapajus libidinosus* *Sapajus xanthosternos*	Brazil	Invasive	HI	[246] [240]
Zika virus	*Callithrix* sp. *Leontopithecus chrysomelas* *Sapajus* sp.	Brazil	Invasive	HI	[246] [264]
Dengue virus	*Alouatta caraya*	Argentina	Invasive	RT-nested PCR	[247]
Bussuquara virus	*Alouatta caraya* *Leontopithecus chrysomelas* *Leontopithecus chrysopigus* *Saguinus bicolor*	Argentina Brazil	Invasive	RT-nested PCR NT HI	[246] [247] [265]
Cacicapore virus	*Leontopithecus chrysomelas*	Brazil	Invasive	HI	[246]
Orthobunyavirus	*Leontopithecus chrysomelas* *Sapajus apella*	Brazil	Invasive	HI	[246] [266]
Oropouche orthobunyavirus	*Alouatta caraya* *Callithrix* sp. *Sapajus apella* *Sapajus libidinosus*	Brazil	Invasive	HA HI Neutralization assays CF confirmed by RT-PCR	[238] [267] [240] [268]
Apeu virus	*Alouatta caraya* *Sapajus apella*	Brazil	Invasive	PRN T70	[269]
Tacaiuma orthobunyavirus	*Leontopithecus chrysomelas*	Brazil	Invasive	HI Real time PCR	[246]
Phlebovirus	*Leontopithecus chrysomelas*	Brazil	Invasive	HI	[246]
Icoaraci phlebovirus	*Alouatta caraya* *Leontopithecus chrysomelas*	Brazil	Invasive	HI NT	[246,261]

[1] HA = Hemagglutination test; HI = Hemagglutination inhibition test; NATb = Neutralizing antibody (NTAb) survey; BS = Blood smears; ELISA = Enzyme-Linked Immunosorbent Assay; IHC = Immunohistochemistry; PRN = Plaque-reduction neutralizing; DFA = Direct Immunofluorescence Assay; IFAT = Indirect Fluorescent Antibody Technique; IIF = Indirect Immunofluorescence Assay; NT = Neutralization test; MNT = Mouse neutralization test; CF = Complement fixation test.

2.3. Risk Factors Associated with the Transmission of Disease

There are several risk factors that favor the transmission of diseases such as socioecological and ecological factors (Table 5) [270,271].

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Table 5. Risk factors associated with the transmission of diseases in neotropical non-human primates.

Risk Factors	Factors	Examples
Socioecological factors	Animal behavior and social organization	Group size Movement between groups Sexual selection number of mating partners Type of contact and contact rate
	Sleeping site ecology	Host age Habitat
	Migration	Animal Humans
Ecological factors	Host density	High vs. low
	Climate change	Habitat shifts Host switching
	Human activities	Primate behavior Agricultural practices Land-use changes
	Others	Malnutrition Pollution

2.3.1. Socioecological Factors

- Animal behavior and social organization

Non-human primates are social, and as social animals they are at risk of infectious or parasitic diseases [272]. Factors such as group size, movement between groups, and sexual selection (number of mating partners) are among the variables of host–parasite interactions in NHPs that are considered to be drivers of parasite transmission [273]. Regarding group size, this variable is a risk factor for some infectious and parasitic diseases. In Amazonian primates, a larger group size will attract more mosquitos, and a higher risk of malaria infection was observed [274,275]. However, this is not true for other vectors [276]. There are strategies such as fission where subgrouping can act as a dilution effect for vectors [277]. The type of contact such as grooming [278] and the contact rate might also influence parasite transmission [279]. For example, lice transferred to other lemurs across several seasons [280] may increase the risk of infection of parasitic diseases [281,282]. Host age may also have an influence on the prevalence of hemoparasites in non-human primates [283]. For *Plasmodium*, the innate immune system plays a role in protecting young non-human primates from it and the parasite can benefit from an immune system weakened by age [284]. In Springer, et al. [285], *Plasmodium* sp. were more likely to infect older individuals of Verreaux's sifakas (*Propithecus verreauxi*), whereas *Babesia* sp. infected the younger ones.

- Sleeping site ecology

Sleeping behavior has been described as a risk factor for parasite transmission. It has been suggested that Amazonian NHPs sleeping in microhabitats are less likely to be infected with malaria [275]. In Milne-Edwards' sportive lemur (*Lepilemur edwardsi*), they are at greater risk of infection of ectoparasites and thus hemoparasites as well because they sleep in tree holes [286]. On the contrary, chimps (*Pan troglodytes schweinfurthii*) build their sleeping sites in *Cynometra alexandri* trees, which are known for having insect-repellent properties [287], decreasing the risk of infection.

- Migration

The OIE has not declared the presence of the West Nile virus in South America; however, three horses have died in Argentina and they were diagnosed with the WNV close to a North American cluster [288]. It has been suggested that wild bird migration could spread the virus to South America [289–291]. This scenario is not only valid for animals [292], but for humans as well [293]. The origins of *Plasmodium falciparum* and *Plasmodium vivax* in Central and South America are related to the migration of enslaved Africans and Australasian people, respectively [294].

2.3.2. Ecological Factors

- Host density

Host density is another risk factor that can increase parasite transmission [295,296]. However, lower densities such as those from orangutans (*Pongo pygmaeus*) (around two individuals per km^2) can harbor as many as two species of malaria [297].

- Climate change

Climate change influences the emergence of infectious and parasitic diseases in several types of environments [298,299]. Several studies have described potential scenarios with models of climate change to describe distribution patterns of hosts and their pathogens and/or the vector [300,301]. As for the impact of climate change on NHPs, some studies have described habitat shifts at the altitudinal gradient [302] or at the latitudinal gradient [303]. Nunn, et al. [304] published a study on the latitudinal gradient of parasite species richness, which can give us an idea of how this distribution can impact NHPs if these ones change their distribution. In the neotropics, according to the modeling of the IPCC [305], some forests will shift to savannah woodlands, where this process could influence the host–parasite interaction [306,307]. The density and diversity of pathogens might be different in these savannas than in the forest [308,309]. Climate change could lead to host switching in NHPs [310] and an increase in the distribution of vectors [311–315]. For example, in avian malarias, host specificity was found in regions with pronounced rainfall seasonality [316]. However, pathogens can also adapt to new temperatures [317]. Finally, climate change can indirectly influence the behavior of primates, which can influence how pathogens can spread across populations. For example, climate change has a direct influence on the phenology of plants (e.g., fruiting, flowering) [318], and as a consequence, groups of NHPs forage differently for food [319–321], whether they fission into subgroups or whether they increase their home range for more food [322]. The implications of these changes are reflected on the host and might change the host–parasite relationship. According to HobergandBrooks [323], the primary sources of emerging infectious diseases will be those that are going to be able to survive climate change.

2.3.3. Human Activities

Human activities such as agricultural practices [324] and land-use changes (e.g., deforestation) [325,326] can increase the risk of parasite transmission [327]. For example, in Malaysian Borneo, macaque hosts and mosquito vectors are having more contact with humans due to these human activities [325]. In South America, there are also NHPs that survive in human-disturbed environments; thus, they can maintain the sylvatic cycle close to humans [69].

2.3.4. Others

Malnutrition [328,329] due to poor habitats (e.g., fragmentation) [307] and toxic chemicals and pollution are other factors that also increase parasite transmission [328].

2.4. Surveillance Networks

2.4.1. World Organization for Animal Health (OIE)

The animal health situation is monitored in each country and each country is responsible to declare to the OIE (World Organization for Animal Health). However, there are no notifications on NHPs for the Neotropical region or elsewhere. However, there are publications of some diseases such as yellow fever present in non-human primates [330]. For other diseases present in the OIE portal, the Ebola virus disease, for example, is not listed; however, they recommend it to be voluntarily reported [331,332]. In addition, the OIE have a guideline and a training manual on wildlife disease surveillance [333,334], which could be applied as guidelines in Ecuador and other neotropical countries.

2.4.2. International Organizations from the United Nations System and Wildlife Monitoring

The World Health Organization (WHO) work in collaboration with the FAO (Food and Agriculture Organization of the United Nations) and OIE to deal with zoonotic diseases. However, they also work with local governments, academia as well as non-governmental organizations (NGOs). The FAO has several programs such as Vmergem, PAATS and LinkTads that have as objectives to help and to develop technical capacities for local governments.

2.4.3. Local Networks

The Ministry of Environment in Ecuador does not have a program on wildlife disease monitoring but it has workshops on wildlife health [335]. The National Institute of Research on Public Health (INSPI) has a program on parasites and infectious diseases, and they make guidelines for zoonotic wildlife diseases and wildlife groups such as NHPs. As for NGOs and management plans, there are none working on specific wildlife disease surveillance in Ecuador. However, the IUCN (International Union for Conservation of Nature) have international guidelines for each taxonomic group and their diseases, which can be applied by specialists all over the world [336–338]. Brazil is the only neotropical country with a guideline and a manual on epizootics in NHPs [339,340]. It is no coincidence that it is the country with the highest number of studies on neotropical NHP diseases (Tables 2–4).

3. Discussion

Diseases in NHPs are of conservation and medical importance because they may threaten both NHP populations [105,252] and humans [341,342]. That is why monitoring and long-term surveillance in NHPs [343,344] can enhance the knowledge of diseases and the risks associated with them. However, we should pay attention to the choice of methods to detect NHP diseases. For example, for neotropical NHPs, just one study used a non-invasive method to monitor protozoa [9] (Tables 1–3). Invasive techniques such as serological tests, blood smears, and tissues are used to detect arthropod-borne diseases and blood pathogen diseases [345]. Instead, you can use fecal [8,346,347], urine [348] or saliva [349,350] samples to monitor viruses, bacteria and other blood pathogens and obtain as much information as the other techniques as long as you only need to have an idea of the prevalence and the presence of the disease. Once you have a general idea of the current situation, in order to characterize the disease, you can move forward to an invasive technique but with fewer samples.

It is important to use non-invasive samples in wildlife studies since there are studies that determined the diagnostic sensitivity of molecular tests for the study of blood-borne pathogens, and obtained data close to invasive samples [351]. For example, for *Plasmodium falciparum*, a study determined by PCR the limit of detection at 6.5 parasites/µL in fecal samples from NHPs from the Brazilian Amazon [9]. In human blood samples, the limit of detection of *Plasmodium falciparum* ranges from 0.03 parasites/µL to 9 parasites/ml using methods such as qPCR [352] and RT-PCR [353]. The sensitivity of parasite DNA extraction for both stool and blood samples will depend on sample storage [354], DNA extraction methods [355] and parasite densities in the population and in individuals [356,357]. Studies aim to improve molecular techniques to increase the sensitivity of these techniques in the diagnosis of pathogens [352,353,358,359].

Socioecological and ecological risk factors are associated with the transmission of blood-borne pathogens in NHPs. Factors such as human activities and climate change are identified as factors in the emergence of infectious diseases [360]. However, vectors must be considered to evaluate the transmission of these pathogens. For example, vector density and longevity would also increase the transmission rate of these pathogens [361]. Studies have even identified the feeding preferences of vectors and their connection to disease transmission [362,363]. Another study found an effect between habitat fragmentation and the infection rate of vectors with *Plasmodium* sp. [364].

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Methods of surveillance should be adapted to wildlife populations. In captive settings, monitoring is easier than in wild populations. Additionally, the risk of infection can change whether they are captive or wild. Captive settings are an environment under control most of the time (depending on the captive conditions in neotropical countries), while monitoring free-ranging populations can be difficult for several reasons (poaching or legal hunting for meat, illegal pet trade, among others). However, long-term studies on NHPs may help to mitigate the effect of hunting [365]. NHPs from captive settings are most of the time from unknown origin [366], which makes it more difficult to know the biohazard threat involved. Sometimes the quarantine period is not respected, and diagnostic tests are not performed (either because they do not have the budget or because they are not aware of them), increasing the risk of infections. In addition to these conditions, the contact rate with humans such as care takers and tourists can introduce human pathogens to those populations (reverse zoonoses) [367], increasing the chances that an NHP can be infected. It is not unusual to see on social media, even during a pandemic, rescue center personnel or tourists taking pictures of themselves with primates without adequate biosecurity measures. In the other direction, pathogens can be transmitted to humans through primate biting (contact with body fluids) or scratches [368]. Cases of monkey bites in Ecuador are not unusual; however, local health services do not follow strict protocols such as taking samples from the patient and the monkey for further analysis or applying prophylaxis treatments against NHP bacteria or rabies.

In order to reduce the risks associated with the diseases, local governments should implement control measures adapted to NHPs. There are high risk activities such as NHP translocations [369] (from one geographical region to another or from one captive setting to another), reintroductions [370], among others, that can be a health risk for local populations of NHPs and humans. The success of these high-risk activities depends not only on NHP health but also on NHP socioecology, the support from local communities and the presence of environmental education programs [371]. NHP local populations and translocated groups should be monitored constantly. The costs of these activities are really high and losing individuals would be a step backwards. If the risk is too high, maybe the budget associated with this activity should be implemented in other types of conservation programs that could help primate populations more than the same translocation or reintroduction.

4. Materials and Methods

This systematic review was carried out using PRISMA guidelines for reporting systematic reviews and meta-analyses [372,373] and to identify bibliographic research from 1927 until 2019 about blood parasites, hemoparasites and arboviruses present in neotropical non-human primates. In several databases, we used the following search string (keywords and Boolean operators) “blood and parasites and primates”, “Hemoparasites and Primates”, “Haemoparasites and Primates”, “Arbovirus and Primates” or “Parasites and Primates”. The databases that we used were Scopus, Google Scholar and Pubmed. We also included grey literature such as theses and abstract presentations (Figure 1). Once the results were obtained, we made a selection by eliminating studies according to the following criteria: (1) the parasite was not a hemoparasite, (2) the published studies were in a language that the authors do not understand, (3) the study was not from a neotropical non-human primate, and finally (4) duplicate studies. We included all articles that clearly indicated the name of the parasite and the species of the host. We also included studies in captive and wild habitats.

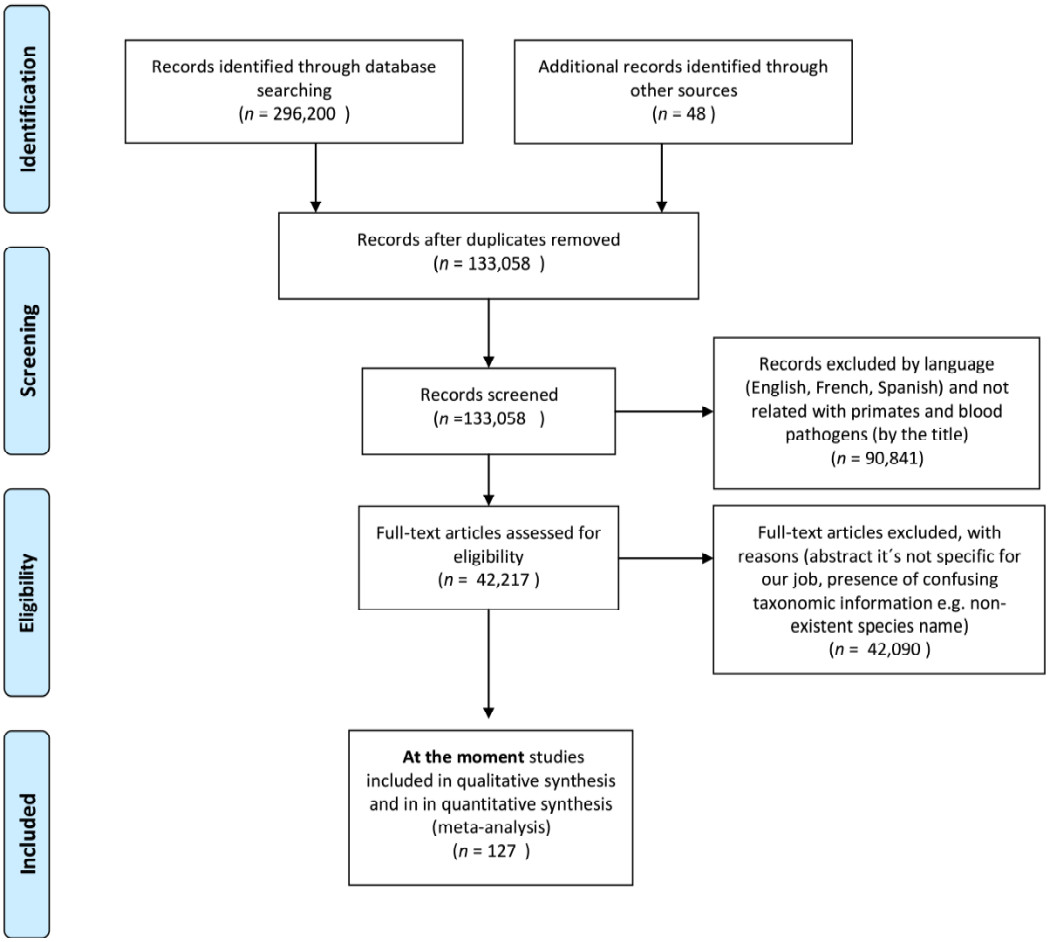

Figure 1. Flowchart (modified from PRISMA 2009) describing the literature search and study selection.

5. Conclusions

In this study, we found that NHPs are reservoirs for a large number of blood-borne pathogens. In addition, socioecological and ecological risk factors facilitate the transmission of these blood-borne pathogens either between NHPs or between NHPs and humans. The genus *Alouatta* is the one that records the highest number of blood-borne pathogens. This genus has the widest range of distribution from Mexico to Argentina. However, bacterial and viral pathogen groups have not been studied in depth in South America and especially in Ecuador, so these data will allow decision makers to decide where to focus their research efforts.

The Ministries of Health and Environment should prioritize the implementation of infection prevention and control measures in countries with a high risk of disease transmission. The Ministry of Environment should have a protocol to protect workers who are exposed to zoonotic diseases, for example, park rangers and zoo care takers, but also ecotourism. Ecotourism is considered a vulnerable group but also a group that exposes NHPs to infections [374,375]. A guideline should establish measures to prevent the introduction and spread of infection among NHP and human populations [376]. Some measures include reducing the frequency and duration of field visits as well as the number of visitors. Another biosecurity measure is to increase the viewing distance to NHPs [125,377]. Additionally, we should consider surveillance in national programs [378] as a tool for public

health [333] and NHP conservation [3,338,379,380]. Finally, there are a large number of diseases that are under-surveyed. A large number of studies support surveillance programs as they improve the early detection of diseases [381–384]. These surveillance programs must have regular and effective monitoring protocols adapted to non-human primates. In order to implement these control programs, Ministries of Environment, Universities, and Health and wildlife researchers must collaborate with each other to determine monitoring strategies and to identify priority diseases for the country.

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Experimental section

Study 2:

Molecular identification of *Plasmodium falciparum* from captive Non-Human Primates in the Western Amazon Ecuador

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Preamble

Malaria is a disease caused by the pathogen of the genus *Plasmodium*. This disease is found in humans and other vertebrates among which are the NHPs. This study sought to identify *Plasmodium* is NHP species in the Ecuadorian Amazon.

A total of 26 faecal DNA samples were analysed from captive and free-ranging NHP, in the Western Amazon region of Ecuador. We were able to identify *Plasmodium falciparum* in a sample from a female of the species *Leontocebus lagonotus*

This is the first report at national level of *Plasmodium falciparum* using non-invasive samples to detect bloodborne pathogens. Additionally, this study allowed us to update the protocols of collection, storage and transport of the samples obtained at national level.

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Article

Molecular Identification of *Plasmodium falciparum* from Captive Non-Human Primates in the Western Amazon Ecuador

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Abstract: Background: Malaria is a disease caused by hemoparasites of the *Plasmodium* genus. Non-human primates (NHP) are hosts of *Plasmodium* sp. around the world. Several studies have demonstrated that *Plasmodium* sp. emerged from Africa. However, little information is currently available about *Plasmodium falciparum* in the neotropical NHP and even less in Ecuador. Indeed, the objective of our study was to identify by molecular phylogenetic analyses the *Plasmodium* species associated with NHP from the Western Amazon region of Ecuador, and to design a molecular taxonomy protocol to use in the NHP disease ecology. **Methods:** We extracted DNA from faecal samples (n = 26) from nine species of captive (n = 19) and free-ranging (n = 7) NHP, collected from 2011 to 2019 in the Western Amazon region of Ecuador. **Results:** Using a pan-Plasmodium PCR, we obtained one positive sample from an adult female *Leontocebus lagonotus*. A maximum likelihood phylogenetic analysis showed that this sequence unequivocally clustered with *Plasmodium falciparum*. **Conclusions:** The identification of *Plasmodium* sp. in NHP of the Ecuadorian Amazon would be essential to identify their role as potential zoonotic reservoirs, and it is also important to identify their origin in wildlife and their transmission in captive NHP.

Keywords: *Leontocebus lagonotus*; malaria; faecal samples

1. Introduction

Malaria is a disease transmitted by mosquitoes and caused by a hemoparasite of the genus Plasmodium. The protozoan *Plasmodium* spp. belongs to the Apicomplexa groups; they have the ability to parasite cells thanks to a group of organelles called the apical complex. *Plasmodium* inhabits red blood cells and hepatocytes [1]. It causes high fever, anemia, headaches, and diarrhea, among others. This mosquito-borne disease is found in humans and in several vertebrates such as birds [2,3], bats [4], antelopes and reptiles [5], and non-human primates (NHP) [6–9]. The latter are an important source of infections for *Plasmodium* sp. in humans. In OW (Old World) monkeys, *Plasmodium* sp. has been detected in chimpanzees (*Pan troglodytes*) [10], baboons (*Papio anubis*), Tantalus monkeys (*Chlorocebus aethiops*), red patas monkeys (*Erythrocebus patas*) [6],

long-tailed and pig-tailed macaques (*Macaca fascicularis* and *Macaca nemestrina*) [7], and recently in a lemur species (*Propithecus verreauxi)* and *Macaca radiata* [11], to name a few. In the neotropics, there is evidence of natural infection in humans with *Plasmodium brasilianum* in Venezuela [12] and *Plasmodium simium* in Brazil [13]. These two species of Plasmodium naturally infect NHP. *Plasmodium brasilianum* has been identified in at least 49 species of New World (NW) NHP [14–20] (Table 1). *Plasmodium simium* has been found in less species, around 4 NW NHP species [18,21–23]. There are 25 known species of Plasmodium and some of those (*P. vivax*, *P. malariae*, *P. falciparum*, *P. ovale*, *P. knowlesi*) are responsible for human malaria [24–26]. *Plasmodium falciparum* is one of the most malignant species of malaria and it originated from human migration into the NW [10,27,28].

Several genes are used for molecular identification and phylogenetic studies of *Plasmodium* species [29,30]. However, the small subunit ribosomal RNA gene is widely used for molecular characterization and phylogenetic studies [31–33]. Indeed, it has both highly conserved and very variable domains. This gene was used to study the phylogenetic relationships [34–36] and host specificity of *Plasmodium* sp. [19]. More than 49 species of NW monkeys are known to be infected with *Plasmodium* sp. [37,38]. In Ecuador, only a few studies on *Plasmodium* sp. were realized [39]. Avian Plasmodia were studied in the Galapagos [40,41]. In humans, several studies with molecular markers have identified population origins of *P. falciparum* in the Northwest of Ecuador [42,43], but only one study yielded sequence information [44]. The aim of this study was to monitor *Plasmodium* sp. in the Amazon region of Ecuador, to identify potential zoonotic reservoirs, and to identify the origins of malaria parasites in wildlife and potential human–monkey transmission with captive NHP.

Table 1. *Plasmodium* species found in neotropical non-human primates.

Host	Location	Plasmodium Species	Sampling (Invasive Non-Invasive)	Detection Methods	References
Alouatta seniculus.	Brazil	*Plasmodium* sp.	Invasive	Conventional microscopy (GIEMSA) PCR	[17]
Alouatta caraya *Alouatta guariba clamitans* *Alouatta seniculus macconnelli* *Sapajus apella*	Brazil French Guiana	*Plasmodium vivax*	Invasive	Microscopy Enzyme-linked Immunosorbent assay IFA ELISA PCR Real-time PCR	[22,45,46]

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Table 1. *Cont.*

Host	Location	Plasmodium Species	Sampling (Invasive Non-invasive)	Detection Methods	References
Alouatta sp. *Alouatta seniculus* *Alouatta seniculus straminea* *Alouatta caraya* *Alouatta guariba clamitans* *Alouatta guariba guariba* *Aotus nigriceps* *Alouatta g. clamitans* *Ateles* sp. *Ateles belzebuth* *Ateles chamek* *Ateles paniscus* *Aotus nigriceps* *Bracytheles arachnoides* *Cacajao calvus* *Cacajao rubicundus* *Callicebus bruneus* *Callicebus dubuis* *Callicebus moloch* *Callicebus personatus* *Callicebus torquatus* *Callithrix geoffroyi* *Cebus* sp. *Chiropotes albinasus* *Chiropotes chiropotes* *Chiropotus* sp. *Chiropotes satanas* *Lagothrix cana cana* *Lagothrix lagotricha lagotricha* *Lagothrix lagotricha poeppigii* *Leontopithecus chrysomelas* *Leontopithecus rosalia* *Mico humeralifer* *Pithecia monachus* *Pithecia irrorata* *Pithecia pithecia* *Saguinus martinsi martinsi* *Saguinus martinsi ochraceous* *Saguinus midas niger* *Saguinus midas* *Saimiri* sp. *Saimiri sciureus* *Saimiri sciureus sciureus* *Saimiri sciureus boliviensis* *Saimiri ustus* *Sapajus apella apella* *Sapajus apella macrocephalus* *Sapajus robustus* *Sapajus xanthosternos*	French Guyana Brazil Venezuela	*Plasmodium brasilianum*	Invasive	Blood smears Conventional microscopy (GIEMSA) PCR ELISA	[14–20]
Alouatta guariba clamitans *Cebus* sp. *Sapajus robustus* *Sapajus xanthosternos*	Brazil	*Plasmodium simium*	Invasive Non-Invasive	Blood smears PCR PCR from faecal samples Nested-PCR	[18,21–23]
Alouatta caraya *Alouatta guariba* *Alouatta puruensis* *Alouatta seniculus macconnelli* *Ateles chamek* *Callicebus bruneus* *Lagothrix cana cana* *Sapajus apella*	Brazil French Guyana	*Plasmodium falciparum*	Invasive	ELISA IFA PCR	[14,45]

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2. Results

A total of 26 faecal DNA samples were analysed from captive and free-ranging NHP, in the Western Amazon region of Ecuador. After DNA extraction and using a pan-*Plasmodium* PCR, one positive sample was obtained from an adult female *Leontocebus lagonotus* (representing 3.85%). This animal came from a rescue centre in Pastaza. As with the other rescue centres in Ecuador, most NHP from this rescue centre have been donated by families or confiscated by the police during roadside checks, with this individual's information being uncertain.

We sequenced the amplicon and then aligned it with sequences from other *Plasmodium sp.* species available in the GenBank. The maximum-likelihood phylogeny (ML) tree (Figure 1) yielded a topology with the different *Plasmodium* species clades and an internal (derivated) monophyletic clade comprised by *P. falciparum* including the sequence obtained from the *Leontocebus lagonotus* plus *P. reichenowi* sequences and *P. gaboni*. The ML recovered a clear reciprocal monophyly between *falciparum* and *reichenowi* as sister groups both with a strong support group by bootstrapping values of 74%. The arrangements of other sister clades were recovered: *P. simium* + *P. vivax*; *P. inui* + *(P. fragile* + *P. knowlesi)*; *P. ovale* + (*P. brasilianum* + *P. malariae*). All sister groups were strongly supported by bootstrapping values of above 96%. In the analysis performed, the sequence of the amplified *Plasmodium* from the NHP *L. lagonotus*' faeces is located within the *P. falciparum* clade, supporting its molecular identification with this species in the phylogenetic species sense.

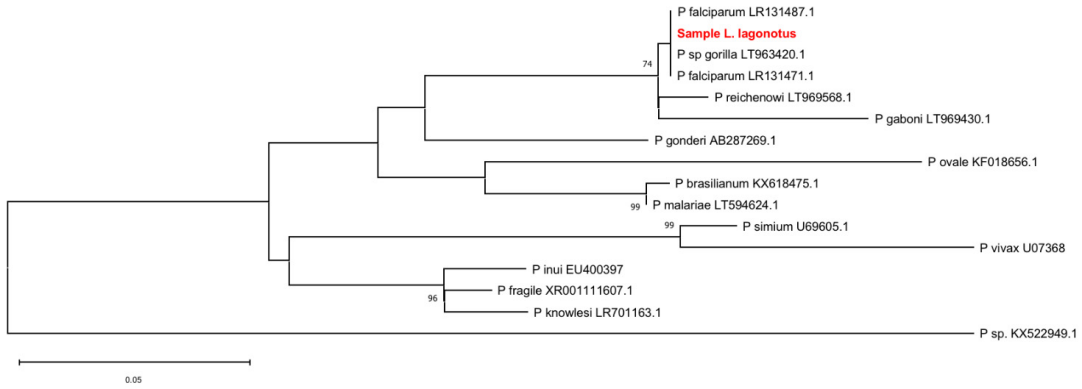

Figure 1. Evolutionary relationships of the *Leontocebus lagonotus* isolate described in this study (bolded and highlighted in red) compared to representative members of the Plasmodium genus. The tree is based on the maximum-likelihood phylogeny of the partial small subunit ribosomal RNA gene. The phylogenetic analysis was performed using the Tamura 3-parameter substitution model implemented in MEGA X. Bootstrap percentages > 70% (1000 resamplings) are indicated at the nodes. GenBank accession numbers are indicated for each strain. The scale bar indicates nucleotide substitutions per site.

3. Discussion

DNA from only 26 of the 109 faecal samples collected from 109 NHP was analysed because the quality of the DNA from the other 83 samples was insufficient for the detection of *Plasmodium*, possibly due to the presence of some inhibitors that prevent the amplification of DNA. We detected *Plasmodium falciparum* in a faecal sample from *Leontocebus lagonotus* (3.85%), contributing to the parasite ecology of NW NHP. The percentage of detection is not high, but it is in line with previous published results in scientific literature in Latin America (e.g., [23]).

Malaria parasites display host specificity [47], such as avian malaria in birds [48,49], and malaria in apes [50]. However, it is estimated that the diversification of *Plasmodium* was rapid 16–24 million years ago (MYA) or 26–38 MYA, which differs from the divergence times of the hosts (reptiles to mammals) 75–310 MYA. This difference in the divergence

times between parasites and hosts suggests that there has been no co-divergence with the hosts [51]. In primates, two groups of *Plasmodium* have been identified. The first includes the species *P. malariae/P. ovale/P. hylobatid*, which infect Old World primates. The second group includes the species *P. falciparum/P. reichenowi*, which infect humans and NHP. In NW monkeys, *P. brasilianum* and *Plasmodium simium* are known to be the cause of primate malaria. *Plasmodium falciparum* is known to be a cause of human malaria, a result of a recent cross-species transmission of a parasite between gorillas and humans [52]. However, *P. falciparum* is also known to naturally infect at least eight species of NW NHP [14,45]. Our sample is located in the clade of *P. falciparum*, and close to *P. recheinowi*. This relationship was already observed in previous studies [51,53,54]. It has been reported that *Plasmodium falciparum* and *P. reichenowi* are part of a monophyletic clade [55].

Non-human primates in captivity from this study are in close contact with care takers and tourists, which may increase the possibility of parasite transmission [56]. However, the origin of these captive animals is unknown. This assumption makes us wonder whether the infection occurred in a sylvatic environment or whether it occurred during the captive period of this NHP. If it occurred during the sylvatic environment, the transmission could be the result of a natural infection via mosquitoes. *Leontocebus lagonotus* is known to be widely distributed in Western Amazonia in Ecuador, i.e., in Pastaza and Morona Santiago provinces [57], which according to the Center for Disease Control and Prevention (CDC) [58], are areas of malaria (*Plasmodium vivax* and *Plasmodium falciparum*). In 2019, Pastaza and Morona Santiago where the two provinces with the highest number of malaria (*Plasmodium falciparum* and *Plasmodium vivax*) cases in humans [59]. If it occurred during the captive period, further studies should be carried out to determine if the vector responsible for the human–NHP transmission is present in this region because there are no reports of the vector in the area.

4. Materials and Methods

4.1. Study Sites and Sampling

This study was performed in Puyo (Pastaza), Tena (Napo), and Macas (Morona Santiago), three cities in the Western Amazon region (Figure 2). We examined *Plasmodium* spp. in two populations, one captive and one free-ranging. The captive population was studied in wildlife refuges. Most NHP from wildlife refuges had been donated by families or confiscated by the police during roadside checks. The free-ranging population lived in the small town of Misahualli (Tena, Napo) (1°2′7.0″ S, 77°39′59.4″ W) (Figure 3).

Faecal samples are an important source of information about pathogens (viruses, prokaryotes, or eukaryotes) that infect primate species. The analysis of molecular faecal samples offers a non-invasive option that becomes a valid alternative to the traditional sampling methods (blood and tissue samples) of primates [60–77]. Studies in conservation genetics have used faecal samples for DNA extraction prioritizing species that are in some category of protection or threat [78,79]. In this study, we collected 109 faecal samples from 109 NHP. However, the DNA quality was not good enough on 83 samples to detect *Plasmodium*. Indeed, we analysed faecal samples (n = 26) from nine species of captive (n = 19) and free-ranging (n = 7) NHPs (Table 2), between 2011 and 2019, in the Western Amazon region of Ecuador from Proyecto Primates Ecuador. Individuals were followed daily from 8 a.m. to 6 p.m. and we collected the samples immediately after defecation to avoid getting confused with samples from other species. All animals were individually identified to facilitate the results analysis. For the molecular analyses, samples were stored in 50 mL Falcon tubes in 99% alcohol at −20 °C to prevent the degradation of DNA. In addition, 600 µL of faeces suspension (1:3; 1 part of faecal sample and 3 parts of ethanol 96–100%) was centrifuged for 2 min at 239 g and the pellet was washed with 1 mL of PBS Buffer (Oxoid, Hampshire, England). This solution (pellet +PBS) was centrifugated for 5 min and the supernatant was discarded. This washing step was repeated three times. Next, the pellet was re-suspended in 600 µL of 2% PVPP (polyvinylpolypyrolidone—Sigma), and frozen overnight at −20 °C to facilitate the capture of phenols in the sample.

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DNA extraction was performed twice on different days using the QIAamp Stool FAST Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. To prevent cross-contamination, sample preparation, DNA extraction, and the PCR were performed in completely different and separated rooms. Furthermore, the master mix was assembled in a DNA-free room. Ultraviolet light sterilization was performed before and after each procedure. The PCR was carried out using primers targeting the small subunit of 18S ribosomal RNA. The primers used were described by dos Santos et al. [80] (Table 3).

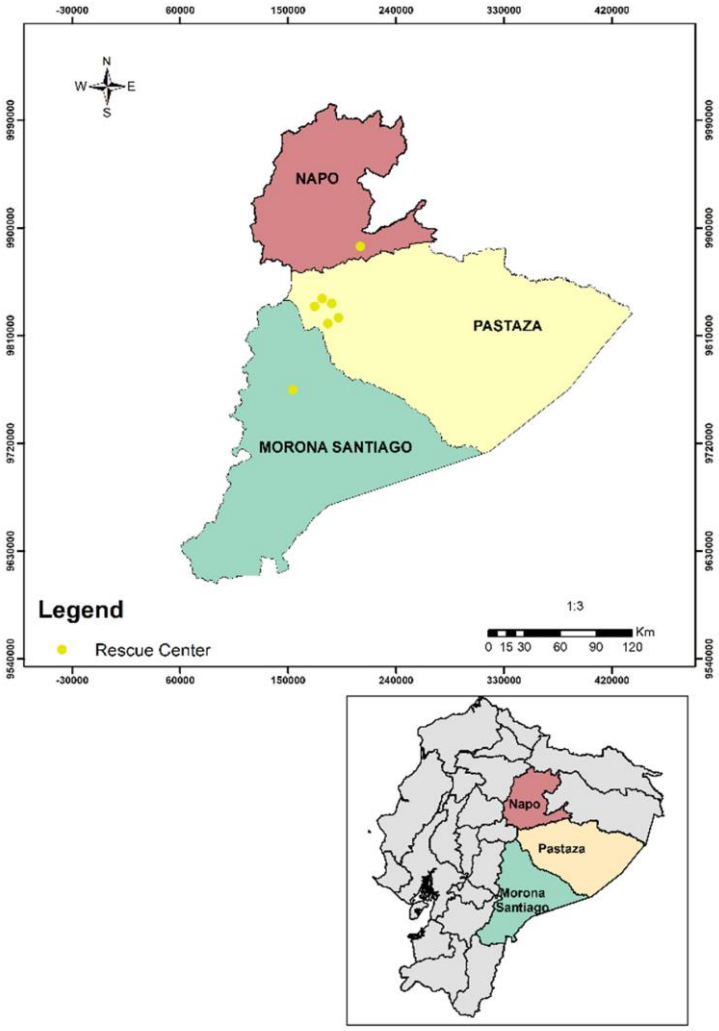

Figure 2. Rescue centres surveyed in the Amazon region of Ecuador.

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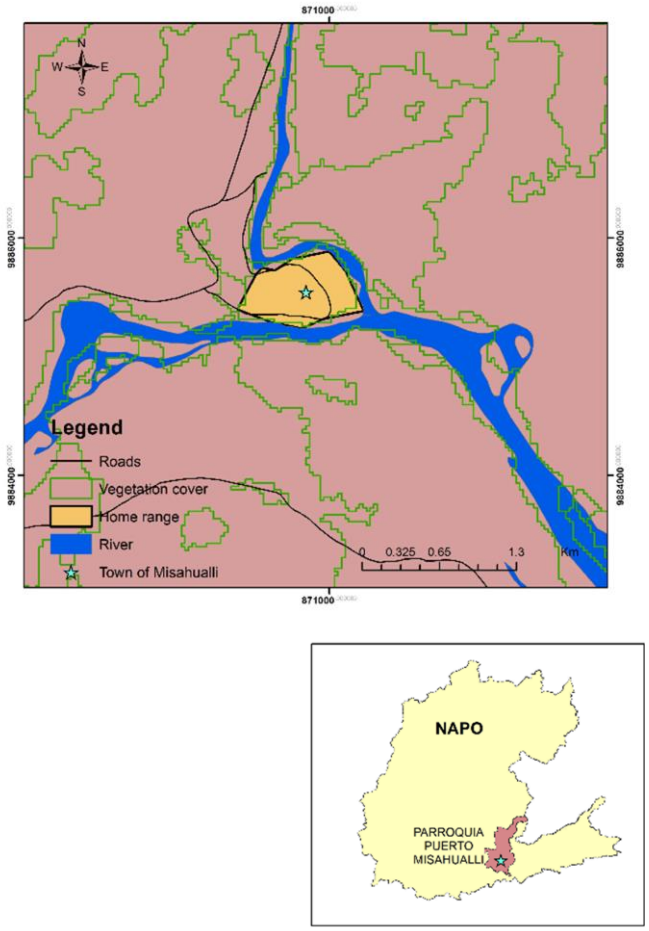

Figure 3. Location of free-ranging population of *Cebus yuracus* surveyed in the Amazon region of Ecuador.

The molecular identification was performed in two reactions (nested PCR) as described by [80], with adaptations. The amplification in the first reaction consisted of 95 °C for 5 min, 95 °C for 30 seg; 50 cycles of 55 °C for 30 seg, 72 °C for 1 min; and a final extension step at 72 °C for 5 min, with a product of 600 bp. In addition, the amplification in the second reaction consisted of an initial denaturation at 95 °C for 5 min, 95 °C for 30 seg; 50 cycles of 58 °C for 30 seg and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The products of the second reaction (240 bp) were observed using the electrophoresis of an agarose gel under UV light. Amplicons were cut, extracted using NucleoSpin gel and the PCR clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced (Sanger sequencing) by Eurofins (Hamburg, Germany). Every PCR reaction contained a negative and a positive control. Sterile filtered pipette tips were used in all stages of the methodology to prevent contamination. We changed the pipette tip after each sample to avoid false-positive reactions/cross-contamination and all laboratory consumables were not reused.

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Table 2. Non-human primate species sampled in the Ecuadorian Amazon.

Habitat Settings	Non-Human Primate Species	n	Sex		Age		
			Male	Female	Adult	Subadult	Juvenile
Captive	*Alouatta seniculus*	4	0	4	1	2	1
	Ateles belzebuth	1	1	0	1	0	0
	Callicebus lucifer	1	1	0	1	0	0
	Cebuella pygmaea	1	1	0	1	0	0
	Cebus yuracus	2	1	1	1	1	0
	Lagothrix lagotricha	2	0	2	1	0	1
	Leontocebus fuscicollis	3	2	1	2	1	0
	Saimiri sciureus	3	2	1	1	1	1
	Sapajus apella	2	1	1	1	0	1
Free ranging	*Cebus yuracus*	7	5	2	4	1	2

Table 3. Sequences of the primers.

Reaction	Primer	Oligonucleotide Sequence	
First reaction	rPLU1	5′-TCAAAGATTAAGCCATGCAAGTGA 3′	forward
	rPLU6R	5′-CGTTTTAACTGCAACAATTTTAA-3′	reverse
Second Reaction	rPLU3	5′-TTTTTATAAGGATAACTACGGAAAAGCTGT-3′	forward
	rPLU4	5′-TACCCGTCATAGCCATGTTAGGCCAATACC-3′	reverse

A positive control for *Plasmodium* spp. was obtained using DNA extracted from the spleen of a Belgian blackbird (*Turdus merula*) collected in 2018 [81,82].

4.2. Molecular Identification

The sequence was uploaded to GenBank under the accession number submission MZ156589. Sequence reconstruction was performed using Assembler by MacVector software 17.5.5. The first sequence identity was confirmed by BLAST in NCBI resources. A total of 15 sequences from 14 species of *Plasmodium* were retrieved from GenBank and included as sister groups in order to obtain a wide geographic diversity and taxonomic representation [83] (Table 4). DNA sequences were aligned using MacVector 17.5.5 [84] by the ClustalW algorithm with high gap creation and extension penalties by 30.0 and 10.0, respectively, searching for a strong positional homology. The evolutionary history was inferred by using the maximum likelihood method and the Tamura 3-parameter model. The tree with the highest log likelihood (−932.64) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.3352)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences. There was a total of 241 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 4. GenBank accession numbers of *Plasmodium* species sequences.

Plasmodium Species	ID Genbank	Host	Country
Plasmodium sp.	LT963420.1	*Gorilla* sp.	Unknown
P. falciparum	LR131487.1	Unknown (Genome assembly)	Unknown (Genome assembly)
P. falciparum	MZ156589	*Leontocebus lagonotus*	Ecuador
P. falciparum	LR131471.1	Unknown (Genome assembly)	Unknown (Genome assembly)
P. reichenowi	LT969568.1	Unknown (Genome assembly)	Unknown (Genome assembly)
P. gaboni	LT969430.1	Unknown (Genome assembly)	Unknown (Genome assembly)
P. gonderi	AB287269.1	*Cercocebus atys*	Central Africa
P. ovale	KF018656.1	*Homo sapiens*	China
P. brasillianum	KX618475.1	*Sapajus flavius*	Brazil

Table 4. *Cont.*

Plasmodium Species	ID Genbank	Host	Country
P. malariae	LT594624.1	Unknown (Genome assembly)	Unknown (Genome assembly)
P. fragile	XR001111607.1	Unknown	Unknown
P. inui	EU400397	*Macaca fascicularis*	Thailand
P. simium	U69605.1	*Saimiris sciureus*	Colombia
P. vivax	U07368	Unknown	CDC Strain
Plamodium sp.	KX522949.1	*Anopheles nuneztovari*	Brazil
P. knowlesi	LR701163.1	Unknown (Genome assembly)	Unknown (Genome assembly)

The robustness for all the analyses was estimated using bootstrapping with 1000 pseudoreplicates and shown in percentage.

5. Conclusions

The results of this study provide evidence of *Plasmodium falciparum* in a species of NW NHP, and the potential risk of zoonotic malaria transmission. The present study, by identifying the presence of the parasite in NHP, suggests the need to promote continuous and systematic diagnoses and monitoring of malaria in these animals. In addition, wildlife trafficking and management should be incorporated into public health policies for the prevention of malaria as an emerging zoonotic disease.

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Experimental section

Study 3

First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon

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Preamble

Trypanosomes are a group of pathogens with a wide distribution (Europe, Asia, Africa and America) and are widely distributed as mammalian pathogens in the Americas, but their pathology and biology are not well known in the Neotropical region. *Babesia* is a genus of pathogens that some species are considered tick-borne pathogens and are reported to affect birds and mammals, including humans.

We identified two distinct species of trypanosomes in two non-human primate species, *Cebus albifrons*, a free-living individual, and *Leontocebus lagonotus*, a captive individual, both with distinct sociecological characteristics.

This is the first study at national level and one of the few at regional level using non-invasive samples, becoming the first record of trypanosomes for the species of *Leontocebus lagonotus* and the first report for Ecuador of *Trypanosoma* in *Cebus albifrons*. These results highlight the need for specific management protocols for Wildlife Management Units to avoid the transmission of blood pathogens such as *Trypanosoma* sp.

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First Molecular Identification of Trypanosomes and Absence of *Babesia* sp. DNA in Faeces of Non-Human Primates in the Ecuadorian Amazon

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Abstract: Trypanosomes are a group of pathogens distributed in the continents of Africa, America, Asia and Europe, and they affect all vertebrates including the neotropical primate group. Information about the trypanosome's diversity, phylogeny, ecology and pathology in non-human primates (NHPs) from the neotropical region is scarce. The objective of the study was to identify *Trypanosoma* and *Babesia* molecularly in NHPs under the phylogenetic species concept. We extracted DNA from a total of 76 faecal samples collected between 2019 and 2021, from a total of 11 non-human primate species of which 46 are from captive NHPs and 30 are free-living NHPs in the Western Amazon region of Ecuador. We did not detect DNA of *Babesia* sp. by polymerase chain reaction test in any of the faecal samples. However, the nested-PCR-based method revealed *Trypanosoma* parasites by ITS gene amplification in two faecal samples; one for the species *Leontocebus lagonotus* (from the captive population) and a second one for *Cebus albifrons* (from the free-ranging population). Maximum parsimony and likelihood methods with the Kimura2+G+I model inferred the evolutionary history of the two records, which showed an evolutionary relationship with the genus *Trypanosoma*. Two sequences are monophyletic with *Trypanosoma*. However, the number of sequences available in GenBank for their species identification is limited. The two samples present different molecular identifications and evolutionary origins in the tree topology. We are most likely referring to two different species, and two different localities of infection. We suggest that health management protocols should be implemented to prevent the transmission of blood-borne pathogens such as *Trypanosoma* sp. among captive populations. In addition, these protocols also protect the personnel of wildlife rehabilitation centers working in close proximity to NHPs and vice versa.

Keywords: DNA; non-human primate; *Trypanosoma*; *Babesia*; faecal samples; Ecuadorian Amazon; wildlife rehabilitation center

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1. Introduction

Neotropical non-human primates (NHPs) are threatened by habitat loss or habitat fragmentation (agricultural activities, logging, oil drilling, new road networks), hunting and the wildlife trade [1–4]. These activities increase contact between people and NHPs (increase the human–wildlife interface), but also enhance the prevalence of pathogens [5–8].

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Captive and free-ranging neotropical NHPs harbour a large diversity of pathogens [9]. Several factors influence the richness, prevalence, and transmission of pathogens in NHPs. Some individuals are more susceptible than others depending on their sex, age, NHP species, behaviour and social status, population density and geographic location.

Trypanosomes are a widespread group of pathogens that infect all kinds of vertebrates across America, Africa, Asia and Europe [10–15]. Trypanosomes are intracellular or extracellular pathogens found in blood, lymph and tissues [16–19]. Although trypanosomatids are widespread pathogens of mammals in the Americas, their biology, taxonomy and pathology are not well known for neotropical NHPs [20]. Natural infections of trypanosomes are common in neotropical NHPs. The prevalence of trypanosomes among NHPs can vary from one species to another [21]. According to Aysanoa et al. [22], prevalence was higher in free-ranging NHPs than in captive NHPs [23]. Some species such as *Trypanosoma cruzi* are common among NHPs' trypanosomes and can cause myocarditis, haemorrhage, and encephalitis to NHPs [24–26]. In addition to *Trypanosoma cruzi*, NHPs can be infected by 12 more species (Appendix A). Some species are non-pathogenic to the host such as *Trypanosoma minasense* [27]. *T. minasense* does not infect blood-sucking triatomines such as other species of trypanosomes [28], and information of vector is scarce [29]. However, it is suggested that some species of *Trypanosoma* sp. can be transmitted by oral contamination [30,31]. *Trypanosoma minasense*, *T. devei* and *T. lambrechti* are considered the most primitive species infecting neotropical mammals among the other species belonging to the genus *Trypanosoma* [32].

Babesia is the genus of several species of tick-borne pathogens affecting the red cells of several mammals and birds [33–40]. Three species of *Babesia* sp. are zoonotic: *B. divergens*, *B. duncani* and *B. microti*. *Babesia microti* in America is a blood pathogen, this apicomplexan can infect humans (serve as accidental host) and causes babesiosis. Rodents are natural reservoirs [41], and ticks of the family Ixodidae that infect deer are the primary vectors of *Babesia microti* [42–44]. In old-world NHPs, *Babesia microti* has been observed in several species of NHPs [45,46]. In neotropical NHPs, *Babesia* sp. can be found at least in five different NHP species [47,48]. However, the species of *Babesia* sp. are uncharacterised [49].

The internal transcribed spacers (ITS) are widely used for molecular characterization and phylogenetic studies [50–53]. The ITS region includes the ITS1 and the ITS2. These ITS vary in size between species and subspecies [54] and show more variation than the ribosomal coding region, with high evolutionary rates [55]. This gene was used to molecularly characterize trypanosomes and to study the phylogenetic of trypanosomes [56–62]. The Cathepsin L-like (CatL-like) protein is a cysteine protease important in the life cycle and pathogenicity of trypanosomes, involved in mechanisms such as tissue damage, invasion, and recovery of metabolites from host proteins [63–66]. The active site sequence of the gene encoding this protein, being a conserved, multi-copy gene, has been widely used as a targetable marker for *Trypanosoma* spp. diagnosis, in some cases in combination with other molecular markers such as ITS and 18S [67–71].

Molecular genetic analysis has had its limitations in wild populations due to the difficult access to blood or tissue samples for DNA extraction [72], which is why non-invasive sampling has been chosen worldwide. The main drawback is mainly due to the low amount of genetic material obtained [72]. However, in 1990, Boom et al. [73] presented the first study that succeeded in isolating DNA from epithelial cells that were mixed with faeces. Since then, with improvements included in their protocols, conservation genetics studies use faecal samples for DNA extraction [74–76]. Molecular analysis of faecal samples has already been used to decipher the origins of major pathogens in human and non-human primates [77–87], even in blood pathogens [61,88,89]. Successful amplification of *Trypanosoma* sp. [90] and *Babesia* sp. DNA in previous studies from stool samples in mice, dogs, and foxes [91] offers a non-invasive option as a valid alternative to traditional sampling methods.

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In Ecuador, information regarding blood pathogens in NHPs is scarce. Therefore, this study aimed for the first time to detect *Trypanosoma* and *Babesia* in captive and free-ranging NHPs from the Ecuadorian Amazon through molecular techniques (PCR and sequencing).

2. Materials and Methods

2.1. Sampling Location

This study was performed in Puyo (1°2′9′1.3′ S 78°0.154′ W) and Mera (0°10′0″ S and 78°28′0″ W), Tena (Napo) (0.9938° S 77.8129° W), and Macas (Morona Santiago) (2.3087° S 78.1114° W), four cities in the Western Amazon region. We collected samples from a free-ranging population in Misahualli, Tena (1°2′7.0′ S, 77°39′59.4″ W) and from captive individuals located in 5 different wildlife rehabilitation centers of Ecuador. Captive NHPs have been donated by families or confiscated by the police during roadside checks; individual information such as location origin is uncertain (Figure 1).

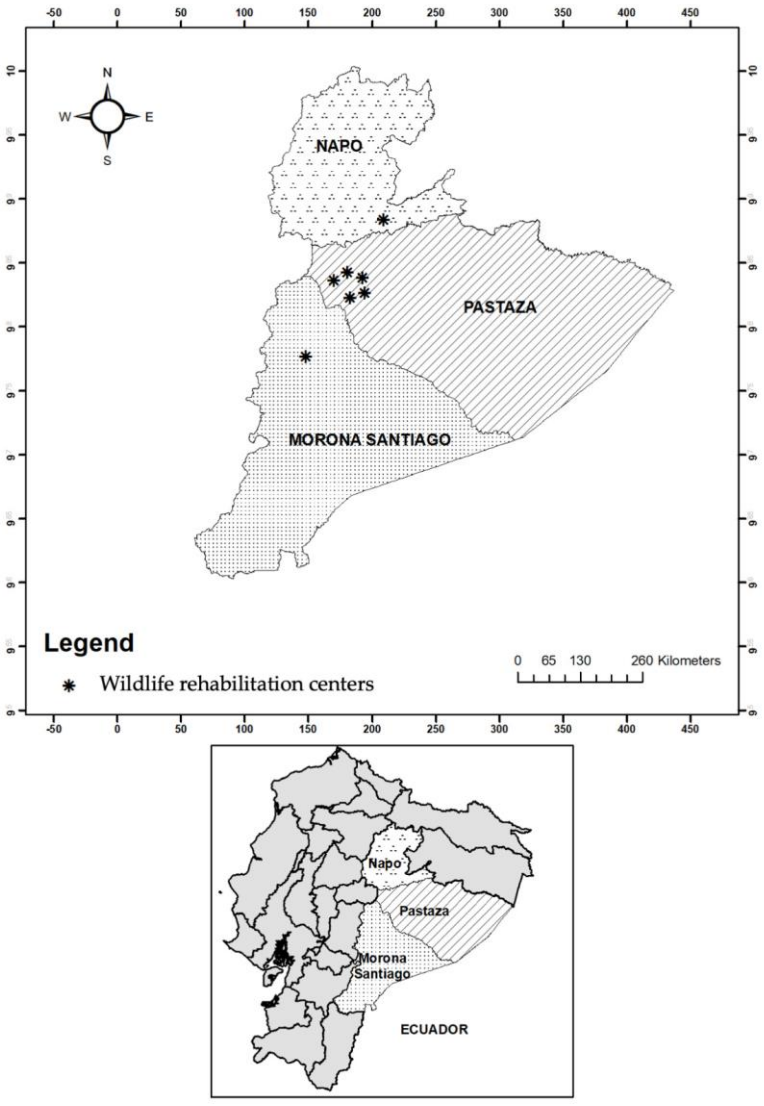

Figure 1. Sampling location of wildlife rehabilitation centers with non-human primates in Ecuador.

2.2. Sample Collection and Ethics Statement

We collected a total of 76 fecal samples of 11 species of NHPs (Table 1) between 2019 and 2021. The sample collection and ethical procedures were approved by the local authorities, the Ministerio del Ambiente, Agua y Transición Ecológica, MAATE (No. MAE-DNB-CM-2015-0028-M-002). Individuals were followed daily from 08:00 h to 18:00 h to avoid multiple sampling. In addition, all animals were individually identified to avoid confusion between individuals and to facilitate species, sex and age association [92]. Finally, we collected the faecal samples immediately after defecation to avoid a possible contamination from the environment and were taken at least 24 h without disturbing the animals. In primatology, when birth dates are unknown, age as well as sex, is generally assigned in categories based on physical characteristics including body size, dentition and gland development in species where these are evident, and behavioral characteristics [93–95]. All samples were processed at the International Centre of Zoonoses at the Central University of Ecuador and examined at the Biotechnology Animal Lab at the Universidad de las Fuerzas Armadas ESPE.

Table 1. Non-human primate's species screened for *Trypanosoma* sp. and *Babesia* sp.

Habitat	Non-Human Primate Species	n	Sex		Age		
			Male	Female	Juvenile	Subadult	Adult
Free-ranging	*Cebus albifrons*	18	8	10	7	4	7
Captive	*Alouatta seniculus*	4	0	4	2	1	1
	Ateles belzebuth	4	4	0	2	1	1
	Aotus vociferans	1	0	1	0	0	1
	Cebuella pygmaea	2	1	1	1	0	1
	Cebus apella	1	0	1	0	0	1
	Cebus albifrons	10	9	1	3	1	6
	Lagothrix lagotricha	18	7	11	0	6	11
	Leontocebus lagonotus	9	4	5	0	0	9
	Plecturocebus discolor	4	3	1	3	0	1
	Saimiri sciureus	3	3	0	1	1	1
	Sapajus apella	2	1	1	1	0	1

2.3. Storage and DNA Isolation Protocol

For the molecular evaluation, samples were stored in 50 mL Falcon tubes in 99% alcohol at −20 °C to prevent the degradation of DNA. In addition, 600 µL of faeces suspension (1:3; 1 part of faecal sample and 3 parts of ethanol 96–100%) was centrifuged for 2 min at 239 g and the pellet was washed with 1 mL of PBS Buffer (Oxoid, Hampshire, England). This solution (pellet + PBS) was centrifuged for 5 min and the supernatant was discarded. This washing step was repeated three times. Next, the pellet was resuspended in 600 µL of 2% PVPP (polyvinylpolypyrolidone—Sigma), and frozen overnight at −20 °C to facilitate the capture of phenols in the sample. DNA extraction was performed twice on different days using the QIAamp Stool FAST Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. To prevent cross-contamination, sample preparation, DNA extraction, and the polymerase chain reaction (PCR) were performed in completely different and separate rooms.

2.4. Molecular Amplification and Sequencing of *Trypanosoma* sp.

The molecular identification was performed with two PCR assays: (i) A CatL-PCR, according to reaction conditions by Cortez et al. [70] (Table 2), with adaptations in the reaction mixture, that consisted of a final volume of 25 µL with 1× of Buffer, 1 µM of

each primer DTO154 and DTO155 (Table 3), 1.5 mM of $MgCl_2$, 0.2 mM of dDNT Mix, 1.25 U of Taq Platinum Polymerase (Invitrogen) and 2 µL of DNA. (ii) A nested ITS-PCR as described by [96,97], the reaction mixture consisted of 1× of Buffer, 1 µM of each primer (ITS1 and ITS2 in the first reaction, ITS3 and ITS4 in the second reaction) (Table 3), 2.5 mM of $MgCl_2$, 0.2 mM of dDNT Mix, 1.25 U of Tap Platinum Polymerase (Invitrogen) and 2 µL of DNA in the first reaction and 1uL of first reaction PCR product in the second reaction; the amplification consisted of an initial denaturation of 95 °C for 7 min; 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 2 min in the first reaction and 1.5 min in the second reaction; and a final extension step at 72 °C for 10 min (Table 4). The final PCR products of the CatL-PCR and ITS-PCR were observed using the electrophoresis of an agarose gel under UV light. ITS-PCR amplicons were cut, extracted using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced (Sanger sequencing) by Macrogen (South Korea). Every PCR reaction contained a negative (nuclease-free water) and a positive control. A positive control for CatL-PCR was a positive DNA sample of *Trypanosoma vivax* [98] and for ITS-PCR a positive DNA sample of *Trypanosoma theileri*, available in the Laboratorio de Biotecnología Animal of the Universidad de las Fuerzas Armadas ESPE. Two sequences belonging to the 5.8S and ITS-2 were recovered from Sanger sequencing Macrogen Korea.

Table 2. Three steps CatL-PCR cycles, temperature, and time for *Trypanosoma* sp.

Step		Temperature	Time	Number of Cycles
Step 1	Pre-denaturation	94 °C	5 min	1 cycle
Step 2	Denaturation	94 °C	1 min	35 cycles
	Annealing	56 °C	1 min	
	Extension	72 °C	1 min	
Step 3	Final extension	72 °C	5 min	1 cycle

Table 3. Sequences of the primers for *Trypanosoma* sp. and *Babesia* sp.

	Reaction	Primer	Oligonucleotide Sequence	
Trypanosoma	CatL-PCR	DTO154	5′-ACAGAATTCCAGGGCCAATGCGGCTCGTGCTGG-3′	Forward
		DTO155	5′-TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA-3′	Reverse
	ITS-PCR First Reaction	ITS1	5′-GATTACGTCCCTGCCATTTG-3′	Forward
		ITS2	5′-TTGTTCGCTATCGGTCTTCC-3′	Reverse
	ITS-PCR Second Reaction	ITS3	5′-GGAAGCAAAAGTCGTAACAAGG-3′	Forward
		ITS4	5′-TGTTTTCTTTTCCTCCGCTG-3′	Reverse
Babesia		Piro A	5′-AATACCCAATCCTGACACACAGGG-3′	Forward
		Piro B	5′-TTAAATACACGAATGCCCCCCCAAC-3′	Reverse

Table 4. Three steps nested ITS-PCR cycles, temperature, and time for *Trypanosoma* sp.

Step		Temperature	Time	Number of Cycles
Step 1	Pre-denaturation	95 °C	7 min	1 cycle
Step 2	Denaturation	95 °C	1 min	35 cycles
	Annealing	59 °C	1 min	
	Extension	72 °C	2 min (first reaction) 1.5 min (second reaction)	
Step 3	Final extension	72 °C	10 min	1 cycle

2.5. Molecular Amplification and Sequencing of Babesia sp.

The molecular identification by PCR was performed with the primers designed by Olmeda et al. [99] and using the reaction condition described by Medina Naranjo et al. [100], which consisted of: 1× of Buffer, 0.25 µM of each primer Piro A and Piro B (Table 3), 1.5 mM of $MgCl_2$, 0.2 mM of dDNT Mix, 0.5 U of Tap Platinum Polymerase (Invitrogen) and 2 µL of DNA; the amplification of an initial denaturation of 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 30 sec; and a final extension step at 72 °C for 5 min (Table 5). The PCR products were observed using the electrophoresis of an agarose gel under UV light. Every PCR reaction contained a negative and a positive control. A positive control for *Babesia* sp. was a DNA sample obtained from the study performed by Chávez-Larrea et al. [101].

Table 5. Three steps PCR cycles, temperature, and time for *Babesia* sp.

	Step	Temperature	Time	Number of Cycles
Step 1	Pre-denaturation	94 °C	5 min	1 cycle
Step 2	Denaturation	94 °C	1 min	35 cycles
	Annealing	55 °C	1 min	
	Extension	72 °C	30 s	
Step 3	Final extension	72 °C	5 min	1 cycle

2.6. Molecular Analysis: Sequence Assembled, Alignments and Phylogenetic Analyses

Sequences were uploaded to GenBank under the accession number OP683488.1 for *Trypanosoma* sp. detected in *Leontocebus lagonotus* and OP683532.1 for *Trypanosoma* sp. detected in *Cebus albifrons*. Our two 5.8S-ITS-2 sequences of 400 bp length were contig assembly and consensus sequences of ITS-2 were performed and edited using Assembler by MacVector software 18.2.5 [9], then the sequence identity was confirmed by BLAST in NCBI resources. The two sequences were first aligned with the unique complete sequence 18S-ITS1-5.8S-ITS2-28S available in GenBank that included ITS2 (*T. minansense* AB362411.1 by Sato et al. [102], recovered from new-world NHPs from South American tamarins) to corroborate the portion of rDNA and matching or sequences (from 2636 bp to 3019 bp). The 5.8S-ITS-2 sequences deposited in GenBank NCBI from other species of *Trypanosoma* were included as sister groups and *Leishmania* was selected as the outgroup *sensu* [103] to get a wide geographic diversity and taxonomic representative and to test the phylogenetic species monophyly.

A phylogenetic analysis was performed using a total of 23 ITS-2 sequences from 13 species of *Trypanosoma* that were retrieved from GenBank and included two species of *Leishmania* as outgroup to corroborate the Blast identity close to *Trypanosomatidae* and to search the evolutionary relationships with other trypanosomes (Table 6).

The DNA sequences were aligned using MacVector 18.2.5 by the ClustalW algorithm with high gap creation and extension penalties by 30.0 and 10.0, respectively, searching for a strong positional homology.

The evolutionary history was inferred by using the maximum parsimony and likelihood methods with the Kimura2+G+I model. Maximum Parsimony analyses were implemented in PAUP 4.0a (169 build) [104] using the heuristic search option with a Tree Bisection Reconnection branch-swapping algorithm with at-random stepwise addition of 10 replicates for each search and 100–1000 replications per analysis. Gaps were treated both as missing data. The characters were treated as unordered, and equally weighted, after the characters were weighted by consistency index. The robustness of the trees was estimated using parsimony bootstrap with 1000 pseudoreplicates after excluding uninformative characters [105]. We also performed a Maximum Likelihood (ML, and substitution model estimated on MEGAX).

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Table 6. Sequences obtained from GenBank to elaborate the cladograms.

Species/Sequences	GenBank ID
Leishmania infantum (outgroup)	LC459327.1
Leishmania donovani (outgroup)	LC459329
Trypanosoma vivax	KX584847.1; KX584884.1
T evansi	LC199490.1; KY014244
T theileri	KY412803.1; JX178183.1
T congolense	JN673388.1
T simiae	JN673382.1; JN673379.1
T cruzi	JN673388.1; GU991802.1
T minasense	AB362411.1
T brucei	KU552340.1
T godfreyi	JN673383.1
T equiperdum	KU552342.1
T rangeli	AY230233.1; AY230240.1
Trypanosomatidae sp.	JN673400
T cf. *cervi*	JX178169.1

3. Results

We have performed a PCR and a Nested PCR to detect the gene *Trypanosoma* from faecal samples of 11 species of NHPs. The PCR with the Catepsine L-Like gene failed to amplify DNA from *Trypanosoma* sp., although this gene can be easily amplified in other samples from wild mammals [106]. On the other hand, the Nested PCR of 35 cycles successfully amplified the ITS1 gene.

The nested PCR results showed a total of 8/76 (10.53%) samples positive for *Trypanosoma* sp. Positive samples belong to *Alouatta seniculus* (n = 1), *Ateles belzebuth* (n = 1), *Cebus albifrons* (two captive and two free-ranging individuals), *Lagothrix lagotricha* (n = 1) and *Leontocebus lagonotus* (n = 1). Among these positive samples, two (2.63%) yielded amplicons for trypanosomes species in *Leontocebus lagonotus* (from the captive population) and *Cebus albifrons* (from the free-ranging population). The sequences presented a first Blast identity with *Trypanosomatidae*.

We did not observe positive samples for *Babesia* sp. for any of the samples.

From the 458 *Trypanosoma* sequences in GenBank that partially or completely included ITS-2 belonging to 16 species, we aligned the two ITS sequences amplified from the faecal samples with 21 ITS sequences from 13 species (Table 3) (three species from Russia were not included) to elaborate the cladogram.

The cladogram showed that our two sequences belong to the trypanosomes genus. Our results revealed two unexpected novel sequences. The topology of the cladogram (Figure 2A) shows two clades (A and B). The clade shows subclades A.1 with *T. cruzi* as a sister group of the *Trypanosoma* group of *Leontocebus lagonotus* ME001 Ecuador + *T. brucei*, *T. evansi* and *T. equiperdum* in derived position and subclade A.2 with *T. rangeli* + [*minasense* (cf.*cervi* + *theileri*)]. However, there is no sequence available with which to identify monophyly.

Clade B shows the sequence of *Cebus albifrons* PM020 from Ecuador, internally and closely related to *T. congolense* and basal to *T. vivax* + [(Trypanosmatidae sp (*T. godfreyi* + *T. simiae*)], showing its close relationship with these species. Likewise, there is no ITS-2 sequence in GenBank that shows monophyly with our sequence for a specific identification.

The topologies using maximum parsimony (MP) and maximum likelihood (ML) under the Kimura2+G+I model showed identical relationships (Figure 2B), the two *Leishmania* species as outgroup allow corroborating the monophyly of *Trypanosoma* (100% bootstrap), as well as our sequences showing their evolutionary relationship within the genus.

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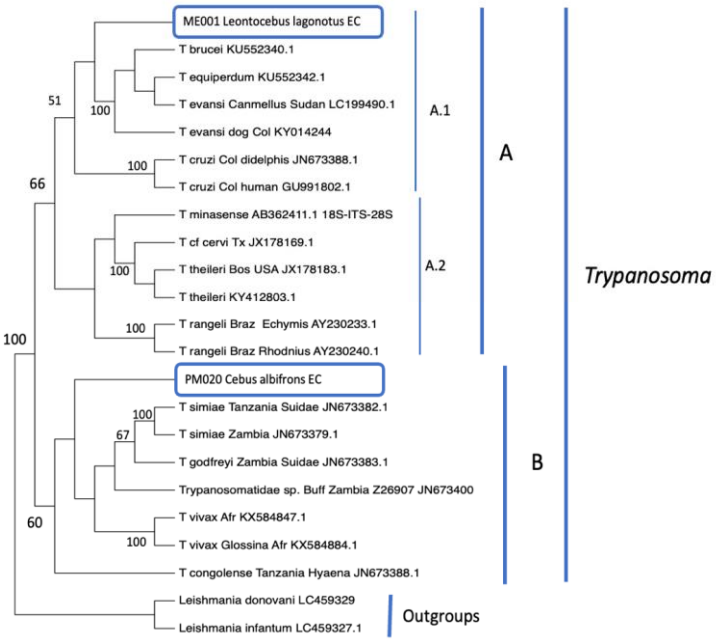
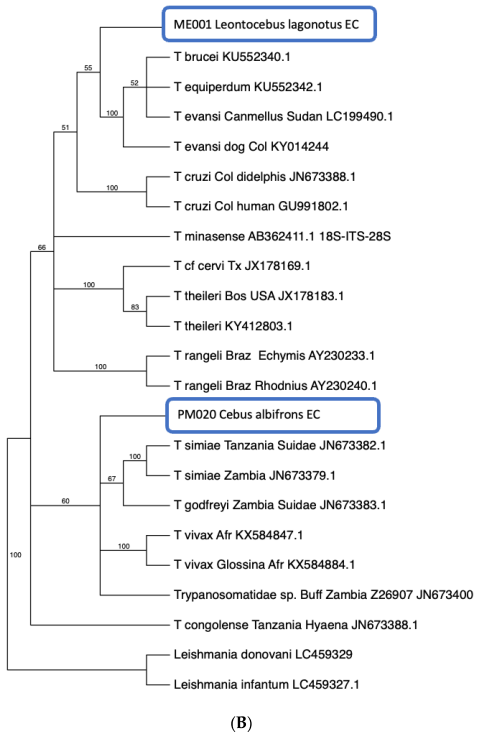


(**A**)



(**B**)

Figure 2. (**A**). Cladogram of the two species of trypanosomes found in NHPs and (**B**). Bootstrap Consensus Tree. Legend: Numbers are percentages of homology. The circled samples are from the study. For (**A**), A and B are clades; B.1 and B.2 are subclades.

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4. Discussion

The present study is the first at the national level and one of the few at the regional level to identify two species of *Trypanosoma* sp. using non-invasive techniques. Although the ITS region was successfully amplified in wild gorillas (*Gorilla gorilla gorilla*) and chimpanzees (*Pan troglodytes troglodytes*) for trypanosomes, this is the first study in the neotropical region to use the ITS region to amplify trypanosomes in faecal samples. We identified the first record of *Trypanosoma* for the NHP species *Leontocebus lagonotus* and the first report for Ecuador of *Trypanosoma* in *Cebus albifrons*. We detected 10.53% of positive samples, whereas only 2.63% yielded a positive sequence. This prevalence is lower than other studies [107,108]. In Aysanoa et al. [22], they found a lower prevalence in captive NHP individuals than in wild individuals. Captive animals may be subject to liberation projects, and they can introduce new trypanosomatids to liberation sites. Triatomines were found in a Brazilian zoo, infesting neotropical NHPs. This indicates that the same pattern could be possible in Ecuadorian wildlife rehabilitation centers where vegetation could facilitate the presence of trypanosomes vectors. Common vectors of trypanosomes like triatomine bugs (*Panstrongylus geniculatus, Triatoma dimidiata, Rhodnius pictipes and Rhodnius robustus*) can be found in the Ecuadorian Amazon [17,109] and the proximity of the forests to the centers would facilitate the maintenance of the forest cycle.

Previous studies have suggested that trypanosomes tend to have harmful effects on the health of infected hosts [110–114]. However, information on the effect of triatomines in NHPs is scarce. The individuals who tested positive had no obvious symptoms that would allow us to make a statement about their health condition, noting that trypanosome records have been made in healthy individuals as well as in sick individuals.

After phylogenetic reconstruction, we identified two large groups: the first, in which we found the sample of *Leontocebus lagonotus* within the same cladogram as the species of *T. brucei*, *T. equiperdum*, *T. evansi*, *T. cruzi*, *T. minasense* and *T. theileri*. In the second cladogram, we found the sample of *Cebus albifrons* together with the species of: *T. simiae*, *T. godfreyi*, *T. vivax* and *T. congolense*. This distribution coincides with several authors [102]. The two samples show different molecular identifications and evolutionary origins, certainly two species, and two localities of infection.

The use of molecular tools for the detection of *Trypanosoma* spp. is crucial because of the unreliability of detection methods based on the observation of their morphology [115,116]. The two sequences are shown to be in monophyly with *Trypanosoma;* however, there are not enough sequences available in GenBank for their specific identification.

As mentioned before, the gene from the Cathepsin L-like protein failed to amplify in the faecal samples. However, past studies diagnosed *T. rangeli*, *T. cruzi* and *T. theileri* with this gene [71,106,117,118]. This protein is a lysosomal cysteine proteinase. The Cathepsin L-like protein is found in several stages of cell multiplication and differentiation as well as cell metabolism and virulence (host cell invasion, immune evasion) in protozoan parasites such as trypanosomes [119,120]. However, according to Cortez et al. [70], this gene has a different number of copies depending on the trypanosome species and therefore is species-specific, and because we were surveying trypanosomes in general, the protocol failed to amplify for all trypanosome species and specifically for neotropical NHPs' trypanosomes, given what was observed with the amplification of the ITS gene.

Based on the findings, the ITS gene is a useful molecular marker to detect trypanosomes; however, for further studies, it is suggested to amplify with 18S-ITS-1 (higher availability); this combination would support us in defining in more specific detail the molecular characterization of these two records. Unfortunately, only *T. simiae*, *T. rangeli*, *T. cruzi* and *T. minasense* (included) of the NHPs-associated *Trypanosoma* have ITS2 sequences available in GenBank from the list in Appendix A.

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We did not record the presence of *Babesia* sp., a protozoan pathogen with a worldwide distribution restricted to tropical and subtropical areas [101]. Non-human primates are a group of mammals that have generated strategies to prevent pathogens [121–123]. One of these strategies is grooming. Grooming is a behaviour that directly supports health-related aspects of different primate species, including the removal of ectopathogens such as leeches in *Macaca fuscata* [124] and ticks [125,126]. For *Papio cynocephalus* (Africa), it was recorded that the amount of grooming received, sex, age and hierarchical level affected the tick load of an individual. However, the primary function of grooming contributes to social aspects in different old=world primate species [127], whereas, in new-world primates, it is suggested that the main function of grooming is hygienic [128]. For this reason, this type of grooming can explain the absence of *Babesia* sp. in our study. It is important to conduct long-term studies that allow us to relate the presence of ticks to the prevalence/absence of tick-borne diseases in non-human primates.

5. Conclusions

This is the first study to amplify trypanosomes in Ecuadorian NHP species.

Even if the prevalence was low, we suggest the implementation of health management protocols to avoid the transmission of blood-borne pathogens such as *Trypanosoma* sp. among captive populations. In addition, these protocols protect the personnel of wildlife rehabilitation centers working in close proximity to NHPs and vice versa. Socioecological aspects are of utmost importance to understand pathogen–vector–host relationships in different species of NHPs. In Ecuador, research activities should be focused on blood pathogens to fill the gap of information and to implement surveillance programs with regular and effective monitoring protocols adapted to NHPs. We suggest to increase the monitoring of free-ranging groups across Ecuador to clarify the role of NHPs as reservoir hosts of novel trypanosomes.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

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Appendix A

Trypanosomes in neotropical non-human primate species (updated from Carrillo-Bilbao et al. [129]).

Trypanosome	Non-Human Primate Species	Reference
Leishmania sp. *Leishmania (Viannia)*	*Alouatta guariba* Atelidae (unknown species) *Aoutus azarai azarai* *Aotus nigriceps* *Callicebus nigrifrons* *Callithrix jacchus* *Callithrix penicillata* *Cebus macrocephalus* *Lagothrix cana* *Leontopithecus crysomelas* *Pithecia* sp. *Pithecia irrorata* *Saguinus imperator* *Saimiri ustus madeirae* *Sapajus apella* *Sapajus xanthosternos*	[130–134]
Leishmania amazonensis	*Ateles paniscus*	[135]
Leishmania braziliensis	*Saguinus geoffroyi*	[136]
Leishmania chagasi *Leishmania infantum*	*Callicebus nigrifrons* *Callithrix jacchus*	[133] [137]
Leishmania mexicana	*Alouatta palliata* *Alouatta pigra*	[138]
Leishmania (Viannia) shawi	*Chiropotes satanus* *Sapajus apella*	[139]
Trypanosoma sp.	*Alouatta palliata* *Alouatta seniculus* *Ateles paniscus* *Pithecia pithecia* *Saguinus leucopus* *Saimiri sciureus*	[27,140]

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Trypanosome	Non-Human Primate Species	Reference
Trypanosoma cruzi	*Alouatta palliata* *Alouatta pigra* *Alouatta caraya* *Alouatta seniculus* *Ateles belzebuth* *Ateles chamek* *Ateles geoffroyi* *Ateles fusciceps* *Ateles paniscus* *Aotus sp.* *Aotus azarai* *Aotus nigriceps* *Cacajao calvus* *Callicebus personatus* *Callicebus nigrifrons* *Callithrix geoffroyi* *Callithrix jacchus* *Callithrix penicillata* *Cebuella pygmaea* *Cebus albifrons* *Cebus capucinus* *Cebus olivaceus* *Cheracebus torquatus* *Chiropotes satanas* *Lagothrix lagotricha* *Lagothrix cana* *Leontopithecus chrysopygus* *Leontopithecus chrysomelas* *Leontopithecus rosalia* *Leontocebus fuscicollis* *Leontocebus fuscicollis weddelli* *Leontocebus nigricollis* *Mico chrysoleucus* *Mico argentatus* *Mico emiliae* *Pithecia irrorata* *Pithecia chrysocephala* *Plecturocebus brunneus* *Saguinus niger* *Saguinus geoffroyi* *Saguinus bicolorbicolor* *Saguinus imperator imperator* *Saguinus labiatus* *Saguinus leucopus* *Saguinus midas* *Saguinus mystax* *Saguinus ustus* *Saimiri boliviensis* *Saimiri sciureus* *Saimiri ustus* *Sapajus apella* *Sapajus flavus* *Sapajus libidinosus* *Sapajus robustus* *Sapajus xanthosternos*	[21,26,31,32,58,107,108,136,138,141–148]

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Trypanosome	Non-Human Primate Species	Reference
Trypanosoma devei	*Cebuella pygmaea* *Callimico goeldii* *Leontocebus fuscicollis weddelli* *Leontocebus tamarin* *Saguinus imperator*	[108,149,150]
Trypanosoma diasi	*Alouatta palliata* *Sapajus apella apella*	[27,149]
Trypanosoma forestali	*Alouatta guariba* *Alouatta caraya*	[32]
Trypanosoma hippicum	*Alouatta guariba* *Alouatta seniculus*	[151]
Trypanosoma lambrechti	*Alouatta seniculus* *Cebus albifrons* *Cheracebus torquatus* *Chiropotes satanas* *Pithecia pithecia* *Sapajus apella*	[32,136,151]
Trypanosoma lesourdi	*Ateles paniscus*	[32]
Trypanosoma mycetae	*Alouatta belzebul* *Alouatta belzebul belzebul* *Alouatta palliata* *Alouatta caraya* *Alouatta seniculus* *Chiropotes satanas*	[32,144,148–151]
Trypanosoma minasense	*Alouatta belzebul* *Alouatta caraya* *Alouatta guariba* *Alouatta palliata* *Alouatta seniculus* *Aotus trivirgatus* *Ateles fusciceps* *Ateles geoffroyi griscescens Callithrix jacchus* *Callithrix penicillata* *Cebus albifrons* *Cebus capucinus* *Leontocebus weddelli* *Leontocebus fuscicollis weddelli* *Plecturocebus ornatus* *Saguinus geoffroyi* *Saguinus imperator imperator* *Saguinus midas* *Saimiri sciureus* *Saimiri sciureus macrodon* *Saimiri ustus* *Sapajus apella*	[21,27,32,102,108,136,141,144,148,149,152,153]

Trypanosome	Non-Human Primate Species	Reference
Trypanosoma rangeli like	*Alouatta seniculus* *Alouatta belzebul* *Alouatta caraya* *Cebuella pygmaea* *Cebus albifrons unicolor* *Cebus capucinus* *Callimico goeldii* *Leontocebus fuscicollis weddelli* *Pithecia pithecia* *Saguinus bicolor* *Saguinus bicolor bicolor* *Saimiri boliviensis* *Saimiri ustus* *Saimiri sciureus* *Saguinus geoffroyi* *Saguinus imperator imperator* *Saguinus midas* *Saimiri boliviensis* *Sapajus apella Sapajus libidinosus*	[21,30,108,141,144,148,154,155]
Trypanosoma saimiri	*Saimiri sciureus sciureus*	[150]
Trypanosoma venezuelensis	*Alouatta guariba* *Alouatta seniculus*	[151]

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Experimental section

Study 4:

Perception of biosecurity and risks in Ecuadorian Wildlife Management Units

Submitted

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Preamble

Information related to biosecurity in the Wildlife Management Units (WMUs) at the national level is lacking despite the importance of maintaining good biosecurity practices. The aim of this study was to investigate how tourists, volunteers, and owners of the WMUs perceive the biosecurity measures implemented in these units. The perception of biosecurity of the different stakeholders in the WMUs allowed us to fill gaps in the biosecurity processes.

We developed a biosecurity scoring system to assess biosecurity measures applied by different stakeholders from WMUs including owners, visitors, and volunteers (n = 150). The instruments were applied in person, by telephone or online, in which three domains related to biosecurity in the WMUs were considered, i.e. education, infrastructure and practice. The highest rates for visitors and owners were in education while for volunteers it was infrastructure.

There is a link between biosecurity and biodiversity, therefore biosecurity measures, that prevent the spread of infectious diseases, may be relevant when trying to strengthen the conservation of biodiversityand animal health within the WMUs. The biosecurity conditions reported in this study have room for improvement. Biosecurity must be considered collectively. Specific training on related topics in the study area is not very constant and organized since at the national level there is no initiative to carry them out in a planned and organized framework.

Abstract

Limited information on biosecurity and risks is available within wildlife management units (WMU). This study provides a baseline assessment of biosecurity practices in wildlife refuges and will contribute to efforts to adapt biosecurity programmes to WMUs. To determine the perception of biosecurity in WMUs, three surveys were designed: (i) for owners/administrators, (ii) for visitors and (iii) for volunteers. The online survey was conducted from March 2021 to March 2022, and required 20 min to be completed. A total of 150 complete surveys were answered. A biosecurity index was developed using a multi-criteria decision analysis. Owners/administrators and visitors had a higher index in the domain of education criteria than the other two domains (infrastructure and management practices) while volunteers had a higher score in infrastructure than education and management practices. Most visitors did wash their hands after they left the WMUs. All volunteers and most visitors complied with parking outside the WMU as tourist vehicles can be inanimate vectors of diseases or invasive organisms. In addition to the infrastructure, the presence of a quarantine area was also observed in more than half of the WMUs. The biosecurity conditions reported in this study have room for improvement. Finally, the results obtained in this study should promote biosecurity campaigns and formulate training in biosecurity practices adapted to the reality of WMUs in Ecuador, in order to contribute to the One Health Biosecurity concept.

Keywords: biosecurity; Ecuador; wildlife; management unit; scoring; One Health.

Introduction

Biosecurity means "a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections, or infestations to, from and within an animal population" (WOAH, 2022). According to the Ecuadorian environmental code, 2021, the definition used for the term biosecurity is as follows: "A strategic and integrated approach to the analysis and management of relevant risks to human, animal and plant life and health and related risks to the environment".

Biosecurity issues, as they influence human, animal, plant and environmental health, become a matter of national security. The WOAH (OIE – World Organization of Animal Health) recognizes the importance of wildlife in the transmission of pathogens from both wildlife and domestic animals (Craig, 2021). La Comunidad Andina de Naciones, through its resolutions, establishes the need to monitor wildlife in the different rescue centers (CAN, 2002). The National Agency for Regulation, Control and Phytosanitary Surveillance is responsible for complying with these resolutions in Ecuador (ARCSA, 2020; WOAH, 2015, 2018). In Ecuador, the centres of *ex situ* conservation and management of wildlife species are: nurseries, botanical gardens, zoos, sustainable breeding and reproduction centers, rescue and rehabilitation centers, germplasm banks, aquariums, including biodiversity documentation and registration centres (COA, 2017). In Ecuador, 31 wildlife management units (WMUs) comply with sanitary regulations (ARCSA, 2020).

The regulations governing wildlife in Ecuador fall under the Organic Environmental Code. It includes the biosecurity measures that should be applied to wildlife. These measures must be included in the different management plans that ensure the proper functioning of WMUs (COA, 2017). In addition, inspectors from the Ministry of the Environment, Water and Ecological Transition undertake inspections to verify compliance with regulations, including the implementation of the approved management plan (MAATE, 2015). The environmental licenses granted to WMUs are issued by the national regulated entity after compliance with a series of requirements, including the environmental management plan of the centre, which incorporates biosecurity-related provisions, both for the wildlife species housed and for the visitors and staff of the unit (COA, 2017; MAATE, 2015). The WOAH has declared that 'surveillance of wildlife diseases must be considered equally as important as surveillance and control of diseases in domestic animals' (Craig, 2021).

Worldwide, protocols and structures exist to minimize pathways for the transmission of pathogens and other agents that can affect animals, visitors, volunteers, collaborators and other members of WMUs (CDC, 2023; Wildlife-Health-Australia, 2018). For example in Australia, a zero tolerance biosecurity program seeks to protect the biodiversity of the Barrow Island (Scott *et al*., 2017). Wildlife management units are created out of the need to protect wild animals from species trafficking, hunting,

poisoning and other anthropogenic interactions (Chaves *et al*., 2021). In Ecuador, a first study (Carrillo Bilbao *et al*., 2021) showed that wildlife could pose a risk for the spread of blood borne pathogens in captive settings such as wildlife management units (WMUs). However, information on the perception of biosecurity and to a greater extent of biological risks in WMUs is completely lacking. The perception of biosecurity and biological risks will allow to better understand the related behaviour (Shannon *et al*., 2019). In this case, WMUs biosecurity skills should be built upon good practices (Shannon *et al*., 2019). It is important to recognize the role of wildlife units in the conservation of biodiversity, and at the same time, to consider the risk of exposure to zoonotic diseases involved. Above all, the interactions with wildlife species, which are generally poorly understood, increase the risk of pathogen introduction, and spread (Chaves *et al*., 2021; Karesh, 1995).

In WMUs, the need for adequate and rigorous biosecurity practices is imminent in the face of the growing threat of (re)emerging infectious diseases, primarily zoonotic, such as Lyme disease, monkeypox, severe acute respiratory syndrome, avian influenza and West Nile fever (Boyes *et al*., 1994; CFPSH, 2008; Kelly, 2005; Patterson *et al*., 2005; Vitazkova *et al*., 2006). The emergence of these pathogens and the diseases they can cause in animal and human populations is an inevitable consequence of population demographic changes, globalization, air travel, poverty, pollution, and climate change (Allen *et al*., 2017; Gibb *et al*., 2020; Gould *et al*., 2009; Kelly, 2005; Muthayya *et al*., 2013; Newton-Fisher, 1999; Pollard *et al*., 2000; Saker *et al*., 2004; Schmeller *et al*., 2020; Slingenbergh *et al*., 2004; UNEP, 2020). Many pathogens are highly contagious and poor biosecurity practices, specially regarding handling and confinement practices of wild animals, may facilitate their transmission between infected and uninfected individuals (CDC, 2021; Gerhold *et al*., 2016; Officer *et al*., 2014). Poor biosecurity practices increase the transmission of pathogens (Gray *et al*., 2018). This is one of the reasons for encouraging increased biosecurity measures in recent years (Renault *et al*., 2021). In addition, poor biosecurity could lead to diseases outbreaks in animal and human populations (Gray *et al*., 2018; Silva *et al*., 2013).

The individuals of different species found in WMUs in Ecuador, as in most South American countries, come from different anthropogenic pressures including hunting, illegal trade, pets, and poisoning. Therefore, in Latin America these wildlife management units mainly rescue, rehabilitate, and release native fauna (Karesh, 1995; Romero *et al*., 2019). However, in Latin America, scarce economic resources, lack of information and support from government authorities affect the proper functioning of these WMUs. The COVID-19 pandemic had an impact on the reduction of personnel and the number of visits by veterinary staff (since in many cases their staff are non-permanent). In addition, maintenance costs (such as feeding, nursing, caging) of the species received in the WMUs increased, and the number of animal releases increased as well. These conditions create an environment conducive to the transmission of several diseases (Chaves *et al*., 2021; Sales *et al*., 2019; Stidworthy, 2016). Proper

biosecurity measures and procedures in WMUs are effective in protecting the health of people, wildlife populations and thus ecosystems. It is also important to minimize the risk of cross-contamination of samples collected from trapped wildlife to ensure the validity of epidemiological studies that rely on such samples (Hillman *et al*., 2016).

Therefore, the objective of this study was to determine the perception of biosecurity knowledge and its application in WMUs, as a prerequisite to create an input to promote the correct application of biosecurity measures in these units (Unwin *et al*., 2009).

Material and methods

General Aspects

To determine the perception of biosecurity in WMUs, three surveys were designed and validated by experts from the University of Liege and the University of the Armed Forces of Ecuador, one for each actor: owners/administrators (first survey), visitors (second survey) and volunteers (third survey).

Target Population

Due to COVID-19 pandemia, a first online survey (**Appendix 1**) was created using SurveyMonkey, to gather information on the perception of biosecurity among owners/ administrators from WMUs in Ecuador. A second survey (**Appendix 2**) allowed gathering information from visitors (tourists who visit WMUs) and a third survey targeted volunteers (people who collaborate to the different daily activities within the facilities of the WMUs) (**Appendix 3**). The three surveys were pre-tested by one owner/administrators of the WMUs, a visitor and volunteers of a WMU, where the respective corrections were made with the objective of improving clarity and including the missing aspects.

Visits were made to the different WMUs to determine at first-hand whether they were open to implement the survey on the perception of biosecurity in their facilities, as the WMUs were going through different complications (social and economic) due to the COVID-19 pandemic. In addition, there was little knowledge of biosecurity and its correlation with wildlife, so awareness needed to be raised prior to the implementation of the survey.

Questionary Development

In the three surveys, owners/administrators, visitors and volunteers, we sought to collect demographic information, biosecurity knowledge, biosecurity practices, included training in biosecurity, infrastructure, related to biosecurity in the WMU. They were conducted in person and online using the Surveymonkey® application, from March 2021 to March 2022. They required 20 min to be completed. In the invitation request, we reminded the participants that all data would be collected and analysed anonymously.

Invitations to complete the surveys were sent through their personal and institutional e-mails, by telephone and through social networks and WMUs on an individual basis. Some surveys were sent to volunteers and visitors we already knew from past visits. In parallel to the online surveys, on-site

visits (n = 8) to the WMUs were performed: visitors and volunteers were selected randomly according to their presence at the moment of the visit.

The first survey (owners/administrators) included a total of 68 questions, of which 61 were closed questions and seven were open questions. It was applied both in person (1) and online (7), due to mobility and COVID-19 logistical restrictions, after previous explanation to the 31 owners/administrators of the WMUs.

This survey has a total of fourth sections, the first section corresponds to the identification of the respondent and general activities and included 13 questions. The second section included questions on general biosecurity knowledge (N = 4 questions). The third section corresponded to the biosecurity practices (N = 22 questions). Finally, the fourth section focused on the infrastructure of the WMUs (N = 29 questions).

The second survey (visitors) was applied mostly online (n = 119) and two were conducted in person which included a total of 46 questions; of which 39 were closed questions and seven were open. The first section which corresponds to the identification of the respondent included 11 questions. The second section included two questions on general biosecurity knowledge. The third section corresponded to the biosecurity practices and included 20 questions. Finally, the fourth section focused on the infrastructure of the WMUs and included 13 questions.

The third survey (volunteers) was applied online (n = 7) and in person (n = 14), and included a total of 71 questions; of which 62 were closed questions and nine were open. This survey has a total of fourth sections. The first section corresponds to the identification of the respondent, and included 12 questions. The second section included two questions on general biosecurity knowledge. The third section corresponded to the biosecurity practices and included 32 questions. Finally, the fourth section focused on the infrastructure of the WMUs and included 25 questions.

The third survey was only sent and applied during visits to the Ecuadorian Amazon region (Pastaza and Nueva Loja Province) because this region was the only one to have a large number of WMUs to respond the survey.

The different variables considered in the questions of the survey were based on previous on site visits (past fieldwork in the WMUs), as well as the bibliographic support developed in Shannon *et al*. (2019).

Additionally, during eight physical visits, previous to the surveys, the observation of aspects related to biosecurity was considered; among them, we determined personal hygiene, cleaning activities, control, and prevention of aspects that can spread diseases: quarantine, sterilization (e.g. for surgical

equipment), protective clothing, disinfection of material/equipment (food handling tools, rubber boots) and areas (Varela-Arias *et al*., 2014).

Data Analysis.

Questionary distribution.

The survey for the owners/administrators was applied to all 31 WMUs registered in Ecuador in 2020 by Agrocalidad (ARCSA, 2020).

We visited eight units whom participants responded to the questions. During this visit, one looked at whether the WMU provided hand washing stations at the animal exhibit, or whether it trained staff and volunteers on how to prevent illness after being in proximity to animals. We also observed whether the center had signs for visitors about when and how to wash their hands, where they can eat, and if animal areas were identified with appropriate signage. Finally, we looked at whether they separated animal food handling areas from the animals, and whether they provided staff with resources on animal exhibit safety, especially if their staff included veterinary health care providers.

Scoring system

Questions on biosecurity related to WMUs were classified into three groups depending on the domains of criteria, i.e. education on biosecurity, practices, and infrastructure. Only answered questions were scored (e.g. presence/absence; ordinal ranking within several modalities). The maximum was calculated for each question based on the response options and categorizing these options as ¨best¨ if they adhered to best modalities and ¨worst¨ if they were far from it. Because a question with more modalities has more chance to have more points in a question and the fact that a question should have more importance to another question in terms of biosecurity, each question was weighted relatively to the other. This weighting process of each question was obtained by consensus between the authors. A pondered score for each of the three groups of questions (education, practices, and infrastructure) and an overall pondered score for each respondent were calculated. All pondered scores were standardised on a scale ranging from 0 to 100. For each of the three groups of respondents (owners/administrators, visitors and volunteers), an example of the scoring applied was presented in Appendixes 1, 2 and 3.

Statistical Analysis and graphical representation:

A spider tool provided a visual comparison of the domains of criteria in relation with the perception of biosecurity in the WMUs of the Amazonian region, in Ecuador. The domains of criteria we used were: education on biosecurity, biosecurity-related practices, and infrastructures. A positive score from a respondent was considered if he complies with the positif aspect related to biosecurity.

The spider web tool assessed the different weighted responses for each domain of criteria (i.e. education, practices, and infrastructure), with the average of respondents. The barometer index tool was used to aggregate the three domains of criteria according to an index ranging from 0 to 100.

A Shapiro-Wilk test was performed to assess the normal distribution of data. A Kruskal-Wallis (H) test investigated the differences between the domains of criteria in the index score for visitors and owners/administrators. An Anova test (F) assessed the differences between domains of criteria in the index score for volunteers. All statistical tests were done using SPSS® software. A significance level of alpha <0.05 was set for all statistical analyses. All data was collected, stored, and analysed anonymously.

Definition of incident and accident

An accident is defined as an event that has occurred unintentionally and results in damage, injury or harm: when someone is cutting the fruits for the animals and cut himself with a knife. An incident is defined as an event that has occurred unintentionally, but may not result in damage, injury, or harm: when an animal escape from the cage but is trapped again without anyone being hurt.

Results

A total of 150 complete surveys were completed by, respectively, eight owners/administrators, 121 visitors and 21 volunteers.

General descriptive analysis

The first survey (owners/administrators) was sent to a total of 31 WMUs, distributed nationwide, but only eight of them completed the survey, i.e. the Quito City Zoo, and seven WMUs located in the Ecuadorian Amazon region (six in the Province of Pastaza and one in the province of Nueva Loja).

The number of people working in WMUs ranges from 2 to 48. In six WMUs, owners/administrators had only indirect contacts with animals (no direct contact). Only one owner considered that he had a high knowledge of biosecurity measures, while six of them consider their level of knowledge as intermediate. Only half of the WMUs had a year-round education or training program on biosecurity and environmental related topics for their personal. Most WMUs (N = 6) release wildlife.

The second survey was only sent to persons known as visitors (119) to the Ecuadorian Amazon region (Pastaza and Nueva Loja Province), either by the administrators or by the surveyors, according to the register kept in the WMUs; on site visits were organised (2). Visitors consider their level of biosecurity knowledge to be high to intermediate whereas owners/administrators and volunteers considered their level of biosecurity knowledge as intermediate (Table 1).

Table 1. Profiles of the respondents

Variable		Owners/ Administrators (first survey)	Visitors (second survey)	Volunteers (third survey)
Number of Respondents		8	121	21
Gender	Female	3	60	8
	Male	5	61	13
Education	Elementary	0	5	0
	High school	1	45	13
	Graduate	3	51	3
	Posgraduate	4	20	5
Age (years old)	17-30	0	33	16
	>30-40	3	44	5
	>40-50	4	28	0
	>50	1	16	0
Biosecurity knowledge	High	1	52	6
	Intermediate	4	54	15
	Low	1	15	0
	Do not know	1	0	0

According to the third survey, volunteers conduct a range of activities including: from work planning and supervision to cage cleaning while volunteers are there for tree specific roles: cage cleaning, animal care and handling, and food handling (**Fig 1**).

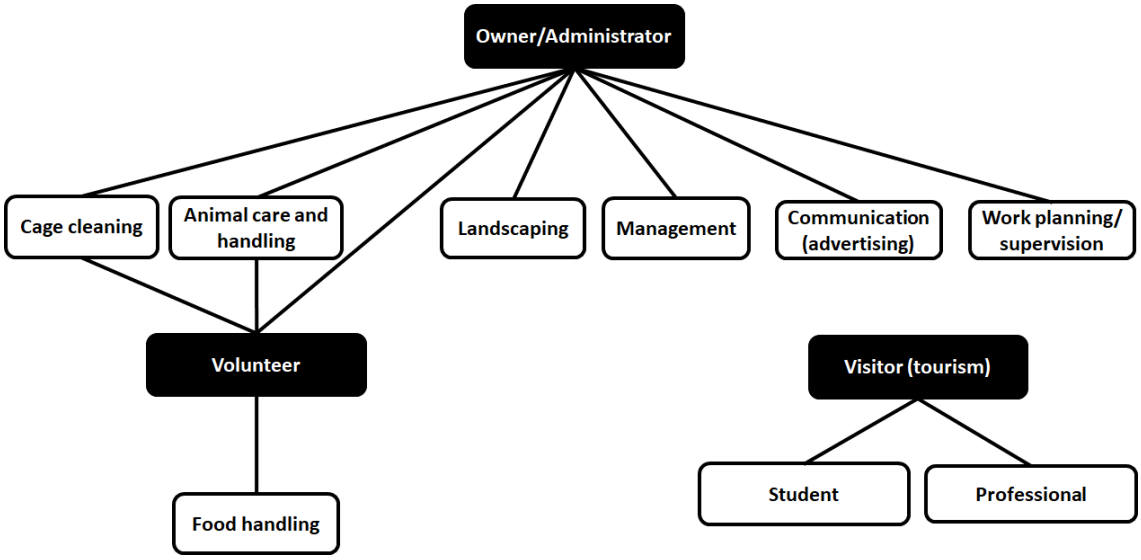

Figure 1. Social Network Analysis showing the roles respondent play in the Wildlife Management Unit according to the responses obtained in the survey.

Perception of the risk towards infectious biological fluids perception

The majority of visitors considered fluids such as blood (N = 85; 70.3 %) and sweat (N = 71; 62.8 %) as potentially infectious. Among volunteers, the majority considered semen, feces and saliva a risk (**Fig 2**). Owners/administrators perceived blood and feces as potentially infectious.

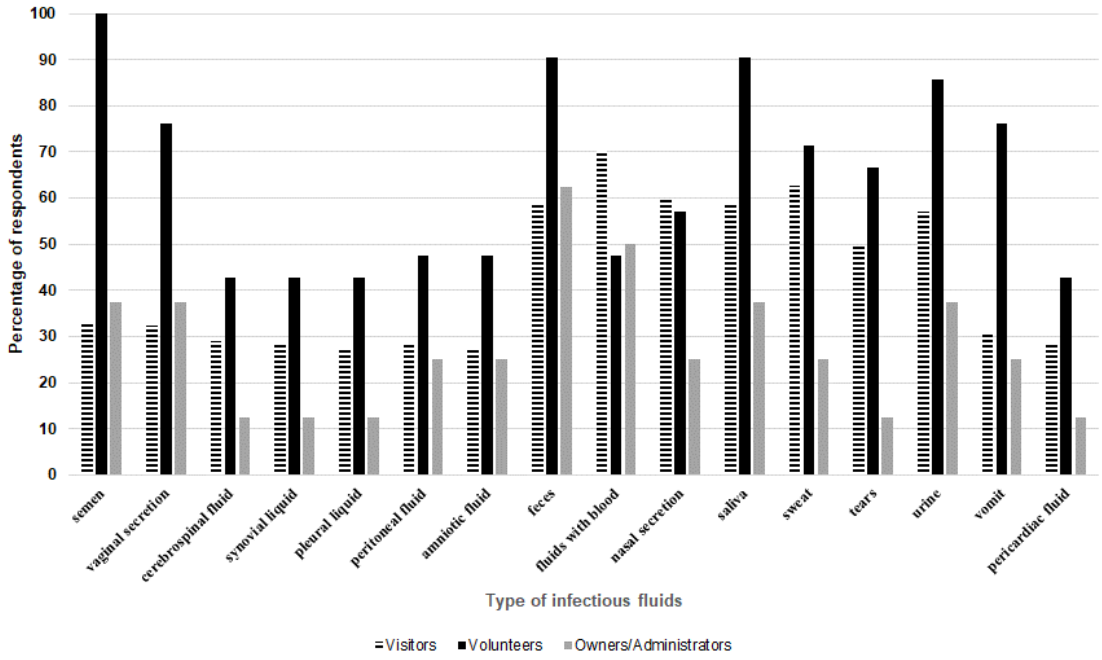

Figure 2. Perception of the risk represented by the type of infectious fluids from the respondents' point of view based on their profile.

Perception according to the three domains of criteria

Owners (69.6%, H = 27.443, $p < 0.05$) and visitors (63.6%, H = 149.408, $p < 0.05$) had a higher index score in education domain of criteria than the other two domains of criteria (infrastructure and practices) (**Fig 3**), while volunteers had a higher score in the 'Infrastructure' domain of criteria (56.1 %, F = 9.643, $p < 0.05$) vs. the education and practices domains of criteria.

However, in the overall average score with the barometer index, visitor had a significant higher score, i.e. 57.1 %, while owners/administrators and volunteers had 40.6 % and 40.7 %, respectively (H = 43. 359, $p < 0.05$) (**Fig 4** and **Fig 5)**.

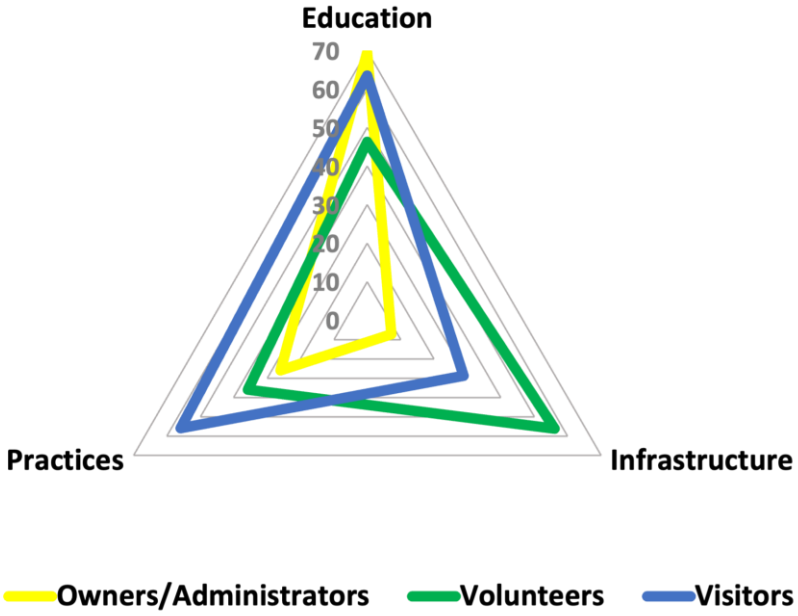

Figure 3. Spider score for each domain of criteria (Education, Practices, Infrastructure) according to the different categories of respondents (Owners/Adminstrators, Volunteers and Visitors).

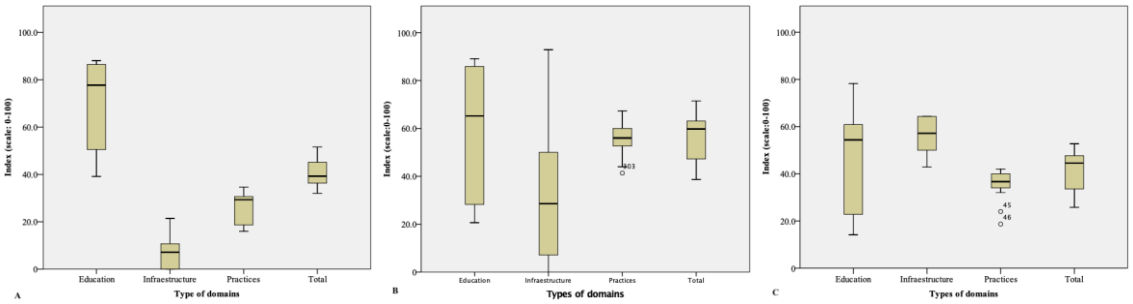

Figure 4. Overall average score for owners/administrators (A), visitors (B) and volunteers (C) for the three domains of criteria, i.e. education, infrastructure, and practices.

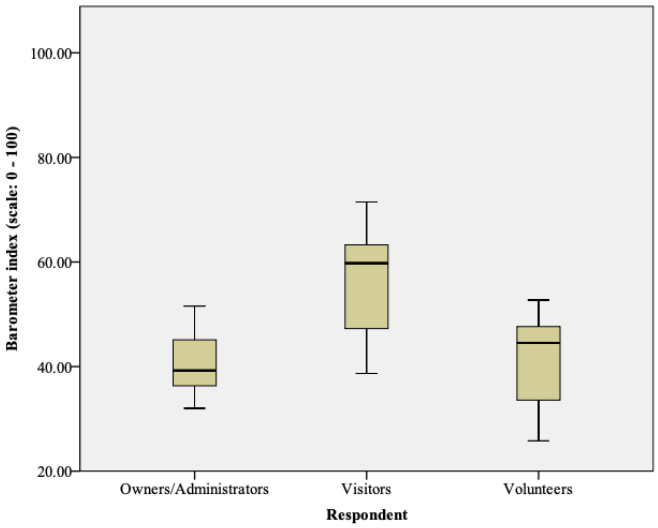

Figure 5. Barometer index per category of respondents (Owners/Adminstrators, Volunteers and Visitors).

Biosecurity incidents and accidents

Only two visitors and one volunteer suffered accidents, i.e. animal bites. All volunteers did, however, witness biosecurity problems related to animal bites (accidents) and animal escapes (incidents). Visitors had direct (N = 38; 31.4 %) and indirect contacts (N = 83; 68.6 %) with animals, most of them (N = 75; 62.0 %) being attracted to touching the animals.

Protection practices

Most visitors (N = 95; 78.5 %) mentioned they had not received an introductory talk on biosecurity before entering the WMU circuit. Furthermore, 55.4 % of them (N = 67) did not wash their hands before entering the WMU. Nevertheless, 79.3% of them (N = 96) did wash them after or upon leaving the WMU (**Fig 6**). A mask, gloves and rubber boots were not commonly worn during their visits, as illustrated in Figure 6.

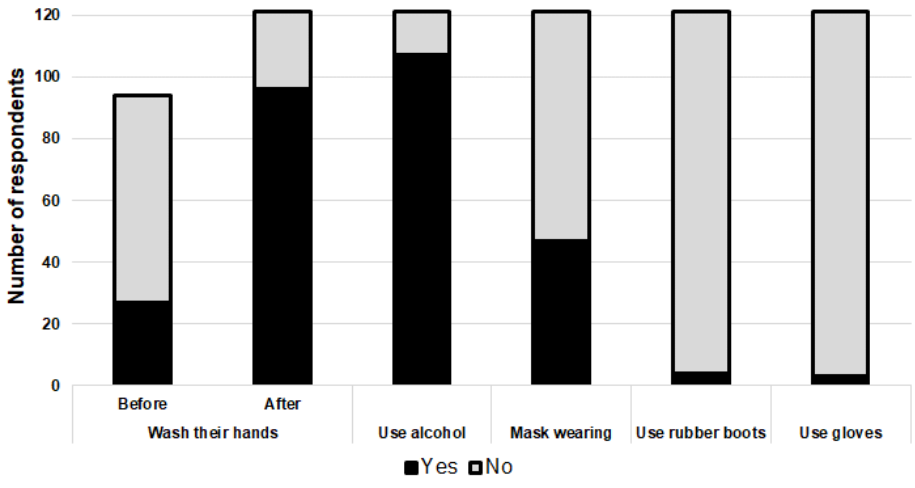

Figure 6. Biosecurity practices applied by visitors who visited wildlife management units.

All volunteers washed their hand before and after their day at the WMU. Only one volunteer did not wash his/her hands during the day. Just a few volunteers (N = 5; 23.8 %) did not use alcohol to disinfect their hands (**Fig 7**). Of those who did use alcohol, all of them (N = 16) used it for food handling, seven of them to disinfect enclosures and ten of them before handling animals. Masks were not worn by volunteers during their workday; however, they did use rubber boots and gloves.

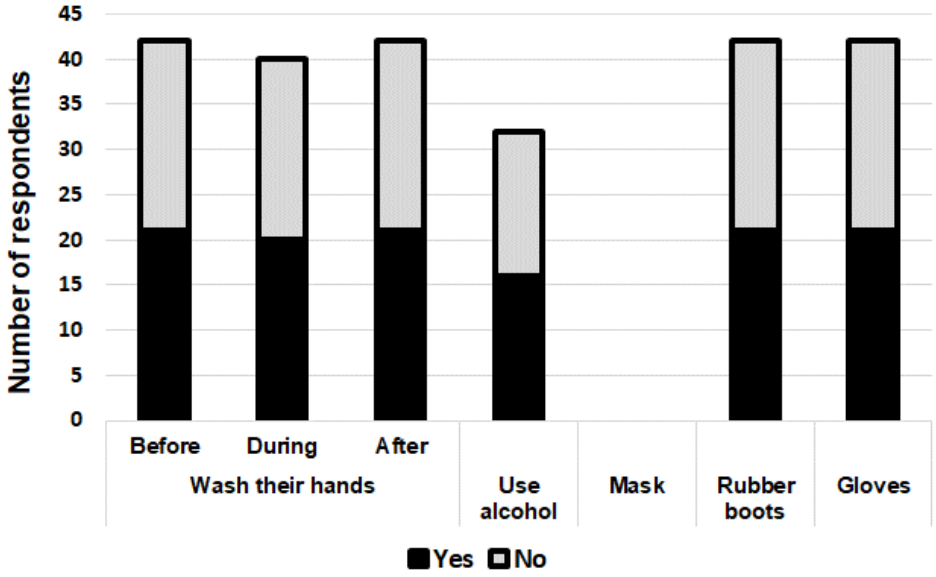

Figure 7. Biosecurity practices applied by volunteers who worked in wildlife management units during their stay.

Infrastructures

All volunteers and most visitors observed a parking lot outside the WMU (N = 117; 96.7 %) and the delimitating fence (N = 80.1 %) surrounding the WMU. However, 14.9 % of visitors (N = 18) did not respect the fence surrounding animal cages, an observation that 57.1 % of the volunteers (N = 12) made as well. In addition to the infrastructure, the presence of a quarantine area was also observed by more than half of the volunteers, but four of them were not aware of the existence of a quarantine zone in the WMU. Finally, all volunteers worked in a specific area for food handling.

Discussion

It is important to note that the results of this study were influenced by the poor management of the pandemic in this sector where there is a link between public health, animal health and the environment. Of the WMUs visited, four had workers with COVID-19 symptoms, and others had a limited number of workers. In addition, most had limited information on COVID-19, and had no government support. This situation was compounded by the limited financial resources of the WMUs. This pattern was identified in several WMUs in Latin America (Chaves *et al*., 2021) .

Surveillance of wildlife diseases is an essential link between biosecurity and biodiversity (Astorga *et al*., 2023; Bartlow *et al*., 2021). All WMUs play an important role: they are actors within the One Health perspective as they contribute to the different areas of conservation (Godinez *et al*., 2019; Spiezio *et al*., 2022), education (Gusset *et al*., 2011; Miranda *et al*., 2023) and monitoring of wildlife diseases (Caballero-Gómez *et al*., 2022; Carrillo Bilbao *et al*., 2021; Kvapil *et al*., 2021).

Biosecurity measures help preventing the introduction and spread of infectious diseases. On livestock farms, it helps enhancing productivity by improving animal health (Maye *et al*., 2020; Renault *et al*., 2020). In WMUs, biosecurity helps improving *ex situ* and *in situ* conservation programmes (Stephen *et al*., 2018). Many wildlife pathogens are specific to wildlife (Hoyt *et al*., 2021; Koutsoumanis *et al*., 2023; Upshall *et al*., 1987). However, some pathogens can be transmitted to humans and/or domestic animals (Revilla *et al*., 2018) under certain conditions. Considering the zoonotic outbreaks worldwide (Casimiro-Soriguer *et al*., 2021; Cohen, 2019; Ferraris *et al*., 2019; Wang *et al*., 2021) and especially the COVID-19 pandemic, the importance of biosecurity has increased constantly (Hulme, 2020). Tourist could potentially spread pathogens between species in a same WMU, or between populations of different WMUs, if biosecurity protocoles are not enforced, especially for zoonotic diseases. Biosecurity in WMUs includes, not only prevention of disease transmission on the premises, and prevention between WMUs but also look after human and environmental health at a regional level. Biosecurity also limits the introduction of infectious agents. Animals can be translocated from one unit to another, and as such, pathogens can be introduced in a disease-free unit (Reis *et al*., 2020).

Hand washing is a basic hygiene measure that has been first described by Semmelweis in 1847 (Tyagi *et al*., 2020). If WMUs do not have handwashing facilities or alcohol dispensers, it is very likely that the visitor will not wash their hands, nor will volunteers do so during the day. Most of the time, the incentive to wash hands because they are macroscopically dirty (people see their hands are dirty). Hand washing after visiting facilities prevent the spread of pathogens between facilities but also helps preventing the infection of people by zoonotic pathogens. However, the low record of hand washing before and during visits indicates that these recommendations should be given daily. To make this

activity a daily routine, it can be illustrated throughout the WMUs with diagrams, photographs and best practice tips. One option is to have posters with pictograms in the WMUs at different critical locations, for example before the entrance and on the route inside the WMU to encourage visitors to wash their hands. The use of posters with biosecurity information, such as the risks of airborne pathogens, can as well increase the knowledge and enhance the awareness of biosecurity among visitors. This may help decreasing the risk of pathogen transmission between sites and within populations of animals in the WMU. The use of face masks by visitors is thought to be associated with the COVID-19 pandemic. People wore masks maybe because the survey was performed during the pandemic, thus with SARS-CoV-2 in mind only, but not caring about other potentially zoonotic pathogens. Volunteers use gloves, but do not wear masks, so it would be advisable to request their use, as they work directly or indirectly with animals potentially infected by airborne pathogens. Any person suffering from an infectious respiratory disease (SARS-CoV-2, Tuberculosis, Influenza, among others) should avoid any contact with non-human primates.

Regardless of whether volunteers or visitors implement biosecurity or not, the importance lies in the conditions offered by the management units to comply with biosecurity standards, from infrastructure to formal or non-formal training. Differences in practices might be linked with resources availability in terms of budget, personnel, and infrastructures. The presence of parking facilities in the WMUs is a good biosecurity tool, as tourist vehicles can be inanimate vectors of diseases or invasive organisms (Alarcón *et al*., 2019; Eritja *et al*., 2017; Hall, 2011; Lonsdale *et al*., 1994; Meitiv *et al*., 2020; Tasmania, 2016). In terms of training, the WMU could discuss with volunteers the factors affecting biosecurity according to the species present in the WMU, in order to assess or develop appropriate plans adapted to the realities of the WMUs.

Several studies assessed the transport of pathogens or disease vectors by tourists (Barbosa *et al*., 2021; Carne *et al*., 2017; Iaquinto, 2020; Odeniran *et al*., 2018; Sente *et al*., 2022; Wilson, 1995). For example, in Spain, cars transported passively *Aedes albopictus*, mosquitoes from one province to another, facilitating as such the spread/movements of mosquitoes between provinces (Eritja *et al*., 2017). In this study, biosecurity awareness was high, however respondents' answers regarding their perception of the risk represented by infectious biological fluids demonstrated a lack of understanding on how infectious agents can spread through indirect contact. These results should allow to better promote biosecurity among the population. Other countries that analysed the perception of people and tourists were New Zealand and Ireland. In New Zealand (MPI, 2018), they found out that at national level (75%), people understand the mean of biosecurity. In Ireland, however, awareness of biosecurity among tourists was low (Melly *et al*., 2020).

In this study, the education domain had the highest index. In Switzerland, for example, the education of farm owners is considered to be the most important factor for both the protection of animals

and the prevention of disease transmission (Kuster *et al*., 2015). In other studies, the education level was associated with a high value of understanding on risk perception and biosecurity (Hanisch-Kirkbride *et al*., 2013; Racicot *et al*., 2012).

The main limitation of this study is linked to the current un-availability of sanitary records and incidents/accidents in all the WMUs visited/interviewed. The integration of this information in new studies will be a strong added value to validate the proposed scoring system. However, less sanitary/security problems are expected when the biosecurity index is high, as previously demonstrated with other animals' species (e.g. El Agrebi *et al*., 2021; Renault *et al*., 2020; Steinhauer *et al*., 2021).

Conclusions and recommendations

The aim of this study was to investigate how visitors, volunteers and owners/administrators of the WMUs perceive the biosecurity measures in place in these units. Other factors that could be analysed and improved in the future during biosecurity surveys are the availability of alcohol dispensers and/or hand-washing facilities at critical locations of the WMU.

The perception of biosecurity of the different stakeholders allows to fill the gaps in the biosecurity protocols. The idea is to use these data to improve biosecurity in the WMUs and its perception among visitors. The biosecurity conditions reported in this study have room for improvement. Biosecurity must be considered collectively, and be the responsibility of alla, i.e. visitors, volunteers, owners/administrators along with the government support (Heffernan *et al*., 2008). All WMUs have a responsibility to provide appropriate biosecurity conditions but visitors and volunteers must understand their responsibility and be aware of their role as potential disease spreaders. This study can be used as a tool at the local level to identify risk pathways in WMUs, and at the regional level, to assess human actions that could influence the implementation of biosecurity. Finally, the importance of informing visitors and volunteers should be adopted by WMUs and local goverments. Proposing regulations governing biosecurity standards in WMUs to reduce the risk of disease transmission and the risk of outbreaks would be a way to guide biosecurity managers. Finally, the results obtained in this study should promote biosecurity campaigns and formulate training in biosecurity practices adapted to the reality of WMUs in Ecuador, in order to contribute to the One Health Biosecurity concept (Hulme, 2020).

Acknowledgments

To all the Wildlife Management Units that have selflessly opened their doors to us during several years of collaboration.

Appendix 1. Questionnaire survey for owners/administrators and an example of its scoring system

Section	Question	Modalities	Bareme (high= better)	Example of response	Score observed	Score max	Weight of the question	Pondered score	Pondered score max
Practices	During your visit, did you have any contact with wild animals?	Direct	0	1	0	2	20	0	40
		Indirect	1						
		No	2						
Practices	If you had a contact with wild animals, what was the reason for it?	Food handling	3		11	15	20	220	300
		Enclosure cleaning	4	1					
		Attracted to touch animals	2						
		Animal handling	1						
		Other	5						
Education	You consider your knowledge of biosecurity as	High	0		2	2	10	20	20
		Medium	1						
		Low	2	1					
Education	Which of the following animal secretions do you consider to be infectious fluids?	Semen	1		11	16	20	220	320
		Vaginal secretions	1	1					
		Cerebrospinal fluid	1						
		Synovial fluid	1						
		Pleural fluid	1						
		Peritoneal fluid	1						
		Amniotic fluid	1						
		Stool	1						
		Fluids containing visible blood	1						
		Nasal secretions	1	1					

		Saliva	1	1					
		Sweat	1						
		Tears	1						
		Urine	1	1					
		Vomit	1	1					
		Pericardial fluid	1						
Practices	Did you receive a lecture on biosecurity prior to the start of your visit?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						
Practices	In the case of collaborating or having collaborated as a volunteer and/or support staff, can you tell us if you have received clear instructions on the steps to be followed in the quarantine area?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						
Practices	Your visit and your data were recorded at the beginning of this visit?	Yes	0	1	0	1	15	0	15
		No	1						
		I don't known	1						
Practices	Did you wash your hands before your visit?	Yes	0		3	5	20	60	100
		No	1	1					
	Did you wash your hands during your visit?	Yes	0						
		No	1						
	Did you wash your hands after your visit?	Yes	0						
		No	1						
	Did you use alcohol or other disinfectant to sanitize your hands?	Yes	0						
		No	1	1					
	Do you use alcohol or alcohol gel to disinfect your hands in a general way?	Yes	0						
		No	1	1					

Practices	During your visit did you suffer any incident related to biosecurity and wild species?	Yes	1	1	1	1	25	25	25
		No	0						
Education	Of the events listed below, do you consider them related to biosecurity?	Animal bite	1	1	2	3	25	50	75
		Contact with contaminated organic waste	1						
		Animal Corporal Fluids.	1						
Practices	If you suffered an accident related to biosecurity, could you tell us what it was and with which species?	Yes	0		1	1	25	25	25
		No	1	1					
Practices	Was the accident you had reported to the staff of the rescue center?	Yes	0		1	1	20	20	20
		No	1	1					
		I don't known	1						
Practices	Which of the following actions did the rescue center take after the accident?	The center's administrators applied some type of protocol (initial evaluation, instructions on actions to be taken)	0		0	1	20	0	20
Practices		Provided medical supervision	0	1					
Practices		None	1						
Practices		I don't known	1						
Practices	During your visit, did you use gloves?	Yes	0		3	3	15	45	45
Practices		No	1	1					
Practices	During your visit, did you wear rubber boots?	Yes	0						
Practices		No	1	1					
Practices	During your visit, did you wear face masks?	Yes	0						
Practices		No	1	1					
Practices	During your visit, did you witness any incident with the animals on exhibit?	Yes	1	1	5	12	10	50	120
Practices		No	0						

Practices	If the answer is yes, which types of incident(s) did you witness?	Biting	4	1					
Practices		Stinging	3						
Practices		Animal escape	2						
Practices		Other	1						
Practices	The bitten person I have witnessed knows if he/she was reported to the Center Administrator?	Yes	0						
Practices		No	1						
Infrastructure	Is there a parking lot on the perimeter of the facility visited?	Yes	0		1	1	10	10	10
Infrastructure		No	1	1					
Infrastructure		I don't known	1						
Infrastructure	The entire unit has a fence or enclosure surrounding it?	Yes	0	1	0	1	15	0	15
Infrastructure		No	1						
Infrastructure		I don't known	1						
Infrastructure	Do all cages or enclosures visited have a fence separating you from the cage?	Yes	0	1	0	1	10	0	10
		No	1						
Infrastructure	Do you have a map available and visible with all areas and legends?	Yes	1	1	1	1	5	5	5
		No	0						
Education	Have you received training in aspects related to biosecurity during your visit ?	Yes	0	1	0	3	15	0	45
		No	1						
	During your visit, did you participate to any education or training program on biosecurity or environment ?	Yes	0						
		No	1						
	During your visit, did you participate to any education or training program on environment ?	Yes	0						
		No	1						

	During your visit, did you participate to any education or training program on biosecurity?	Yes	0	1					
		No	1						
Practices	During your visit, have you appreciated that animal enclosures were correct?	Yes	0	1	0	1	10	0	10
		No	1						
		I don't remember	1						
Practices	During your visit, did you consider that the animal enclosures you saw maintained the correct characteristics that would prevent any incidents with visitors such as escapes or attacks ?	Yes	0		1		15	15	0
		No	1	1					
Infrastructure	Does the Center you visited have an outdoor parking lot?	Yes	0	1	1	2	10	10	20
		No	1						
		I don't known	1						
Infrastructure	If the answer was yes, can you tell us if the parking lot is	Inside the center	1	1					
		Outside the center	0						
Infrastructure	Does the Center you visited have animal cages or enclosures that are resistant and prevent animals from escaping?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						

Total	**47**	**76**	**Total pondered**	**820**	**1285**

Barometer % using index 100 **63,81**

Spider	**Domain**	**Pondered score**	**Pondered score max**	**Index**
	Education	290	460	**63,04**
	Infrastructure	40	75	**53,33**
	Practices	490	750	**65,33**

Appendix 2. Questionnaire survey for visitors and an example of its scoring system

Section	Question	Modalities	Bareme (high= better)	Example of response	Score observed	Score max	Weight of the question	Pondered score	Pondered score max
Practices	During your visit, did you have any contact with wild animals?	Direct	0	1	0	2	20	0	40
		Indirect	1						
		No	2						
Practices	If you had a contact with wild animals, what was the reason forit?	Food handling	3		11	15	20	220	300
		Enclosure cleaning	4	1					
		Attracted to touch animals	2						
		Animal handling	1						
		Other	5						
Education	You consider your knowledge of biosecurity as	High	0		2	2	10	20	20
		Medium	1						
		Low	2	1					
Education	Which of the following animal secretions do you consider to be infectious fluids?	Semen	1		11	16	20	220	320
		Vaginal secretions	1	1					
		Cerebrospinal fluid	1						
		Synovial fluid	1						
		Pleural fluid	1						
		Peritoneal fluid	1						
		Amniotic fluid	1						
		Stool	1						
		Fluids containing visible blood	1						
		Nasal secretions	1	1					
		Saliva	1	1					

		Saliva	1	1					
		Sweat	1						
		Tears	1						
		Urine	1	1					
		Vomit	1	1					
		Pericardial fluid	1						
Practices	Did you receive a lecture on biosecurity prior to the start of your visit?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						
Practices	In the case of collaborating or having collaborated as a volunteer and/or support staff, can you tell us if you have received clear instructions on the steps to be followed in the quarantine area?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						
Practices	Your visit and your data were recorded at the beginning of this visit?	Yes	0	1	0	1	15	0	15
		No	1						
		I don't known	1						
Practices	Did you wash your hands before your visit?	Yes	0		3	5	20	60	100
		No	1	1					

	Did you wash your hands during your visit?	Yes	0						
		No	1						
	Did you wash your hands after your visit?	Yes	0						
		No	1						
	Did you use alcohol or other disinfectant to sanitize your hands?	Yes	0						
		No	1	1					
	Do you use alcohol or alcohol gel to disinfect your hands in a general way?	Yes	0						
		No	1	1					
Practices	During your visit did you suffer any incident related to biosecurity and wild species?	Yes	1	1	1	1	25	25	25
		No	0						
Education	Of the events listed below, do you consider them related to biosecurity?	Animal bite	1	1	2	3	25	50	75
		Contact with contaminated organic waste	1						
		Animal Corporal Fluids.	1						
Practices	If you suffered an accident related to biosecurity, could you tell us what it was and with which species?	Yes	0		1	1	25	25	25
		No	1	1					
Practices	Was the accident you had reported to the staff of the rescue center?	Yes	0		1	1	20	20	20
		No	1	1					
		I don't known	1						
Practices	Which of the following actions did the rescue center take after the accident?	The center's administrators applied some type of protocol (initial evaluation, instructions on actions to be taken)	0		0	1	20	0	20
		Provided medical supervision	0	1					

		None	1						
		I don't known	1						
Practices	During your visit, did you use gloves?	Yes	0		3	3	15	45	45
		No	1	1					
	During your visit, did you wear rubber boots?	Yes	0						
		No	1	1					
	During your visit, did you wear face masks?	Yes	0						
		No	1	1					
Practices	During your visit, did you witness any incident with the animals on exhibit?	Yes	1	1	5	12	10	50	120
		No	0						
	If the answer is yes, which types of incident(s) did you witness?	Biting	4	1					
		Stinging	3						
		Animal escape	2						
		Other	1						
	The bitten person I have witnessed knows if he/she was reported to the Center Administrator?	Yes	0						
		No	1						
Infrastructure	Is there a parking lot on the perimeter of the facility visited?	Yes	0		1	1	10	10	10
Infrastructure		No	1	1					
Infrastructure		I don't known	1						
Infrastructure	The entire unit has a fence or enclosure surrounding it?	Yes	0	1	0	1	15	0	15
Infrastructure		No	1						
Infrastructure		I don't known	1						
Infrastructure	Do all cages or enclosures visited have a fence separating you from the cage?	Yes	0	1	0	1	10	0	10
Infrastructure		No	1						
Infrastructure	Do you have a map available and visible with all areas and legends?	Yes	1				5	0	0

Infrastructure		No	0						
Education	Have you received training in aspects related to biosecurity during your visit?	Yes	0	1	0	3	15	0	45
		No	1						
	During your visit, did you participate to any education or training program on biosecurity or environment?	Yes	0						
		No	1						
	During your visit, did you participate to any education or training program on environment?	Yes	0						
		No	1						
	During your visit, did you participate to any education or training program on biosecurity?	Yes	0	1					
		No	1						
Practices	During your visit, have you appreciated that animal enclosures were properly labeled?	Yes	0	1	0	1	10	0	10
		No	1						
		I don't remember	1						
Practices	During your visit, did you consider that the animal enclosures you saw maintained the correct characteristics that would prevent any incidents with visitors such as escapes or attacks?	Yes	0		1		15	15	0
		No	1	1					
Infrastructure	Does the Center you visited have an outdoor parking lot?	Yes	0	1	1	2	10	10	20
		No	1						
		I don't known	1						
	If the answer was yes, can you tell us if the parking lot is	Inside the center	1	1					
		Outside the center	0						
Infrastructure	Does the Center you visited have animal cages or enclosures that are resistant and prevent animals from escaping?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						

Barometer				% using index 100	63,67
Spider	**Domain**	**Pondered score**	**Pondered score max**		**Index**
	Education	290	460		**63,04**
	Infrastructure	35	70		**50,00**
	Practices	490	750		**65,33**

Appendix 3. Questionnaire survey for volunteers and an example of its scoring system

Section	Question	Modalities	Bareme (high= better)	Example of response	Score observed	Score max	Weight of the question	Pondered score	Pondered score max
Practices	During your visit, did you have any contact with wild animals?	Direct	0	1	0	2	20	0	40
		Indirect	1						
		No	2						
Practices	If you had a contact with wild animals, what was the reason for it?	Food handling	3		4	15	20	80	300
		Enclosure cleaning	4	1					
		Attracted to touch animals	2						
		Animal handling	1						
		Other	5						

Education	You consider your knowledge of biosecurity as	High	0		2	2	10	20	20
		Medium	1						
		Low	2	1					
Education	Which of the following animal secretions do you consider to be infectious fluids?	Semen	1		11	16	20	220	320
		Vaginal secretions	1	1					
		Cerebrospinal fluid	1						
		Synovial fluid	1						
		Pleural fluid	1						
		Peritoneal fluid	1						
		Amniotic fluid	1						
		Stool	1						
		Fluids containing visible blood	1						
		Nasal secretions	1	1					
		Saliva	1	1					
		Sweat	1						
		Tears	1						
		Urine	1	1					
		Vomit	1	1					
		Pericardial fluid	1						
Practices	Did you receive a lecture on biosecurity prior to the start of your visit/Did you perform cleaning and disinfection activities during your volunteering or collaborating?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						
Practices	In the case of collaborating or having collaborated as a volunteer and/or support staff, can you tell us if you have received	Yes	0		1	1	15	15	15
		No	1	1					

	clear instructions on the steps to be followed in the quarantine area?	I don't known	1						
Practices	Your visit and your data were recorded at the beginning of this visit?	Yes	0	1	0	1	15	0	15
		No	1						
		I don't known	1						
Practices	Did you wash your hands before your visit?	Yes	0		3	5	20	60	100
		No	1	1					
	Did you wash your hands during your visit?	Yes	0						
		No	1						
	Did you wash your hands after your visit?	Yes	0						
		No	1						
	Did you use alcohol or other disinfectant to sanitize your hands?	Yes	0						
		No	1	1					
	Do you use alcohol or alcohol gel to disinfect your hands in a general way?	Yes	0						
		No	1	1					
Practices	During your visit did you suffer any incident related to biosecurity and wild species?	Yes	1	1	1	1	25	25	25
		No	0						
Education	In the case of collaborating or having collaborated as a volunteer and/or support staff, can you tell us if you have clear instructions on the steps to be followed in the quarantine area?	YES	1	1	1	1	25	25	25
		NO	0						
		I DON'T KNOW	0						
Practices	If you suffered an accident related to biosecurity, could you tell us what it was and with which species?	Yes	0		1	1	25	25	25
		No	1	1					
Practices	Was the accident you had reported to the staff of the rescue center?	Yes	0		1	1	20	20	20
		No	1	1					
		I don't known	1						

Practices	Which of the following actions did the rescue center take after the accident?	The center's administrators applied some type of protocol (initial evaluation, instructions on actions to be taken)	0		0	1	20	0	20
		Provided medical supervision	0	1					
		None	1						
		I don't known	1						
Practices	During your visit, did you use gloves?	Yes	0		3	3	15	45	45
		No	1	1					
	During your visit, did you wear rubber boots?	Yes	0						
		No	1	1					
	During your visit, did you wear face masks?	Yes	0						
		No	1	1					
Practices	During your visit, did you witness any incident with the animals on exhibit?	Yes	1	1	5	12	10	50	120
		No	0						
	If the answer is yes, which types of incident(s) did you witness?	Biting	4	1					
		Stinging	3						
		Animal escape	2						
		Other	1						
	The bitten person I have witnessed knows if he/she was reported to the Center Administrator?	Yes	0						
		No	1						
Infrastructure	Is there a parking lot on the perimeter of the facility visited?	Yes	0		1	1	10	10	10
		No	1	1					
		I don't known	1						
Infrastructure	The entire unit has a fence or enclosure surrounding it?	Yes	0	1	0	1	15	0	15
		No	1						

		I don't known	1						
Infrastructure	Do all cages or enclosures visited have a fence separating you from the cage?	Yes	0	1	0	1	10	0	10
		No	1						
Infrastructure	Do you have a map available and visible with all areas and legends?	Yes	1	1	0	1	5	0	5
		No	0						
Education	Have you received training in aspects related to biosecurity during your visit?	Yes	0	1	0	4	15	0	60
		No	1						
	During your visit, did you participate to any education or training program on biosecurity or environment?	Yes	0						
		No	1						
	In the case of collaborating or having collaborated as a volunteer and/or support staff, if so, can you tell us if the veterinary area of the Center has Biosecurity manuals or guides?	Yes	0						
		No	1						
	In the case of collaborating or having collaborated as a volunteer and/or support personnel, can you please mention if there is a protocol for the entry of animals from the quarantine area?	Yes	0	1					
		No	1						
Practices	During your visit, have you appreciated that animal enclosures were properly labeled?	Yes	0	1	0	1	10	0	10
		No	1						
		I don't remember	1						
Practices	Are specific materials used in areas such as the kitchen, food preparation, quarantine or veterinary clinic regularly cleaned and disinfected?	Yes	0		1		15	15	0
		No	1	1					

Infrastructure	Does the Center you visited have an outdoor parking lot?	Yes	0	1	1	2	10	10	20
		No	1						
		I don't known	1						
	If the answer was yes, can you tell us if the parking lot is	Inside the center	1	1					
		Outside the center	0						
Infrastructure	Does the Center you visited have animal cages or enclosures that are resistant and prevent animals from escaping?	Yes	0		1	1	15	15	15
		No	1	1					
		I don't known	1						

Total	**38**	**75**	**Total pondered**	**650**	**1250**

Barometer			% using index 100	**52,00**

Spider	**Domain**	**Pondered score**	**Pondered score max**	**Index**
	Education	265	425	**62,35**
	Infrastructure	35	75	**46,67**
	Practices	350	750	**46,67**

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Chapter 4 – General discussion and perspectives

1. Diversity and Bloodborne Pathogens (BBP)

Ecuador has a total of 21 species of NHPs, 12.3% of 171 from the neotropical region (Estrada *et al*., 2017) and a 4.78 % of 439 species worldwide (Estrada *et al*., 2018). Of those 171 primates, 132 species (77.2%), harbour 56 species of blood pathogens (Carrillo-Bilbao *et al*., 2021). In our study we identified two species of blood pathogens: *Plasmodium* (Fig. 17) and *Trypanosoma*. *Plasmodium falciparum* by 2021 has been recorded in 8 species of neotropical primates: *Alouatta caraya, A. guariba, A. puruensis, A. seniculus, Ateles chamek, Callicebus bruneus, Lagothrix cana cana, Sapajus apella*. However, our record was the first and only registry of bloodborne pathogens that was carried out on primate species in Ecuador. In our study *P. falciparum* was identified in *Leontocebus lagonotus*, which hasn't been listed with *Plasmodium falciparum* or any other species of *Plasmodium*.

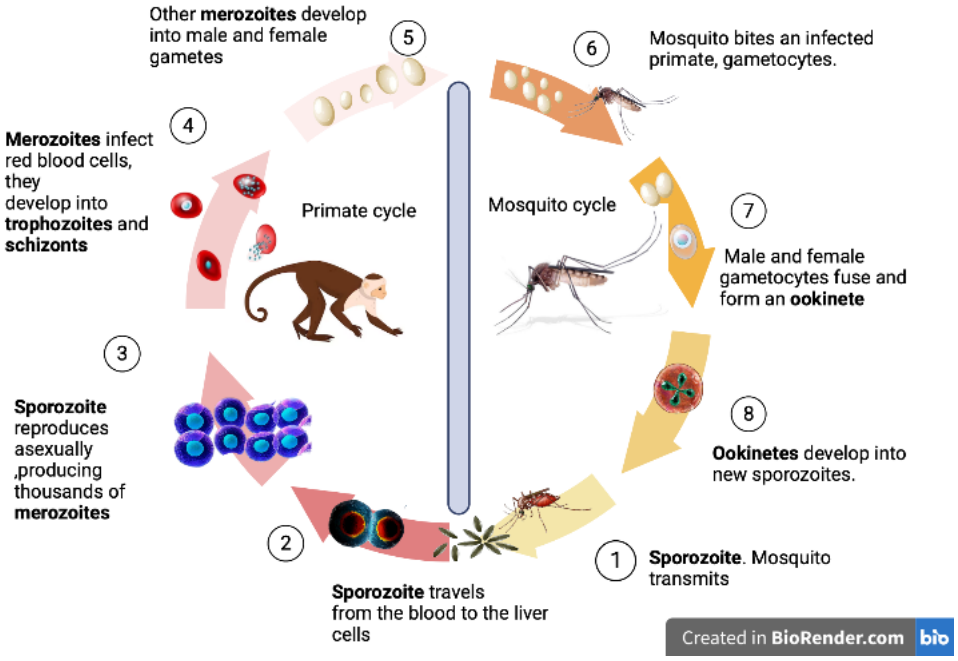

Figure 17. *Plasmodium* sp. life cycle. Adapted from (Venugopal *et al*., 2020).

Other bloodborne pathogens identified in our study were two unknown species of *Trypanosoma* sp. (Fig. 18). All the trypanosomes records (12 species) identified in neotropical NHP according to our review (*Trypanosoma cruzi, Trypanosoma devei, Trypanosoma diasi, Trypanosoma forestali, Trypanosoma hippicum, Trypanosoma lambrechti, Trypanosoma lesourdi, Trypanosoma mycetae, Trypanosoma minasense, Trypanosoma rangeli, Trypanosoma saimiri, Trypanosoma venezuelensis)* were not identified in any of the NHP species from Ecuador, being our results the one that allows us to increase the record of trypanosomes to the results previously mentioned both in the presence of the bloodborne pathogens and in the number of primate species infected with trypanosomes.

Cebus albifrons becomes the first registry at the national level to be infected with *Trypanosoma* sp. in NHP.

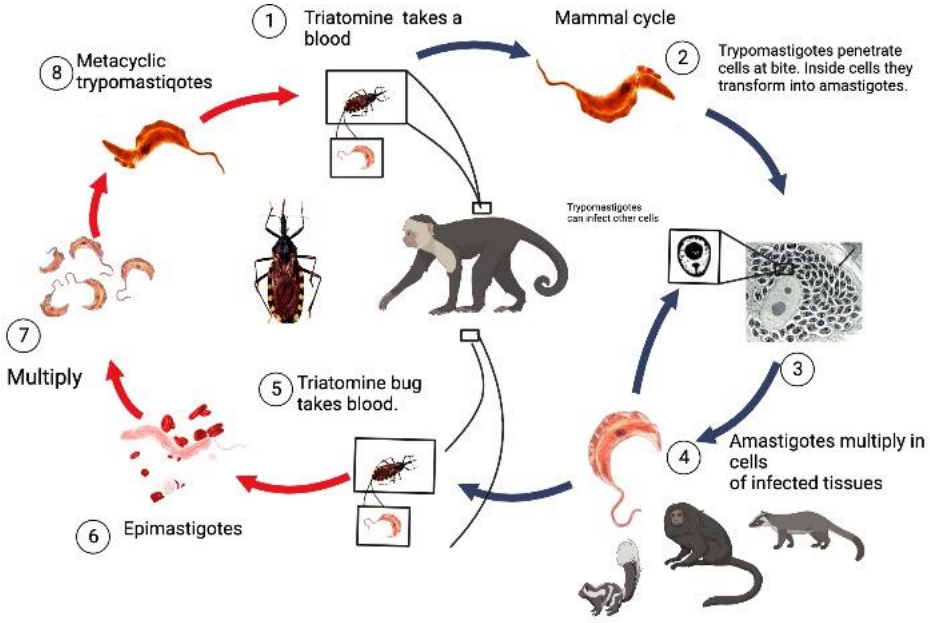

Figure 18. *Trypanosoma* sp. life cycle. Adapted from (Cantey *et al*., 2019)

2. Risk Factors associated with *Plasmodium* and *Trypanosoma*

It has been possible to identify a series of risk factors in the case of bloodborne pathogens in NHP classified into ecological and socio-ecological factors (Carrillo-Bilbao *et al*., 2021; Lederberg *et al*., 1992). Among socioecological we can name animal behaviours, social organization, sleeping site ecology and migration. And among ecological factors there is host density, climate change and human activities. These risk factors are reasons to assess the records found for *Plasmodium* and *Trypanosomes*.

2.1. Socioecological factors:

As it has been described, non-human primates are social animals and physical and non-physical communication is vital for them (Reinhardt *et al*., 2008). Sociality in NHPs is a socioecological factor (Rushmore *et al*., 2017). In our study of Trypanosomes and *Babesia*, we infer the absence of *Babesia* to different strategies developed by the host to prevent pathogens punctually with grooming. This strategy is seen as a hygiene activity (Poirotte *et al*., 2023). In New World primate species, grooming is considered a primary hygienic function of the primate as its presence is related to the health of the infected host (Dunbar, 2008). For example *Leontocebus lagonotus*, being one of the smallest neotropical primates in the neotropics, has been studied in the field and laboratory since the 1960s and the number of studies began to increase in numbers in the 1970s (Cosgrove *et al*., 1968; Epple, 1975; Gengozian *et al*., 1966; Lushbaugh *et al*., 1969; Vogt, 1978). This species is generally polyandrous and, in some cases,

monogamous, but there are records showing all types of social structure. The number of individuals per group ranges from 2 to 7 individuals, with interspecific and intraspecific interrelationships with other primate species and other taxa (Goldizen, 1989). Vogt (1978) recorded the highest percentage of social sharing such as grooming (36%), which shows a high interaction between individuals of the same species and the same group.

This argument is also valid for capuchins. *Cebus albifrons* is a species whose studies on ecology, behaviour and health status are scarce in Ecuador and other regions (Defler, 1979; Martin-Solano *et al*., 2017; Matthews, 2009; Pozo R, 2004). Cooperation and coalition characterise their behaviour with high learning-related behaviour (Perry, 2011), including reports of tool use (Araujo *et al*., 2022; Phillips, 1998). Capuchins groups have between 7 to 35 individuals (Terborgh, 1983) and it is one of the species with the highest rates of extraction from their natural forests, either for meat consumption or trafficking. In addition to interspecific and intraspecific interactions, their groups have a social structure where a dominant male and a dominant female dominate the group, and reciprocal grooming is a social behaviour that takes place most of the time of the day (Smuts *et al*., 1987). In other species of capuchin's, grooming is considered also a hygiene activity (Pfoh *et al*., 2021).

We also noticed that the poor enrichment in captivity did not allow primates to carry out their socioecological characteristics similar to those recorded in the wild. Therefore, environmental enrichment captive species should be more elaborate and according to the natural history of the species so that individuals can be kept in good conditions. In the case of non-human primates it is important to ensure that group integrity is preserved, since when environmental enrichment is done correctly, it minimizes health problems, reproductive failure and other abnormal behavioural and physiological responses (De la Ossa V, 2016; Freeland, 1976; Mason *et al*., 2007). For example, a good enrichment increases grooming between individuals (Bortolini *et al*., 2011; Rojas-Carvajal *et al*., 2018; Shoenfeld, 1989) and therefore may decrease the number of vectors.

The socio-ecological aspects are a factor that affects the transmission of diseases and makes it imminent to understand the relationship between disease transmission, pathogens and their hosts (Freeland, 1976) in order to effectively manage the conservation either in situ or ex situ, considering that infectious diseases are one of the reasons that have caused the extinction or extirpation of 4% of all species worldwide (Pedersen *et al*., 2007; Smith *et al*., 2006). Specifically, behavioural interactions between individuals, with the environment and with vectors affect the transmission of pathogens (Herrera *et al*., 2019). For example, in most neotropical primate species, males migrate out of their home groups to other groups (Agoramoorthy *et al*., 1993; Aureli *et al*., 2013; Garber *et al*., 2016; Jack *et al*., 2004; Manson, 2022; Runestad Connour *et al*., 2020), although females can migrate too (Garber *et al*., 1993; Jack *et al*., 2009), making them more vulnerable because they may catch diseases that were not present in their home group (Freeland, 1976).

2.2. Ecological factors:

2.2.1. Host density and the presence of *Plasmodium* and *Trypanosoma*

Host population density is another risk factor that can increase parasite transmission (Candeloro *et al*., 2016; Gómez *et al*., 2013). In the case of the two records of *Plasmodium* and *Trypanosoma*, we can also highlight the size of the group of both species, *Leontoncebus lagonotus* and *Cebus albifrons* (now *Cebus yuracus*). *Leonteocebus lagonotus* lives in groups of up to 12 individuals (Álvarez-Solas *et al*., 2018a) while *Cebus albifrons* lives in groups from 10 to 54 individuals (Álvarez-Solas *et al*., 2018b; Martin-Solano *et al*., 2017; Rylands *et al*., 2013). The individual of *Cebus albifrons* positive for *Trypanosoma* sp. belongs to a free-ranging population. The free-ranging density is high though with 11 individuals per 9.45 hectares ($1/1.16\ x10^{-4}\ m^2$). In other habitats, free-ranging capuchins with less anthropogenic influence, have very low densities, between 90 ha and 240 ha for 9 or 8 individuals respectively (Matthews, 2009; Parr *et al*., 2013). Another study has demonstrated for other species of Trypanosomes and vectors, that a low density of hosts can decrease the number of vectors and the probability of becoming infected with trypanosomes (Lord *et al*., 2017).

2.2.2. Habitat

As vectors don't have a coevolution with the Trypanosomes parasites since you can find Stercorarian (Haag *et al*., 1998) and Salivarians in the same clade (Radwanska *et al*., 2018), both NHPs species could be infested with triatomines (Arias-Giraldo *et al*., 2020) or with flies. In Stercorarian, trypanosomes develop in the posterior part of the digestive tract of the vectors. In the midgut, trypomastigotes transform into epimastigotes. After a week, they transform into metacyclic trypomastigotes which are released in the vector's faeces. Vectors transmit trypanosomes when they feed as they defecate at the same time as they feed(Ibrahim *et al*., 2021). In Salivarians, trypanosomes are transmitted in the saliva of the vector while it feeds on the host (Magez *et al*., 2021; Radwanska *et al*., 2018).In the case of *Leontocebus lagonotus* (Fig.19), both positive individuals belong to a captive population, and we don't know if they acquired both pathogens (*Plasmodium* and *Trypanosoma*) in the wild or in the captive settings. In the case of *T. cruzi*, NHPs can become infected by ingesting triatomines (Hodo *et al*., 2018; Jansen *et al*., 2018). In captive settings, the presence of triatomines was already discussed that could be a risk factor for captive non-human primates (Carrillo-Bilbao *et al*., 2022). For example, for *Trypanosoma cruzi* a wide range of triatomine species can transmit this parasite (Murillo-Solano *et al*., 2021). Therefore, the presence of orders of mammals in the WMU (personal observation), besides NHP, such as coatis (*Nasua* sp.) and wild felids could increase the likelihood of NHP to become infected. Triatomines can live in coati's nest (de Lima *et al*., 2015; Pessanha *et al*., 2023) and coati's are considered to have high rates of trypanosomes infections (Jansen *et al*., 2018). Other mammals that could be a risk factor in WMU are opossums (*Didelphis* sp.), which are very common in the neotropics.

They are an important reservoir of Trypanosomes (Ardila *et al*., 2022; Herrera *et al*., 1992; Hodo *et al*., 2018; Jansen *et al*., 2018; Pineda *et al*., 2022; Travi *et al*., 1994), and they are known to occupy night monkeys (*Aotus zonalis*) nest (González-Hernández *et al*., 2020).

Figure 19. *Leontocebus lagonotus*. (Proyecto Primates Ecuador, 2023)

The population of free-ranging *Cebus albifrons* (Fig. 20) if located in Misahualli, an anthropogenic environment, surrounded also with livestock farms which can be a source of vectors. Livestock farms in the Napo Province are a source of Trypanosomes (Chávez-Larrea *et al*., 2023).

Figure 20. *Cebus albifrons*. (Proyecto Primates Ecuador, 2023)

2.2.3. Climate change

Climate change influences diseases produced by pathogens in different types of environments, including the distribution patterns of vectors and hosts by influencing their distribution patterns (Alfonso *et al*., 2012; Arbo *et al*., 2022; Fouque *et al*., 2019; Pinto *et al*., 2008). For example, the increase in the frequency of the El Niño phenomenon, will generate more droughts and floods at the regional level (Hartinger *et al*., 2023; Magaña Rueda *et al*., 2002). In countries in the neotropical region, such as Colombia, the El Niño phenomenon, which increases the risk of heavy rainfall and droughts, is associated with a higher incidence of malaria and dengue fever (Estefanía *et al*., 2020; Mantilla *et al*., 2009; Poveda *et al*., 2000). In Paraguay, an association of dengue cases with rainfall indexes was found (Arbo *et al*., 2022). In Ecuador, Pastaza was one of the two Provinces with the highest number of human malaria cases (MSP, 2023). And Puyo and Tena, both cities that were sampled, are the rainiest cities in Ecuador. The monthly mean precipitation is 324 mm for Puyo and 407 mm for Tena (Martin-Solano *et al*., 2017). The rainfall degree is a factor related with the increased of malaria infections rates in other regions as well (Odongo-Aginya *et al*., 2005; Oheneba-Dornyo *et al*., 2022). Changes in climate, can also have influence in the vectors. In Brazil, a study of how the changes in climate impact the distribution of the malaria vector was done (Oheneba-Dornyo *et al*., 2022). In Chile, modelling determined that there could be changes in the distribution of *Trypanosoma cruzi* vectors (Garrido *et al*., 2019). A systematic review indicated that the transmission of *T. cruzi* is highly sensitive to climatic factors (Graves, 2019). For Ebola haemorrhagic fever outbreaks, an association with dry seasons has been determined, so climate change will influence the transmission of this virus (Pinzon *et al*., 2004). Finally, modelling of the richness of vector-borne pathogens in primates finds that the tropical region has the highest number of pathogens in primates (Chapman *et al*., 2005). Therefore, alterations in primate distributions that are affected by climate change could increase or decrease disease transmission.

2.3. Host factors: age and sex

Leontocebus lagonotus was the only NHP species that tested positive for *Plasmodium falciparum*, and it was an adult female. We could only sample adults because at the time there were no offspring in the WMU. In Peru, the only individual of *Leontocebus lagonotus* (before *Saguinus fuscicollis*) that tested positive in their sampling was an adult male from a group of four individuals (Erkenswick *et al*., 2017). This is the second study to have a positive sample for *Leontocebus lagonotus* and the 4th study to screen this species. Therefore, it's important to continue with constant and systematic monitoring of this species to understand the role that age, and sex have in the infection of *Plasmodium falciparum*. In other tamarin species, the rate of infection of *Plasmodium falciparum* is also low (Araújo *et al*., 2013). *Plasmodium falciparum* is considered one of the most severe malarias (Otto *et al*., 2018; Zekar L *et al*., 2022).

With regard to trypanosomes, *Leontocebus lagonotus* have being screened but the rate of infection is also low (Ziccardi *et al*., 2000). However in other species of tamarins, the prevalence of trypanosomes was mild (de Thoisy *et al*., 2001) and there was no difference in infection between adults and juveniles. This might be because exposure to vectors is similar in adults and juveniles since juveniles are in the same location as the adults. Half of the samples from our study were juveniles or subadults. Our positive sample from *Cebus albifrons* was from an adult male. In Panama, trypanosomes in *Cebus capucinus* were found in all age classes but mainly infants and juveniles however the number of infants and juveniles sampled by Sousa *et al*. (1974) was lower than adults. Unfortunately, just one study published data on the effect of sex classes on capuchins (Acosta *et al*., 2016). Of the 20 *Sapajus cay* males sampled, only one tested positive for *Trypanosoma cruzi*, whereas no females tested positive. In other species of capuchins, the rate of infection of trypanosomes varies. For example, among all the free-ranging mammals sampled, *Sapajus libidinosus* had the highest rate of infection of *T. cruzi* (Jansen *et al*., 2018).

It is important to highlight that *Trypanosoma vivax* has not been found in wildlife in the neotropics, only in domestic species, and this may be due to its recent introduction to the continent (Fetene *et al*., 2021; Jones *et al*., 2001). However, *Trypanosoma evansi*, which was introduced earlier in the continent (Hoare, 1972), has already been found in wildlife in the neotropics except in non-human primates (Aregawi *et al*., 2019).

3. Health security

3.1. Current situation in Ecuador

Under Ecuadorian environmental regulations, Wildlife Management Units, as ex-situ wildlife conservation facilities, must maintain an operating license, issued by the Ministry of Environment, Water and Ecological Transition. Therefore, the Wildlife Management Unit must present a management plan, which must include a section related to security (Cumba Endara, 2017). However, thanks to the surveys conducted, we found that there are still gaps that can be improved at the level of the Wildlife Management Unit, the Ministry of Environment, Water and Transition, as well as the general public. Biosecurity monitoring is not only important at the level of the WMUS but also at the level of the authorities, since in both of our studies, *Plasmodium* and *Trypanosoma* were observed in a captive population as well as in a free-ranging population.

3.2. Health surveillance strategies

3.2.1. Prioritisation of zoonotic Bloodborne pathogen

Brazil is the country with the highest number of reports of bloodborne pathogens in NHP, followed by French Guyana, Argentina, Panama, Colombia, Mexico, Peru, Bolivia, Venezuela, Trinidad, Guatemala and Paraguay, which differs from Ecuador, where at the time of the review there were no published studies in the databases used. To prioritize the mammals to be screened, it is important to determine the feeding preferences of vectors such as triatomines (Arias-Giraldo *et al*., 2020; Farfán *et al*., 2007; Hodo *et al*., 2018; Silva *et al*., 2017), flies (Muzari *et al*., 2010; Mwakasungula *et al*., 2022), and mosquitos (Takken *et al*., 2013) in these locations. This type of passive vector surveillance can help us to prevent or identify a possible outbreak. It is important to highlight that in the neotropical region the number of studies of host preferences in flies are scarce or missing.

3.2.2. Criteria to prioritize bloodborne pathogens

We also identified the diversity of arboviruses in mammals in the Neotropical region (García-Romero *et al*., 2023), within the list of 17 countries. The greatest sampling effort was recorded in 11 countries (Argentina, Brazil, Colombia, Costa Rica, Guatemala, French Guiana, Mexico, Panama, Paraguay, Uruguay and Venezuela). In both reviews we found that Brazil is the country with the highest sampling effort for blood parasites. However, there are countries where no effort has been made, despite the high biodiversity of the area and the wide range of mammal hosts. Biodiversity hotspots in the Neotropical region such as Ecuador, Colombia, Chile and the Mesoamerican region, should increase their surveillance as their biodiversity correlates with the emergence of zoonotic diseases (Allen *et al*., 2017; Herrera *et al*., 2019). The consequences related to biodiversity loss are recognized worldwide. Conservation biology uses conservation genetics to preserve species, endangered species (including NHPs) and biodiversity with the use of genetic methodologies. In this field, wildlife is the subject of genetic analyses in various fields of research which have increased in recent decades under a ONE HEALTH framework (Taskın *et al*., 2020).

Biodiversity is linked to biosecurity at several levels. First, the introduction or translocation of animals to local populations has a negative impact on these populations through the cointroduction of parasites (Daszak *et al*. 2000), where these parasites can encounter closely related hosts (Cunningham *et al*. 2003, Farrell *et al*. 2013) and pass into these new hosts or populations. Finally, parasites can regulate the populations sizes through their effect on the new host (Grenfell and Gulland 1995, Møller 2005, Preston and Johnson 2010, Claar *et al*. 2021). Therefore, biosecurity measures in wildlife management units that have reintroduction or release programmes must be strictly adhered to in order not to affect the populations where these animals will be introduced. Second, the anthropogenic interventions in the forest, such as tourism, illegal wildlife trade and bushmeat activities can bring humans and wildlife into close contact with foreign parasites. For example, *Toxoplasma gondii* and *Neospora caninum* can be transmitted to wildlife in natural parks (Cândido *et al*. 2022) if dogs used for hunting are allowed to enter in these regions and through footwear from hunters. Some biosecurity

practices we can consider in protected areas (Walshe *et al*. 2021) are good hygiene practices such as cleaning footwear from visitors at the beginning of trails (Gill *et al*. 2020).

The majority of the non-human primates we conducted studies with are individuals in captivity. Most are in close contact with tourists, volunteers, caregivers and in the case of the Misahualli individuals with the human population, which increases the possibility of pathogen transmission (Chapman *et al*., 2005). In many cases there are no formal records on provenance information of the NHP, which makes it difficult to trace back the zoogeography origin of the host and the pathogens such as *Plasmodium* and *Trypanosoma*. However, what was observed in these wildlife management units is a reflection of the situation in the region, as the provinces of Pastaza and Morona Santiago in 2019 were the two provinces with the highest number of human cases of malaria (*Plasmodium falciparum* and *Plasmodium vivax*) (SIVE, 2019). Therefore, biosecurity management aspects should be strengthened and prioritised, incorporating non-invasive random sampling to enable us to continuously monitor the epidemiological situation of bloodborne pathogens. If infection processes are to be determined during the captive period, further studies on vectors could be carried out to determine if they are responsible for infections and to confirm the risk of human-to-NHPs transmission, as there are no reports of the malaria's vector in the area.

Normally for certain Trypanosome species such as *T. cruzi*, a control measure is the elimination of the vector (ex. Triatomines) (Cruz-Pacheco *et al*., 2012), however it is difficult to implement this type of control in WMUs as theire infrastructures are located in the tropical Amazon with many niches for triatomines. Therefore, the solution for WMUs is the continuous screening of mammals for trypanosomes.

Biosecurity plans and practices in Wildlife Management Units should not only consider non-human primate groups, but directly support all animals in maintaining their diets, health status, veterinary care and proper display, where appropriate. This type of management can reduce the risk of exposure to contaminants among these pathogens and diseases to our residents and visitors. Biosecurity is a daily practice and its role is to keep animal residents and humans safe by implementing measures to reduce identified risks and the spread of diseases (Hess *et al*., 2023; Torremorell, 2022).

Today, biosecurity has to be applied considering the aspects of one health, i.e. based on its three pillars: human, animal and environment (FAO, 2023). These practices must be reflected in individual, local and regional biosecurity plans and their various legislative provisions, systems and processes; with their three main elements being: prevention or limiting the introduction of pathogens, monitoring of infectious diseases, and measures to prevent the spread of a disease or a pathogen (Ellwanger *et al*., 2021).

3.2.3. The role of non-invasive samples in health surveillance

Traditional genetic methods are carried out by collecting invasive samples in wildlife species, such as blood (Cosme *et al*., 2013) and other tissues (Morales *et al*., 2006). However, the use of non-invasive samples should be increased in wildlife studies to ensure their welfare (van der Valk *et al*., 2017; Zemanova, 2020). Unfortunately, a recent review (Zemanova, 2019) found that out of 181 genetic studies in amphibians (n = 50), birds (n = 63), carnivores (n = 31), molluscs (n = 12) and rodents (n = 25), only 39 studies used non-invasive techniques. In our review, of the total number of bloodborne pathogens (56 species), only one was determined by non-invasive samples (*Plasmodium simium*). Our two studies on *Plasmodium* and *Trypanosoma* thus join the list of studies using non-invasive samples in neotropical NHPs, highlighting the importance of this type of sampling in wildlife and health studies (Schilling et al., 2022).

Non-invasive samples are used when sampling wildlife in the field because collection of blood samples can be logistically difficult (Kirmaier *et al*., 2009). For example, invasive sampling requires chemical immobilization which can pose a risk to NHPs and humans. Anesthetising arboreal non-human primates is challenging as they move as high as 80 m above the ground (Gorgens *et al*., 2019; Neville *et al*., 1988). The use of primates in a protected category to obtain a blood sample may be prohibited or must be well justified. The number of individuals used should also be minimized (MacKinnon *et al*., 2014). Specific regulations vary by country, but in general all primates are listed in the convention on International Trade in Endangered Species of Wild Fauna and Flora CITES, which requires a special permit from this convention to prevent illegal primate trade (CITES, 2022).

However, it is important to be aware that the use of non-invasive samples in disease monitoring presents certain difficulties. For example, false negatives can occur when there is a low amount of the pathogen's DNA in the non-invasive sample, because the pathogen life cycle can vary or when doing random sampling. When working with individual serial sampling, the number of false negatives can be reduced. Therefore, it is important to note that when working with non-invasive samples, the ID of the individual must be known. Long-term studies are also important to evaluate sampling and analysis techniques, and the ecology of pathogens and their hosts. Finally, Jirků *et al*. (2012) validated the PCR detection of *Plasmodium* in human feces, making this technique efficient for estimating *Plasmodium* prevalence in NHPs.

Also, the use of Beta-Actin improved our molecular essays in non-invasive samples by increasing the reliability of our results. The Beta-Actin is a gene used as an internal control or housekeeping gene (Stürzenbaum and Kille 2001) for amplification variation among samples, to monitor DNA extraction and PCR amplification effectiveness (Agianniotaki *et al*. 2021). It has an annealing temperature of 49C and a size of 432 bp. Actin is a filament protein, with a molecular weight of 42 kDa,

found in most cell types of eucaryotes and is highly conserved among vertebrates (Ng *et al*. 1985). As a loading control it provides a more reliable interpretation of the PCR amplification as it remains stable during this process. This internal control is used not only for PCR amplification but also in western Blot and qPCR (Li *et al*. 2010).

Currently, the impact of research on wildlife should be reduced, as there have been reported cases where specimens have died due to handling for sampling (Braud *et al*., 2019; Vié *et al*., 1998). Ethical and health concerns, especially during the recent pandemic, should also be taken into account (Zemanova, 2020). Non-invasive sampling is a type of sampling that is not yet widely used, but has seen an increase since the 2010s (Piel *et al*., 2022), mainly for sampling terrestrial mammals and primates for diseases such as COVID-19 and Herpevirus-1 (Gillespie *et al*., 2020). In conservation biology, the use of non-invasive sampling is of utmost importance when dealing with threatened or endangered species such as gorillas (Smiley *et al*., 2010). In some cases, it is legally mandated. In addition, there are NHPs species in protected categories where there are gaps in the pathogens and diseases they carry (Carrillo-Bilbao *et al*., 2021; García-Romero *et al*., 2023; Illia *et al*., 2022) and the use of non-invasives samples, should increase the knowledge of their diseases ecology.

4. Perspectives and recommendations

It is extremely important to make efforts to enhance the traceability of animals resulting from wildlife trafficking to identify more clearly the interrelationship between the pathogen and the socioecological aspects of non-human primates in this case. Socioecological aspects can also provide insights into how these affect the ecology of the vector and the host, considering that basically the transmission of pathogens begins with behavioural interactions at the individual, intraspecific and interspecific level (Fig. 21) (Herrera *et al*., 2019).

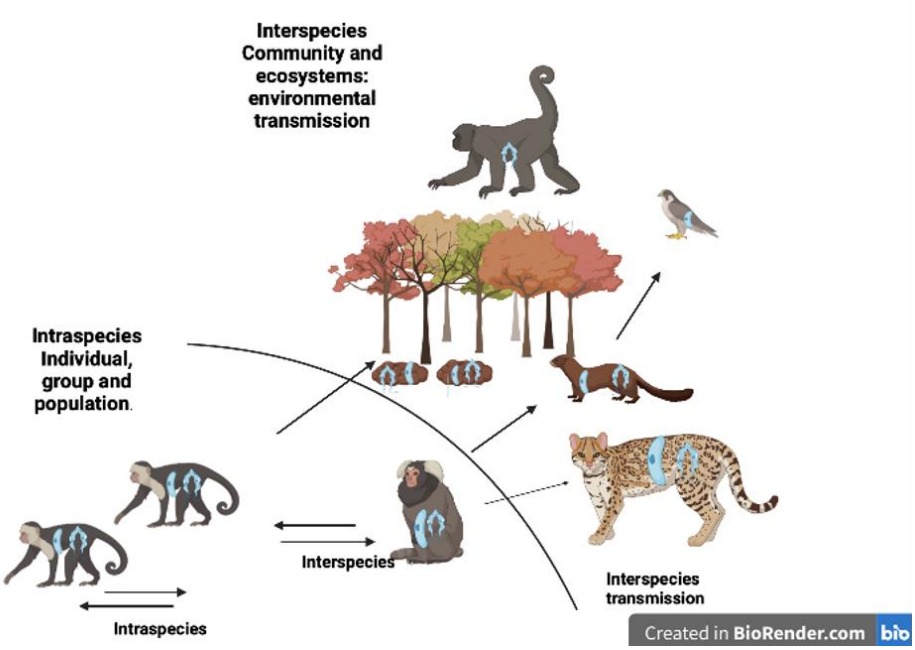

Figure 21. Infectious disease transmission across levels of biological organization, Interspecies-Intraespecies (created in BioRender)**.**

It would also be important to sample the other mammals in the WMUs that can infect vectors that enter in contact with NHPs to understand transmission cycles of bloodborne pathogens in captive environments, especially in tropical regions such as the Amazon (Reis *et al*., 2020). In countries such as Ecuador where the detailed knowledge of vector-borne diseases, pathogens and hosts are poorly known, it is imminent to give priority to an effective quarantine, surveillance systems and contingency plans structured with relevant information on diseases, their vectors, hosts and pathogens.

In Latin America, surveillance of vector-borne diseases is limited and in many cases non-existent, limiting an effective early response (Harvell *et al*., 2009). Currently, in relation to vector-borne diseases and their surveillance, it is necessary to incorporate broad elements of ecology and natural history of the vector, host, pathogen and disease, with the aim of generating effective early warning systems that consider climate change as another actor. We can't neglect climate is an actor that affects several elements of the systems such as vector, host, pathogen and disease behaviour (Fouque *et al*., 2019; Reiter, 2008).

Our reports on bloodborne pathogens *Plasmodium* and *Trypanosoma* in captive and free-living non-human primate species should be considered as suitable surveillance tools to detect environmental and human health hazards in the study area, especially since the vector of malarias has not yet been reported for the Province of Pastaza (Neo *et al*., 2017), even more when more than 60% of emerging infectious diseases in humans originate as zoonoses and there are countries like ours that lack this type

of information (Jones *et al*., 2008). Finally, assessing and mapping the list of bloodborne pathogen at country and regional level would be essential for decision-makers in order to prioritize these pathogens.

This work had, as any other project, some limitations. However, some actions were implemented to counteract those limitations. In the folling table (Table 5), the limitations and actions are presented:

Table 5. Limitations encountered during the PhD project and the implemented actions to counteract those limitations.

Principal Limitations encountered	Actions to counteract the limitation implemented	Observations
Access to Wildlife Management Units	Continue approaches with WMUs in the long term	It is a long-term action that has been implemented in the last few years.
Renewal of Collection and Research Permits	Permanent contact with the public offices in charge	It is a long-term action that is being implemented.
Low number of individuals per NHP species	Extend the temporality of sampling and centers to Amazonian and non-Amazonian provinces.	It is a long-term action that is being implemented (insisting on contacts in the non-Amazonian provinces).
Scarce information on provenance of NHP individuals	Encourage both WMUs and public offices in charge of the importance of traceability of the individual delivered to the WMU.	There is a need for co-operation with all public and private stakeholders in wildlife management.
Pandemic COVID-19 restrictions	Increased the number of visits to provide face-to-face advice on environmental issues of different interests.	It was executed with the support of the different environmental education guides generated in the different aspects (you can review the guides in the following link).
Limitations on the quality of the collected sample.	DNA extraction and quantification procedures were applied and protocols for collecting, transporting and storing samples were improved.	
External contamination of the sample	The fresh sample is collected immediately after defecation.	Behavioural data and sample collection protocol
Identification of the individual from which the sample was collected	The fresh fecal sample is collected immediately only from the individual that can be identified.	Behavioural data and sample collection protocol
Collection of Invasive samples (blood)	We work with fecal sample collection (non-invasive).	Behavioural data and sample collection protocol
Transit time of bloodborne pathogens in the gastrointestinal tract may be limited.	Increase the number of samples per individual in the long term.	It is associated with economic aspects.

Limited technical requirements.	We contacted laboratories with experience in detecting this type of pathogen (Department of Animal Pathology, University of Liège, Group for Animal and Human Health Research (GISAH), Universidad de las Fuerzas Armadas-ESPE, Instituto de Salud Pública y Zoonosis - CIZ (Universidad Central del Ecuador)) and they allowed us to work there.	
Presence of false negatives.	We collaborated with laboratories with expertise in blood pathogens, the number of individuals per species is relatively low, can be complemented with additional tests such as ELISAs.	

Our studies 1,2,3,4, and our collaboratorion with the arboviruses review, provide basic, necessary and indispensable information such as: a list of bloodborne pathogens of non-human primates at the neotropical level and their risk factors, the identification of bloodborne pathogens in two different primate species and the perception of biosecurity in the Wildlife Management Units of the Province of Pastaza.This information should be used as inputs by the public agencies involved and the private sector. Therefore, it will be possible to develop and adopt good biosecurity initiatives and plans, considering the particularities of the Wildlife Management Units, with the objective of updating their instruments, legislation, systems, and processes. Finally, they will be able to link their plans to national, regional and international biosecurity standards, to be considered as an improvement of information tools related to passive and active surveillance of diseases and their pathogens. In addition, the constant updating of information on wildlife management units and their risk assessments, as well as laboratory diagnostics, will facilitate collaboration between the public and private sector as suggested by the FAO (FAO, 2023).

Finally, this work falls under two lines of action of the global action plan of the quadripartite group (WHO, OIE, FAO and UNEP): emerging zoonotic, epidemics and pandemics and neglected zoonotic diseases, that aims at the third line of action, i.e. strengthening One Health collaborative capacity.

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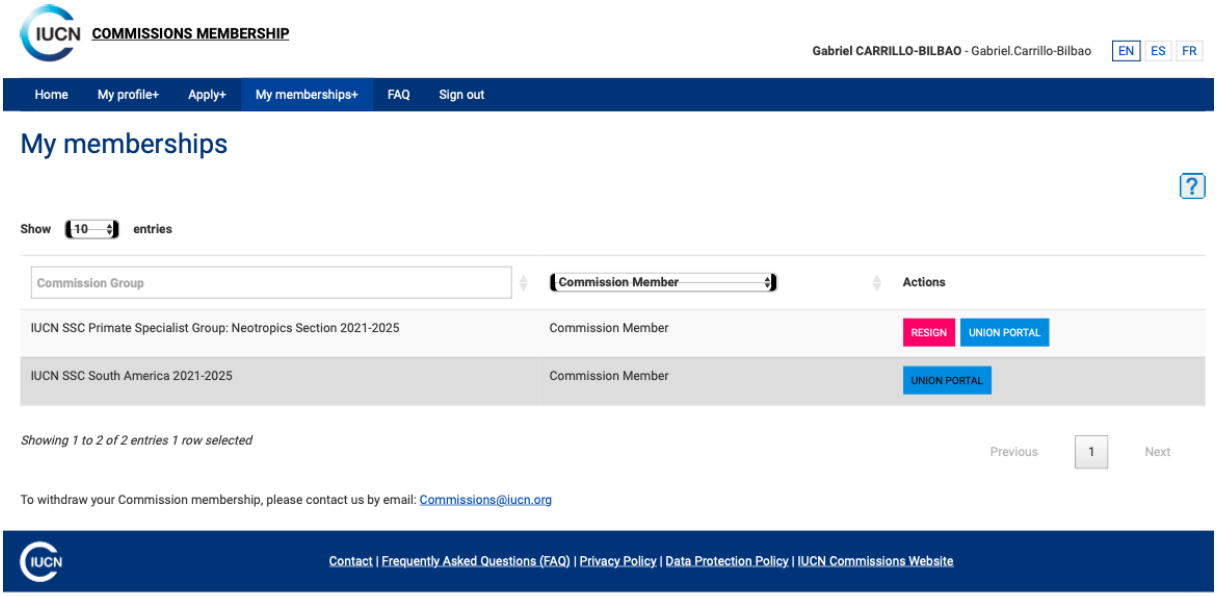
Appendixes

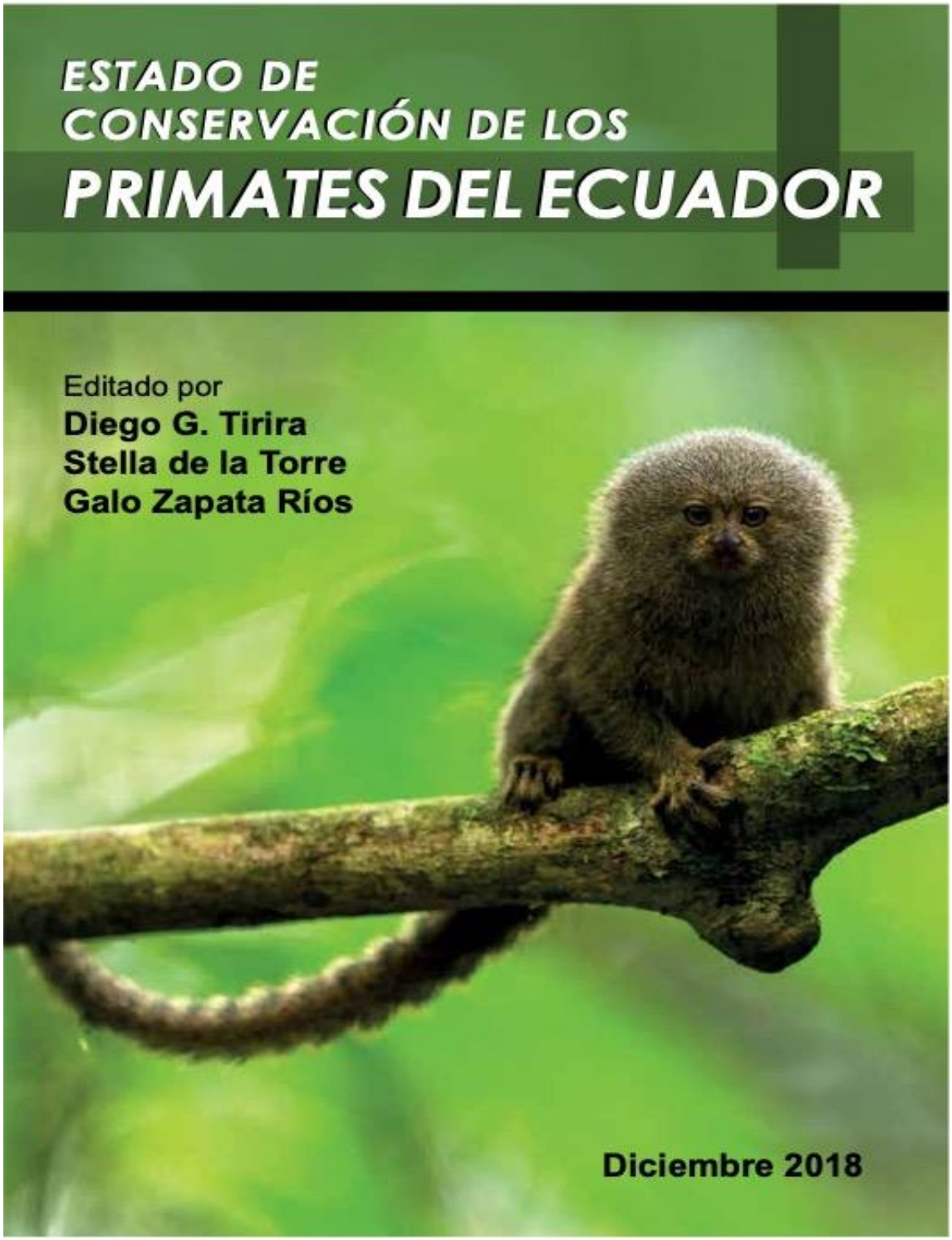
Appendix 1

IUCN Memberships



Appendix 2

Estado de Conservación Primates del Ecuador



FAMILIA ATELIDAE

MONO AULLADOR ROJO DE LINNEO
Alouatta seniculus (Linnaeus, 1766)

Sarah Martin-Solano, Gabriel A. Carrillo-Bilbao,
Stella de la Torre y Diego G. Tirira

OTROS NOMBRES

En español: Mono aullador del Oriente, mono aullador rojo de Colombia, aullador rojo, mono aullador colorado, coto, coto rojo.
En inglés: Linnaeus' Red Howler, Linnaeus' Red Howler Monkey, Red Howler Monkey, Colombian Red Howler Monkey.

COMENTARIOS TAXONÓMICOS

La subespecie correspondiente a la fauna ecuatoriana es *A. s. seniculus* (Rylands *et al.*, 2013b; Tirira, 2017).

DESCRIPCIÓN DE LA ESPECIE

De tamaño grande, una de las mayores en el neotrópico. La cabeza, los hombros, las extremidades, la cola y generalmente el vientre son de color rojo oscuro a rojo púrpura; la espalda y los flancos son más pálidos y suelen tener una coloración de anaranjada a dorada. La cabeza es proporcionalmente grande; el rostro es desnudo y negruzco, con la garganta de apariencia inflada, más notoria en el macho adulto, a manera de bocio, debido al crecimiento exagerado del hueso hioides, agrandamiento que le sirve como caja de resonancia para las fuertes vocalizaciones que emite la especie; el mentón posee una barba larga dirigida hacia adelante, más evidente en el macho adulto. La cola es prensil, gruesa, más larga que la longitud de la cabeza y el cuerpo juntos y a menudo se encuentra enrollada sobre sí misma. Los hombros son robustos y las piernas desproporcionadamente pequeñas y delgadas. Todas sus cuatro extremidades presentan cinco dedos. El macho adulto por lo general presenta las barbas, extremidades y cola de una coloración más oscura que el resto del cuerpo y tiene el escroto

Aldo Fernando Sornoza

Estado de conservación de los primates del Ecuador
Diego G. Tirira, Stella de la Torre y Galo Zapata Ríos (editores),
Grupo de Estudio de los Primates de Ecuador y
Asociación Ecuatoriana de Mastozoología. Quito (2018).

FAMILIA AOTIDAE

MONO NOCTURNO DE SPIX
Aotus vociferans (Spix, 1823)

Gabriel A. Carrillo-Bilbao, Sarah Martin-Solano,
Stella de la Torre y Diego G. Tirira

OTROS NOMBRES

En español: Mono nocturno tropical, mono nocturno, mono de la noche, mico de noche, mico nocturno, tutamono. **En inglés:** Spix's Night Monkey, Night Monkey.

COMENTARIOS TAXONÓMICOS

Se trata de una especie monotípica (Fernández-Duque, Corley y Spence-Aizenberg, 2013). Información taxonómica adicional se menciona en la ficha del mono nocturno lemurino (*A. lemurinus*).

DESCRIPCIÓN DE LA ESPECIE

Es una especie pequeña, con el pelaje denso, suave y lanudo; la coloración general es de marrón amarillenta a marrón grisácea; la región ventral es de color amarillo pálido a amarillento anaranjado, tonalidad que a menudo alcanza el cuello, la cara interior de las extremidades y la base de la cola, pero más oscura alrededor del ano. La cabeza es redonda, con el pelo corto; los ojos son grandes, redondos y conspicuos, de color marrón, con una pequeña mancha de pelo más clara, algo crema, sobre cada ojo; cuando son iluminados por la noche, los ojos producen un intenso reflejo naranja rojizo; las orejas son pequeñas, apenas perceptibles entre el pelaje; el rostro es de color marrón pizarra con manchas de blancas a blanco amarillentas alrededor de los ojos y del hocico; la frente presenta tres franjas oscuras bien evidentes que se extienden hasta la corona; la franja central nace entre los ojos, mientras que las laterales aparecen sobre los lados externos de cada ojo, y a veces se proyectan hacia adelante, hasta las mejillas; las tres franjas convergen en la parte posterior de la corona. La cola es larga y no prensil, nunca enrollada (a menudo

Rubén D. Jarrín

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Grupo de Estudio de los Primates de Ecuador y
Asociación Ecuatoriana de Mastozoología. Quito (2018).

FAMILIA PITHECIIDAE

SAKI DEL NAPO
Pithecia napensis (Lönnberg, 1938)

Gabriel A. Carrillo-Bilbao, Sarah Martin-Solano, Galo Zapata Ríos, Stella de la Torre y Diego G. Tirira

OTROS NOMBRES

En español: Parahuaco, parahuaco común, parahuaco del Napo. **En inglés:** Napo Saki, Saki Monkey.

COMENTARIOS TAXONÓMICOS

Esta especie fue tratada anteriormente como subespecie o sinónimo menor de *P. monachus* (Groves, 2005). Marsh (2014), en su revisión taxonómica, indica que *Pithecia monachus* incluye un complejo de especies, en cuyo caso la especie presente al sur del río Napo, en la Amazonía del Ecuador, es *P. napensis*. Algunos estudios (*e.g.*, Di Fiore, Fernández-Duque y Hurst, 2007; De Luna *et al.*, 2010; Fernández-Duque, Di Fiore y de Luna, 2013; Porter *et al.*, 2015; Van Belle, Fernández-Duque y Di Fiore, 2016) se refieren a esta especie como *P. aequatorialis*. Especie monotípica (Marsh, 2014).

DESCRIPCIÓN DE LA ESPECIE

Se trata de un primate de tamaño mediano. El pelaje es largo, lanudo, ondulado y denso, cuelga del cuerpo a los lados y sobre el vientre, que es casi desnudo. El rostro es casi desnudo, con la corona cubierta por un área de pelo corto y las orejas inconspicuas. La cola es larga, no prensil y con abundante pelaje que le da un aspecto espeso; cuelga recta cuando descansa sentado, o es llevada hacia atrás, no hacia arriba, cuando se desplaza. La locomoción es de tipo cuadrúpedo-caminante y saltadora. En esta especie se evidencia dimorfismo sexual, con las siguientes particularidades para el macho: el dorso es de color negro y canoso con puntas blancas; la cantidad y longitud de los pelos canosos varía, pero en general son más largos en la cola que en la espalda y los brazos; el pelaje es canoso en los brazos, pero no en las piernas; en la cabeza, la corona está

Diego G. Tirira / Archivo Murciélago Blanco

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Asociación Ecuatoriana de Mastozoología. Quito (2018).

FAMILIA PITHECIIDAE

TITÍ ROJIZO
Plecturocebus discolor
(I. Geoffroy y Deville, 1848)

Gabriel A. Carrillo-Bilbao, Sarah Martin-Solano,
Stella de la Torre y Diego G. Tirira

OTROS NOMBRES

En español: Titi, cotoncillo, cotoncillo rojo, songo-songo. **En inglés:** Red-crowned Titi, Dusky Titi Monkey, Red Titi Monkey, Titi Monkey, Discolored Titi.

COMENTARIOS TAXONÓMICOS

Especie anteriormente referida para la fauna del Ecuador dentro del género *Callicebus* y bajo los nombres de *C. cupreus* y *C. moloch* (Jones y Anderson, 1978; Albuja, 1991; Tirira, 1999, 2017; Groves, 2005). Byrne *et al.* (2016) realizaron un análisis filogenético de las especies del género *Callicebus* mediante el uso de ADN nuclear y mitocondrial. Como resultado de este análisis propusieron el nuevo género *Plecturocebus* para las especies del grupo *moloch*, entre las que se encuentra la forma *discolor*. Es necesario revisar la taxonomía de la especie y de las diferentes poblaciones presentes en Ecuador. Especie monotípica (Ferrari *et al.*, 2013).

DESCRIPCIÓN DE LA ESPECIE

Se trata de una especie de primate pequeño. El pelaje es largo y denso; el dorso es de color marrón opaco a marrón rojizo, finamente entremezclado con pelos grises y amarillos; la región ventral es de color rojo anaranjado, al igual que las extremidades y cuello, que le da un contraste notorio con el color del pelaje de su espalda. La cabeza es redondeada; el rostro es pequeño, aplanado y con escaso pelaje de color negruzco a gris oscuro, que contrasta con el mentón, que es rojizo; la parte exterior de las mejillas y el cuello son de color rojo anaranjado; la frente posee una

Rubén D. Jarrín

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Asociación Ecuatoriana de Mastozoología. Quito (2018).

Appendix 3

Arboviruses in Mammals in the Neotropics: A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine

viruses

MDPI

Systematic Review

Arboviruses in Mammals in the Neotropics: A Systematic Review to Strengthen Epidemiological Monitoring Strategies and Conservation Medicine

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Abstract: Arthropod-borne viruses (arboviruses) are a diverse group of ribonucleic acid (RNA) viruses, with the exception of African swine fever virus, that are transmitted by hematophagous arthropods to a vertebrate host. They are the important cause of many diseases due to their ability to spread in different environments and their diversity of vectors. Currently, there is no information on the geographical distribution of the diseases because the routes of transmission and the mammals (wild or domestic) that act as potential hosts are poorly documented or unknown. We conducted a systematic review from 1967 to 2021 to identify the diversity of arboviruses, the areas, and taxonomic groups that have been monitored, the prevalence of positive records, and the associated risk factors. We identified forty-three arboviruses in nine mammalian orders distributed in eleven countries. In Brazil, the order primates harbor the highest number of arbovirus records. The three most recorded arboviruses were Venezuelan equine encephalitis, Saint Louis encephalitis and West Nile virus. Serum is the most used sample to obtain arbovirus records. Deforestation is identified as the main risk factor for arbovirus transmission between different species and environments (an odds ratio of 1.46 with a 95% confidence interval: 1.34–1.59). The results show an increase in the sampling effort over the years in the neotropical region. Despite the importance of arboviruses for public health, little is known about the interaction of arboviruses, their hosts, and vectors, as some countries and mammalian orders have not yet been monitored. Long-term and constant monitoring allows focusing research on the analysis of the interrelationships and characteristics of each component animal, human, and their environment to understand the dynamics of the diseases and guide epidemiological surveillance and vector control programs. The biodiversity of the Neotropics should be considered to support epidemiological monitoring strategies.

Keywords: Latin America; non-human primates; systematic review; arboviruses; Venezuelan equine encephalitis (VEEV); Saint Louis encephalitis (SLEV); West Nile virus (WNV)

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1. Introduction

Tropical forests harbor much of the world's tree diversity [1,2] and more than 1617 recognized mammal species [3,4]. Mammals are important ecological components for

nutrient distribution [5,6], seed dispersal [7,8], and interactive connectors between animal species and habitats [9–11].

The Neotropics region includes much of Latin America, from Mexico to Argentina [12], and is probably the area that harbors the greatest biodiversity on a global scale [13]. Among the species currently recognized in biogeographic regions, the Neotropics harbor the largest number of mammal species (1617 species), followed by the Afrotropics (1572 species), the Palearctic (1162 species), and Australasia–Oceania (527 species) [3].

Within the world list of seventeen megadiverse countries, six of them (Mexico, Venezuela, Colombia, Ecuador, Peru, and Brazil) are part of the Neotropics [14]. In addition, this region includes areas of high "hotspot" diversity and nine areas of endemism or species richness [15,16] that are highly threatened [17].

In the case of mammals, 60% of threatened species are located in hotspots [18], with the highest percentage of declining species concentrated in the Neotropics [19]. For the Neotropics and its diversity, climate change and change use of land are some of the greatest threats [16,20,21], as it influences the occurrence of infectious diseases in various types of the environment [22], as well as the distribution patterns of hosts, and their pathogens and vectors [23–25].

Arboviruses are a group of viruses that are transmitted from infected hosts to susceptible hosts by hematophagous arthropod vectors [26,27]. With the exception of African swine fever virus DNA virus, arboviruses are RNA viruses [28] that belong to one of eight families: *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivirus*), *Peribunyaviridae*, such as genus *Orthobunyavirus*, *Nairoviridae*, such as genus *Orthonairovirus*, *Phenuiviridae*, such as genus *Phlebovirus*, *Reoviridae* genus *Orbivirus*, *Rhabdoviridae* genus *Vesiculovirus*, and *Orthomyxoviridae* genus *Thogotovirus* [29–31]. Approximately 500 arboviruses are known, of which 100 can cause diseases in humans and 40 in domestic animals [32,33].

The success of virus transmission is determined by the interaction between the virus, the vector, the host, and their environment [34]. Establishing the orders of mammals that act as reservoirs is complicated [35], as arboviruses have a wide range of mammalian hosts that could act as potential reservoirs [36] and amplifiers in wild and domestic environments [37].

Vectors serve as reservoirs, amplifiers, and carriers in wild and domestic settings [38]. Hematophagous arthropods are considered active vectors of arboviruses when they are able to ingest a given pathogen by feeding on an infected vertebrate host, followed by the replication of the pathogen in the vector and subsequent transmission to a new vertebrate host [39].

Infection by an arthropod vector is often necessary to maintain the transmission cycle of arboviruses [40]. In tropical and subtropical regions, there is a great diversity and abundance of potential vectors [41]. Therefore, the presence of a diversity of arthropod vectors poses an impact on the health of humans, wildlife, and domestic animal components. Generally, arthropod vectors are insects such as *Aedes* spp. and *Culex* spp. mosquitoes [42,43], sandflies [44,45], and ticks, mainly from the families *Ixodidae* and *Argasidae* [46–48], which circulate with great ease allowing the spread of diseases.

There are several factors that favor the increase in the incidence of arbovirus-caused diseases; for example, the availability of hematophagous vectors [49], urbanization [50,51], global transportation systems [52], deforestation in areas with high levels of biodiversity [53–57], and irrigation systems [58].

Although studies focused on the dynamics of arthropod-borne infectious diseases continue to advance [59], they still pose a conservation risk, mainly in tropical regions, as their prevention and control depend largely on the surveillance of arthropod vectors [60–62]. The great diversity of mammals in the wild, and especially in orders that have a more direct association with human activities, entails special attention because they can act as hosts or reservoirs of arboviruses [63,64].

Some groups of mammals are considered good reservoir hosts and/or amplifying reservoirs; for example, the orders Rodentia and Chiroptera are the most numerous with worldwide distribution and present a variety of locomotor adaptations that allow them to

have a great capacity to adapt to new habitats [65]. Similarly, the order Didelphimorphia presents a wide distribution in the Neotropics in almost all habitat types [66]. These orders involve special attention due to their biological characteristics, as they act as wild reservoirs of arboviruses [36] that affect humans and domestic animals [67,68].

The emergence and spread of emerging infectious diseases are associated with the way humans interact with animal species and the environment [69]. The importance of detecting the zoonotic spread of a vertebrate animal, beyond being a natural reservoir, is to understand a complex process that requires the intervention of environmental, pathogen, and host factors [70,71]. Habitat loss increases biodiversity loss [69]. Better-preserved habitats reduce spillover events, which is known as the dilution effect [72]. Higher diversity often leads to a lower prevalence of infection in hosts [73].

This research proposes to contribute to public health and zoonosis or re/emerging diseases prevention considering that the niche, vector dynamics, hosts, and viruses have been modified in the last decades. In certain habitats, accelerated population growth, quality of life, and sanitary conditions could favor the spread of arboviruses in various mammalian hosts [33,74], posing a global public health problem [47,75].

In order to achieve a better understanding of the ecology of diseases, it is necessary to detect, prevent and control them, and to approach them from a conservation medicine approach given the connection between wildlife and domestic animals, the ecosystem, and humans [76]. This approach will serve as a tool for the understanding, prevention, and management of health problems derived from environmental change [77] in one of the most biodiverse areas of the planet.

This systematic review will allow the identification of studies focused on mammalian arboviruses in the Neotropics excluding the human primate group. The analyses will show the diversity and geographic records of arboviruses, as well as the taxonomic groups of mammals that are most monitored and those that have an information gap. The information obtained on the association of wild and/or domestic mammals as potential hosts and reservoirs of arboviruses is intended to support epidemiological surveillance tasks with a focus on conservation medicine and/or the One Health approach [78,79] to achieve the integration of knowledge and apply it in favor of biodiversity.

2. Methods

The literature review of mammalian arbovirus records in the Neotropics was conducted between 1 June and 31 July 2021 under the Preferred Reporting Items for Systematic Reviews and Meta-Analyses PRISMA 2020 Checklist reporting guide and the PRISMA 2020 flowchart [80,81] that describe the process for literature exploration and justification for the selection of each investigation [82,83].

For the search of indexed articles, we used Google Scholar and PubMed databases by combining the following keywords and Boolean operators: Mammals AND arbovirus AND Neotropical countries AND Argentina OR Belize OR Bolivia OR Brazil OR Chile OR Colombia OR Costa Rica OR Ecuador OR El Salvador OR Guatemala OR Guyana OR French Guyana OR Honduras OR Mexico OR Nicaragua OR Panama OR Paraguay OR Peru OR Suriname OR Uruguay OR Venezuela, including all publications without distinction of the year of publication. Additionally, exclusion criteria included (1) a language other than Spanish, Portuguese, and English, (2) a focus on humans, vaccines, clinical cases, and laboratories, (3) duplicates between the two predetermined databases, (4) lack of information regarding the locality of registration, (5) not including the mammalian class, (6) books with extensive and generalized content, and (7) bibliographic reviews.

All the information obtained from the selected articles was organized in a database to determine the sampling effort, i.e., the number of publications as surrogate data, arbovirus records in neotropical countries (number of samples and prevalence), including orders of mammals (wild and domestic) most monitored, laboratory techniques for sample processing used in arbovirus detection, type of habitat (primary forest, secondary forest, intervened

area, not specified, populated zone, and captive), and state in which the individual was found at the time of sampling.

Data processing was carried out using SPSS version 20 [84] statistical software. The objective was to identify if arbovirus richness is similar between countries or between mammal genera. Arbovirus richness is the number of arbovirus species found in this study. One positive record was considered each time an article recorded one positive order for one arbovirus; e.g., if one article recorded three arboviruses in one order, three positive records are considered.

Additionally, we determinate the principal orders with arbovirus records in different environments (primary forest, secondary forest, intervened area, not specified, populated zone, and captive) and we provide relevant data to be used to support arbovirus management and prevention protocols in order to contribute to the knowledge of arbovirus of public health concern in the region.

This quantitative tool allowed the statistical interpretation of the results presented in each of the independent studies selected within the systematic review process [85].

We performed a negative binomial regression analysis to determine if there is an increase in the monitoring effort number of publications over time [86]. We used the Kruskal–Wallis equality-of-populations rank test to determine if arbovirus richness is similar between countries or between mammal genera [87,88].

The prevalence values for each publication were calculated as follows:

$$\frac{\#\text{ positive records}}{\#\text{ total samples}} * 100 \quad (1)$$

Based on these results, we structured the table in Section 3.1 according to each arbovirus identified, in which the fourth column "n" and the fifth column "prevalence %" contain the minimum and maximum values obtained as a result of the calculation presented.

For the representation of the positive records of arboviruses in mammals of the Neotropics, we generated several maps to identify the areas in each country where the presence of arboviruses was recorded and the orders of mammals monitored in the Neotropics. The data of the geographic points of the sampling sites with positive records for arboviruses were entered into the ArcGIS 10.5 platform [89]. According to the specifications of each investigation, we used coordinates given in the original paper (n = 21) and for the other, we georeferenced based on the locality names (n = 23).

For the graphical representation of viruses recorded in mammalian orders according to habitat, we used GEPHI 0.9.2 software ([90] in which one positive record was considered each time an article recorded one positive order for one arbovirus; e.g., if one article recorded three arboviruses in one order, three positive records are considered).

To determine whether arbovirus records are shared among the countries of the Neotropics, the Jaccard and Sorensen similarity indices were calculated to estimate the compositional similarity of arboviruses among the orders and countries of the Neotropics based on the presence/absence data of positive records identified, using the following formulas [91–93]:

$$JI = \frac{A}{A + B + C} \quad (2)$$

$$SI = \frac{2A}{2A + B + C} \quad (3)$$

where JI = the Jaccard index; SI = the Sorensen index; A = unique species per site one; B = unique species per site two; and C = the number of species in common between two sites.

The two indexes were compared using Pearson's correlation coefficient.

In addition, a Spearman correlation was applied between the two indices for the number of arboviruses per country, as well as for the number of arboviruses per order.

This analysis made it possible to identify the countries and orders that are most closely related due to various factors such as: host mammal distribution, migrations, trade, and even health policies [94].

The World Wide Fund for Nature has compiled and analyzed in the shape file the global data on significantly increasing deforestation and degradation trends [95], which were used for visualization purposes [96]. We added the positive arbovirus records for the Neotropics identified in the literature review. The characteristics of the habitat described in the articles, either in front of deforestation or with vegetation cover, were taken into consideration.

Finally, based on the analysis of the publications obtained as a result of the literature review, the characteristics, conditions, or behaviors that increase the likelihood of encountering the top-3 arboviruses, the Saint Louis encephalitis virus (SLEV), the Venezuelan equine encephalitis virus (VEEV), and the West Nile virus (WNV) in the Neotropics were identified [97]. The parameters considered include factors such as deforestation, habitat use change, human and animal migration, climate change, behavioral patterns, altered interactions, surveillance, and conservation programs. The purpose was to provide relevant data to be used as a tool to support arbovirus management and prevention protocols, and to contribute to the knowledge of arbovirus diversity of public health concerns in the region. We identified the odds ratio of a mammal being exposed to an arbovirus according to the vegetation cover and deforested fronts, as well as the positive and negative records of arbovirus identified in the nine orders of mammals.

3. Results

3.1. Arboviruses Richness

The systematic review was conducted based on PRISMA guidelines (Figure 1) allowed us to quantify the richness of arboviruses (n = 43) present in nine mammalian orders in the Neotropics (Table 1). In the Neotropics region, sampling efforts (n = 46 citations) in the detection of arboviruses in mammals have focused on the following countries: Brazil (n = 15), Argentina (n = 6), Costa Rica (n = 5), Mexico (n = 5), Venezuela (n = 5), Colombia (n = 4), French Guiana (n = 3), Panama (n = 2), Guatemala (n = 1), Paraguay (n = 1), and Uruguay (n = 1). There are several areas harboring the same types of arboviruses, as evidenced in (Table 1). The country with the highest number of arboviruses is Brazil (n = 27). However, no significant differences were found between the arbovirus richness of the countries (Kruskal–Wallis equality-of-populations rank test; Chi2 = 13.474, $p > 0.05$).

Table 1. Diversity of arboviruses in mammalian orders in the Neotropics.

Arbovirus	Mammalian Host	Country	Number	Prevalence (%)	Methods	Type of Sample	Reference
ALPHA: alphavirus undetermined	Perissodactyla	Brazil	985	24.26	PRNT	Blood	[98]
Apeu virus (APEUV)	Artyodactyla	Brazil	607	82.2	PRNT	Serum	[99]
Bunyamwera virus (BUNV)	Perissodactyla Didelphimorphia	Argentina Venezuela	2	100	PRC qPCR	Tissue	[100,101]
Bussuquara virus (BSQV)	Perissodactyla Non-human primates	Argentina Brazil Mexico	108 72	77.33 64.81	PRNT	Serum	[102–104]
Cache Valley virus (CVV)	Carnívora Rodentia	Mexico Panama	2214 80	1.25 1.26	HI	Blood Serum	[105,106]
Cacipacore virus (CPCV)	Non-human primates	Brazil	139	17.98	HI PRNT	Serum	[102]
Caraparu virus (CARV)	Non-human primates	Brazil	139	17.98	HI PRNT	Serum	[102]
Catu virus (CATUV)	Didelphimorphia	Brazil	14	57.14	HI	Serum	[67]
Changuinola virus (CGLV)	Pilosa Non-human primates Rodentia	Colombia Costa Rica Panama	2214 4109	1.26 79.81	HI	Blood Serum	[106,107]
Dengue virus (DENV)	Carnivora Chiroptera Didelphimorphia Non-human primates Rodentia	Argentina Brazil Colombia Costa Rica French Guiana Mexico	616 16	64.81 0.69	ELISA HI PCR PRNT qPCR	Blood Serum Tissue	[102,103,108–118]
Madariaga virus (MADV)	Chiroptera Perissodactyla Non-human primates Rodentia	Brazil Guatemala Mexico Venezuela	2214 14	93.75 1.26	HI PRNT	Blood Serum Tissue	[67,98,102,104,119–122]
Western equine encephalitis virus (WEEV)	Perissodactyla Rodentia	Brazil Mexico	985 75	77.33 1.25	HI PRNT	Blood Serum	[98,105]

Table 1. *Cont.*

Arbovirus	Mammalian Host	Country	Number	Prevalence (%)	Methods	Type of Sample	Reference
Saint Louis encephalitis virus (SLEV)	Artiodactyla Chiroptera Didelphimorphia Perissodactyla Pilosa Non-human primates Rodentia	Argentina Brazil Colombia Costa Rica Guatemala French Guiana Mexico Panama Uruguay	2214 1	100 1.25	ELISA HI PRNT qPCR	Blood Serum	[102–107,110–112,114,116,120,123–131]
Venezuelan equine encephalitis virus (VEEV)	Artiodactyla Chiroptera Didelphimorphia Perissodactyla Pilosa Rodentia	Argentina Brazil Colombia Costa Rica Guatemala Mexico Panama Venezuela	985 64	93.75 0.69	HI PRNT qPCR	Blood Serum Tissue	[98,101,104–107,120–123,126,129,132,133]
Vesicular stomatitis, Indiana virus (VSIV)	Chiroptera Didelphimorphia Pilosa Non-human primates Rodentia	Colombia Costa Rica Guatemala Mexico Panama	2214 80	79.81 1.25	HI PRNT	Blood Serum	[105–107,120]
Vesicular stomatitis, New Jersey virus (VSNJV)	Chiroptera Rodentia	Guatemala Mexico	332 80	26.2 1.25	HI PRNT	Blood Serum	[105,120]
Yellow fever virus (YFV)	Pilosa Non-human primates Rodentia	Brazil French Guiana	574 1	100 8.71	HI PRNT qPCR	Blood Serum Tissue	[102,112,114,128,134]
Undetermined flavavirus	Non-human primates	Costa Rica	86	40.69 44.18	PRNT	Serum	[110,111]
Fort Sherman virus (FSV)	Perissodactyla Non-human primates	Argentina Brazil	222 50	76.8 1.04	HI PRNT qPCR	Blood Serum	[123,135,136]
Gamboa virus (GAMV)	Rodentia	Venezuela	543	5.52	qPCR HI	Blood Tissue	[101]
Guama virus (GMAV)	Rodentia	Colombia Panama	2214	1.26	HI	Serum	[106]
Guaroa virus (GROV)	Artiodactyla	Brazil	607	82.2	PRNT	Serum	[99]

Table 1. *Cont.*

Arbovirus	Mammalian Host	Country	Number	Prevalence (%)	Methods	Type of Sample	Reference
Icoaraci virus (ICOV)	Perissodactyla Non-human primates	Argentina Brazil	222 26	76.8 17.98	HI PRNT qPCR	Blood Serum	[123,124]
Ilheus virus (ILHV)	Pilosa Non-human primates	Argentina Brazil Costa Rica	139 14	79.81 17.98	HI PRNT qPCR	Blood Serum Tissue	[67,102,103,107,124]
Itaqui virus (ITQV)	Perissodactyla	Venezuela	64	93.75	HI	Serum Tissue	[122]
Jamestown Canyon virus (JCV)	Artiodactyla	Mexico	80	1.25	HI	Blood	[105]
Madrid virus (MADV)	Perissodactyla Rodentia	Colombia Panama	2214 194	64.94 1.26	HI	Serum	[106,126]
Maguari virus (MAGV)	Artiodactyla Perissodactyla	Brazil	607	82.2	PRNT	Serum	[99]
Mayaro virus (MAYV)	Perissodactyla Non-human primates	Argentina Brazil French Guiana Panama Paraguay	2214 90	74.44 1.26	HI PRNT	Blood Serum	[98,106,112,137]
Melao virus (MELV)	Lagomorpha Rodentia	Colombia Panama	2214	1.26	HI	Serum	[106]
Mojui dos Campos virus (MDCV)	Chiroptera	Brazil	1	100	HISTOL	Blood	[138]
Murutucu virus (MURV)	Artiodactyla	Brazil	607	82.2	PRNT	Serum	[99]
Oriboca virus (ORIV)	Artiodactyla	Brazil	607	82.2	PRNT	Serum	[99]
Oropouche virus (OROV)	Artiodactyla Non-human primates	Brazil Venezuela	607 1	100 4	HI PCR PRNT	Blood Serum Tissue	[99,128,136,139]
Ossa virus (OSSAV)	Rodentia	Colombia Panama	2214	1.26	HI	Serum	[106]
Punta Toro virus (PTV)	Pilosa	Costa Rica	109	79.81	HI	Blood	[107]
Rio Grande virus (RGV)	Lagomorpha Pilosa	Costa Rica Mexico	109 80	79.81 1.25	HI	Blood	[105,107]
Rocio virus (ROCV)	Perissodactyla Non-human primates	Brazil	753 139	55.11 17.98	ELISA HI PRNT	Serum Tissue	[102,130]

Table 1. *Cont.*

Arbovirus	Mammalian Host	Country	Number	Prevalence (%)	Methods	Type of Sample	Reference
Tacaiuma virus (TCMV)	Artiodactyla Perissodactyla Pilosa	Argentina Brazil	222 75	77.33 17.98	HI PRNT	Blood Serum	[102,123,129]
UNA virus (UNAV)	Non-human primates Rodentia	Argentina Colombia Panama Paraguay	2214 90	74.44 1.26	HI PRNT	Blood Serum	[106,137]
Utinga virus (UTIV)	Non-human primates	Brazil	139	17.98	PRNT	Serum	[102]
West Nile virus (WNV)	Artiodactyla Chiroptera Perissodactyla Pilosa Non-human primates Rodentia	Argentina Costa Rica Mexico Panama Uruguay Venezuela	425 72	79.81 18.57	ELISA HI PRNT	Blood Serum Tissue	[103,104,107,111,115,116,121,123,125,126]
Xingu virus virus (XINV)	Artiodactyla Perissodactyla	Brazil	607	82.2	PRNT	Serum	[99]
Zika virus (ZIKV)	Pilosa Non-human primates	Brazil	132 10	100 6.81	PRNT qPCR	Blood Tissue	[140,141]

Legend: ELISA, enzyme-linked immunoadsorbent assay; HI, hemagglutination inhibition; HISTOL, histology as tissue-based study; PCR, polymerase chain reaction; PRNT, plaque reduction neutralization test; qPCR, quantitative real-time polymerase chain reaction.

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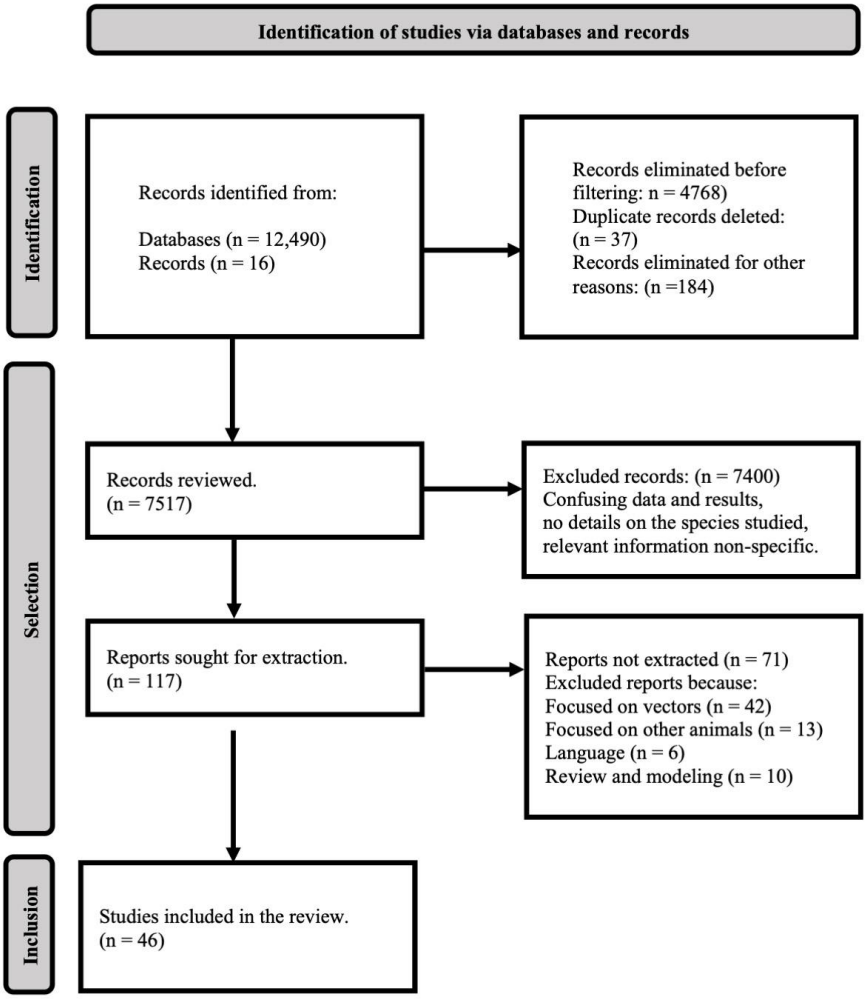


Figure 1. Flow chart modified from (PRISMA 2009) describing the literature search and study selection.

3.2. Prevalence and Detection Methodology

As for the sample size in the studies obtained, it ranges from one single case (case studies) to 2214 individuals. These results show different prevalences among the arbovirus-positive records ranging from 1–100% but, for a small sample size, the 95% confidence interval of the prevalence of arboviruses is wide, so it induced uncertainty.

For the identification of arbovirus-positive records, the most common sample type tested was blood serum (46.67%). Indeed, no study was recorded that performed tests in which animals were not trapped or disturbed. The most used laboratory techniques were hemagglutination inhibition (HI), with 40%, and a plaque reduction neutralization test (PRNT), with 40%, and a real-time polymerase chain reaction qPCR, with 33.33%. A small percentage of studies applied techniques such as a polymerase chain reaction (PCR), with 8.89%, an enzyme-linked immunosorbent assay (ELISA), with 6.67%, and a tissue-based study of histopathology, with 2.22%.

The analysis of the similarity between countries showed that according to the Sorensen and Jaccard indexes, the groups of countries that share more similarity between arboviruses discovered are Colombia and Panama (Sorensen index = 80.00% and Jaccard index = 66.67%), Guatemala and Mexico (Sorensen index = 58.82% and Jaccard index = 41.67%), Guatemala

and Panama (Sorensen index = 50% and Jaccard index = 33.33%), and Mexico and Panama (Sorensen index = 56% and Jaccard index = 38.89%) (Tables 2 and 3). In addition, the relationship between the two indexes was very high (Spearman's correlation coefficient = 0.985, with a p-value < 0.0001).

Table 2. Arbovirus similarity index according to Sorensen's index among Neotropical countries.

	Argentina	Brazil	Colombia	Costa Rica	Guatemala	French Guiana	Mexico	Panama	Paraguay	Uruguay	Venezuela
Argentina											
Brazil	45.00										
Colombia	36.36	16.67									
Costa Rica	47.62	21.05	36.36								
Guatemala	25.00	24.24	37.50	40.00							
French Guiana	37.50	24.24	25.00	26.67	20.00						
Mexico	41.67	30.00	36.36	54.55	58.82	23.53					
Panama	50.00	26.32	80.00	43.48	50.00	25.00	56.00				
Paraguay	30.77	6.67	15.38	0.00	0.00	28.57	0.00	30.77			
Uruguay	30.77	6.67	14.29	33.33	28.57	28.57	28.57	28.57	0.00		
Venezuela	35.29	17.65	11.76	25.00	36.36	0.00	33.33	33.33	0.00	25.00	

Table 3. Arbovirus similarity index according to Jaccard's index among Neotropical countries.

Country	Argentina	Brazil	Colombia	Costa Rica	Guatemala	French Guiana	Mexico	Panama	Paraguay	Uruguay	Venezuela
Argentina											
Brazil	29.03										
Colombia	22.22	9.09									
Costa Rica	31.25	11.76	22.22								
Guatemala	14.29	13.79	23.08	25.00							
French Guiana	23.08	13.79	14.29	15.38	11.11						
Mexico	26.32	17.65	22.22	37.50	41.67	13.33					
Panama	33.33	15.15	66.67	27.78	33.33	14.29	38.89				
Paraguay	18.18	3.45	8.33	0.00	0.00	16.67	0.00	18.18			
Uruguay	18.18	3.45	7.69	20.00	16.67	16.67	16.67	16.67	0.00		
Venezuela	21.43	9.68	6.25	14.29	22.22	0.00	20.00	20.00	0.00	14.29	

The systematic review identified the richness of arboviruses (n = 43) present in nine mammalian orders: non-human primates (n = 20), Perissodactyla (n = 17), Rodentia (n = 18), Artiodactyla (n = 12), Pilosa (n = 11), Chiroptera (n = 8), Didelphimorphia (n = 6), Carnivora (n = 2), and Lagomorpha (n = 2). There are several mammalian orders harboring the same species of arboviruses, as evidenced in (Table A1).

The similarity analysis for mammalian orders evidenced that according to the Sorensen and Jaccard indexes, the orders that share more arboviruses are Chiroptera and Didelphimorphia (Sorensen index = 61.54% and Jaccard index = 44.44%) (Tables 4 and 5).

Table 4. Similarity index according to Sorensen's index among mammalian orders in the Neotropics.

Orders	Artiodactyla	Carnívora	Chiroptera	Didelphimorphia	Lagomorpha	Perissodactyla	Pilosa	Non-Human Primates	Rodentia
Artiodactyla									
Carnívora	0.00								
Chiroptera	30.00	20.00							
Didelphimorphia	22.22	25.00	61.54						
Lagomorpha	0.00	0.00	0.00	0.00					
Perissodactyla	34.48	0.00	32.00	26.09	0.00				
Pilosa	34.78	0.00	42.11	35.29	15.38	28.57			
Non-human primates	18.75	9.09	35.71	23.08	0.00	43.24	45.16		
Rodentia	19.35	19.05	51.85	32.00	9.52	33.33	46.67	46.15	

The results of the literature review identified that monitoring efforts have focused on nine taxonomic orders: mon-human primates (n = 18), Perissodactyla (n = 13), Chiroptera (n = 8), Rodentia (n = 7), Didelphimorphia (n = 7), Artiodactyla (n = 6), Pilosa (n = 4), Carnivora (n = 2), and Lagomorpha (n = 2) (Table A2 and Figure 2).

Table 5. Similarity index according to Jaccard's index among mammal orders in the Neotropics.

Orders	Artiodactyla	Carnívora	Chiroptera	Didelphimorphia	Lagomorpha	Perissodactyla	Pilosa	Non-Human Primates	Rodentia
Artiodactyla									
Carnívora	0.00								
Chiroptera	17.65	11.11							
Didelphimorphia	22.22	14.29	44.44						
Lagomorpha	0.00	0.00	0.00	0.00					
Perissodactyla	20.83	0.00	19.05	15.00	0.00				
Pilosa	21.05	0.00	26.67	21.43	8.33	16.67			
Non-human primates	10.34	4.76	21.74	13.04	0.00	27.59	29.17		
Rodentia	10.71	10.53	35.00	19.05	5.00	20.00	30.43	30.00	

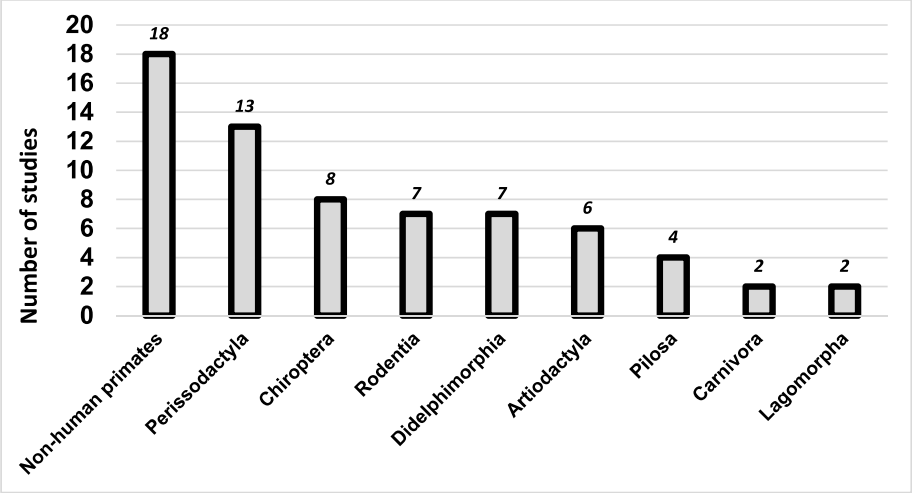


Figure 2. Arbovirus sampling effort in the Neotropics by order of mammal decreasing order.

Sampling efforts were conducted during the period 1967–2021. From the periods 2000–2005 and 2015–2021, there is an increase in research focused on arbovirus detection. It is evident that the sampling effort has increased over the years (negative binomial regression; $p < 0.001$) (Figure 3).

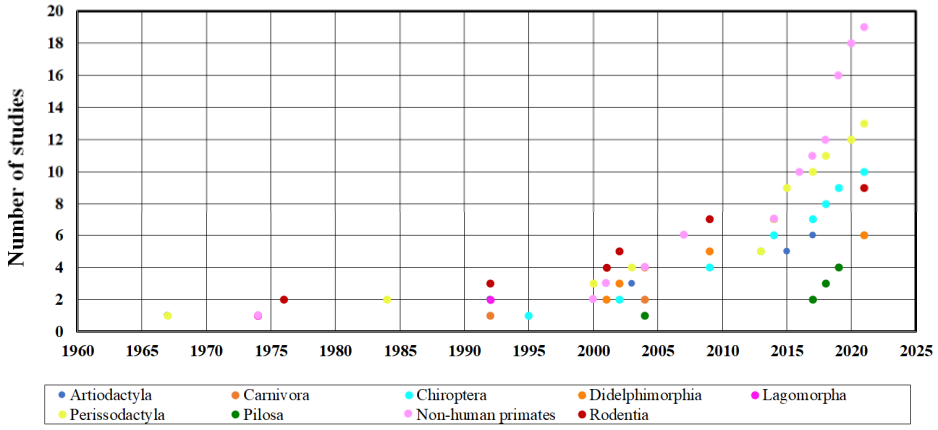


Figure 3. Sampling effort for arboviruses in the different orders of mammals in the Neotropics during the period 1967–2021 (n = 46).

The orders non-human Primates and Rodentia have the highest arbovirus richness (n = 20 and n = 18, respectively). However, no significant difference was found between orders (Kruskal–Wallis equality-of-populations rank test = 14.54, $p > 0.05$). The orders with the highest number of arbovirus-positive records are Primates (n= 54) and Perissodactyla (n = 45) (Figure 4). Wildlife habitat conditions influence arbovirus richness in non-human primates while the domestic environment influences more in the order Perissodactyla.

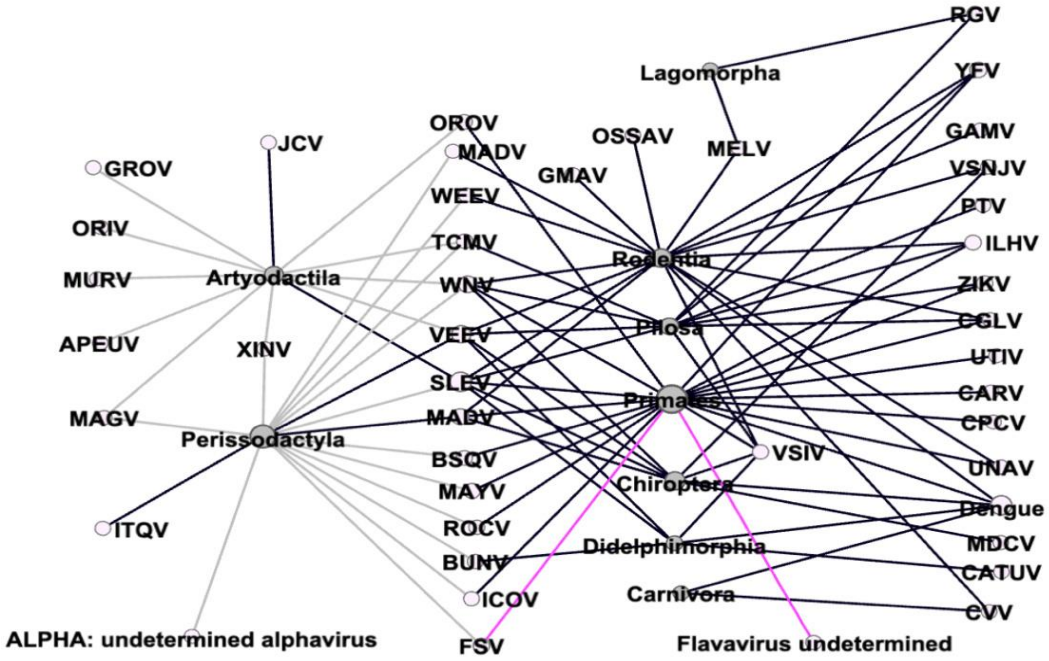


Figure 4. Arbovirus host network in mammals in the Neotropics. Legend: grey link: eomestic environment, black link: wildlife environment, pink link: zoological settings, grey circles: mammal orders, white circles: arbovirus. Note: Apeu virus—APEUV; Bunyamwera virus—BUNV; Bussuquara virus—BSQV; Cache Valley virus—CVV; Cacipacore virus—CPCV; Caraparu virus—CARV; Catu virus—CATUV; Changuinola virus—CGLV; Dengue virus; Madariaga virus—MADV; Western equine encephalitis virus—WEEV; Flavavirus undetermined; Fort Sherman virus—FSV; Gamboa virus—GAMV; Guama virus—GMAV; Guaroa virus—GROV; Icoaraci virus—ICOV; Ilheus virus—ILHV; Itaqui virus—ITQV; Jamestown Canyon virus—JCV; Madrid virus—MADV; Maguari virus—MAGV; Mayaro virus—MAYV; Melao virus—MELV; Mojui dos Campos virus—MDCV; Murutucu virus—MURV; Oriboca virus—ORIV; Oropouche virus—OROV; Ossa virus—OSSAV; Punta Toro virus—PTV; Rocio virus—ROCV; Sant Louis encefalitis virus—SLEV; Tacaiuma virus—TCMV; UNA virus—UNAV; Utinga virus—UTIV; Venezuelan equine encephalitis virus—VEEV; Vesicular stomatitis, Indiana serotype virus—VSIV; Vesicular stomatitis virus, New Jersey serotype—VSNJV; West Nile virus—WNV; Xingu virus—XINV; yellow fever—YFV; Zika—ZIKV; Rio Grande—RGV.

The arboviruses recorded in most mammalian orders in the Neotropics are the Saint Louis encephalitis virus (SLEV) (n = 7), the Venezuelan equine encephalitis virus (VEEV) (n = 6), and the West Nile virus (WNV) (n = 6) (Figure 5).

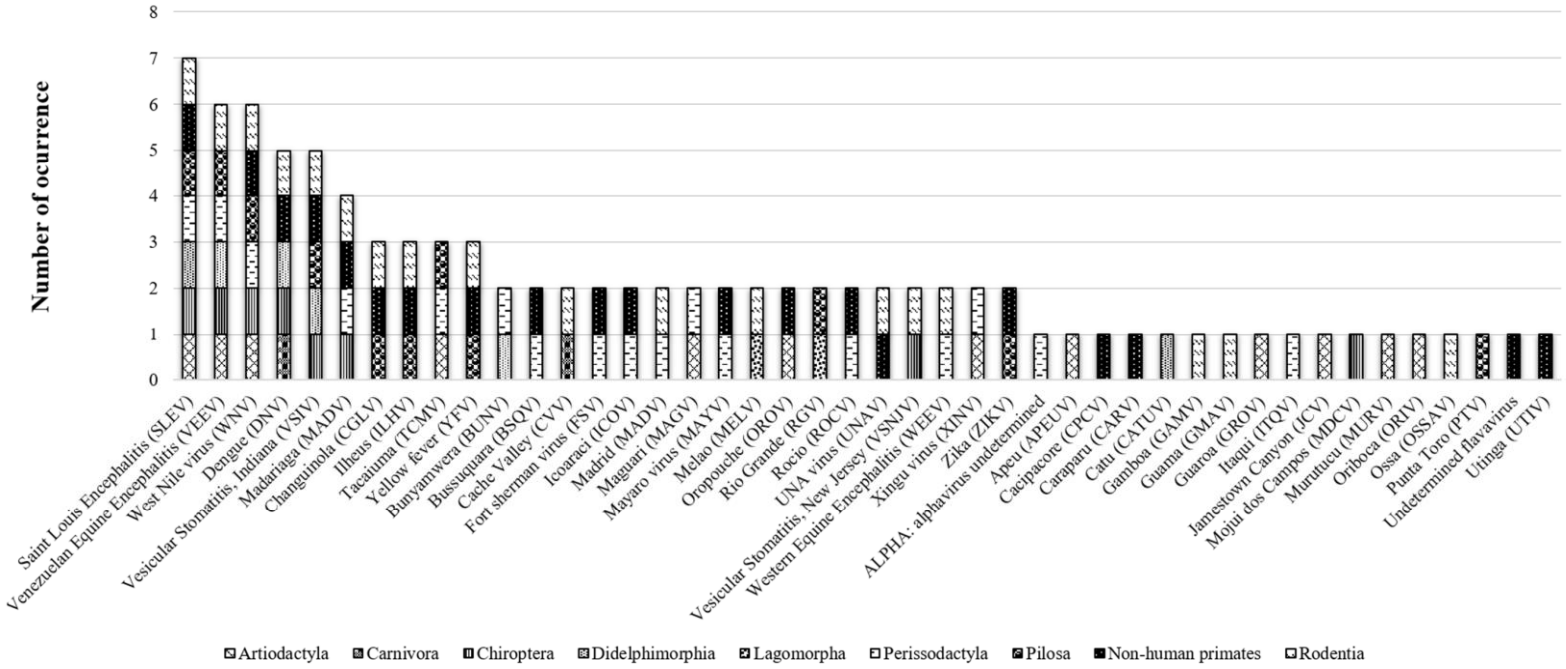
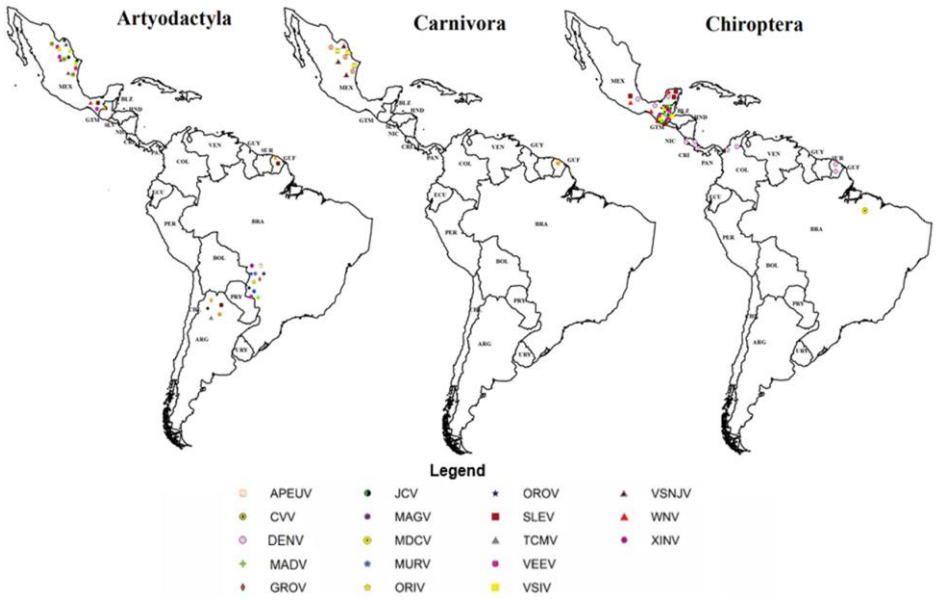
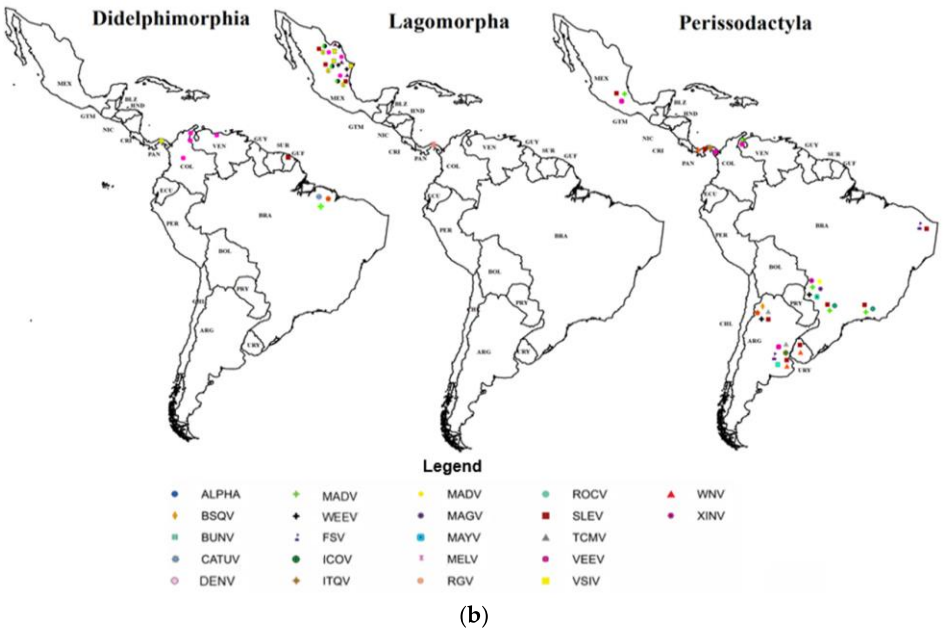


Figure 5. Arbovirus records by mammalian order in the Neotropics.

The map (Figure 6) shows the areas reported with the presence of arboviruses according to the orders of mammals monitored and the types of arboviruses identified for the Neotropics. The country with the highest number of positive orders for arboviruses was Brazil, with 27 arboviruses.

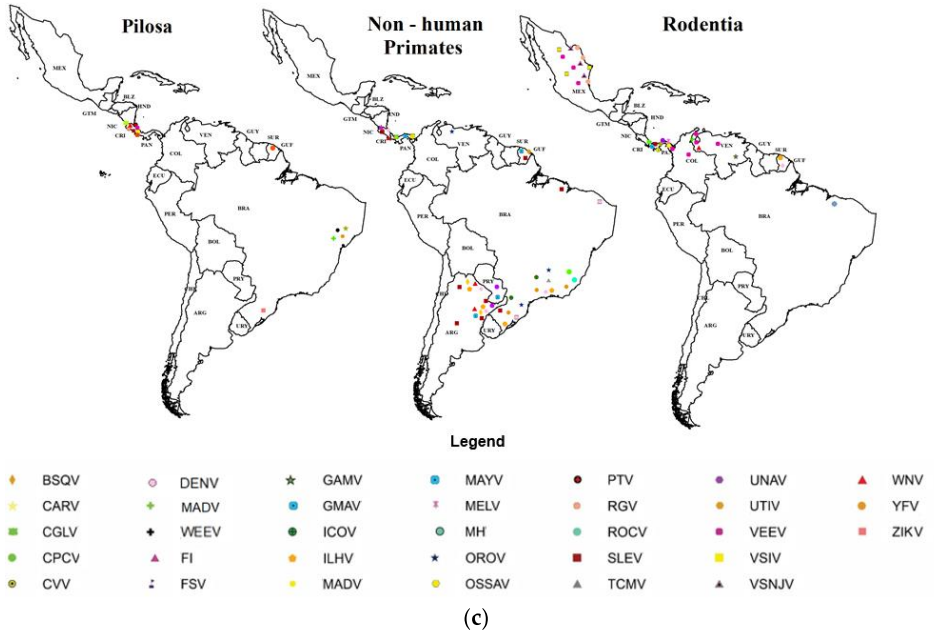


(**a**)



(**b**)

Figure 6. *Cont.*



(**c**)

Figure 6. (**a**) Geographical location of arboviruses recorded by mammalian order in the Neotropics. (**b**) Geographical location of arboviruses recorded by mammalian order in the Neotropics. (**c**) Geographical location of arboviruses recorded by mammalian order in the Neotropics. Note: The concordance between the abbreviation of viruses and their full names is derivated from Table 1.

3.3. Habitat Types with Positive Orders and Sampling Conditions According to Order

It was possible to identify that arbovirus records were found in a wild range of sampling habitat types, i.e., primary forest, secondary forest, disturbed areas, captivity, and even in populated areas (Figure 7). In addition, all mammalian orders that were identified as hosts were found in the wild. However, there were also records both in captive conditions and in the domestic environment (Figure 8).

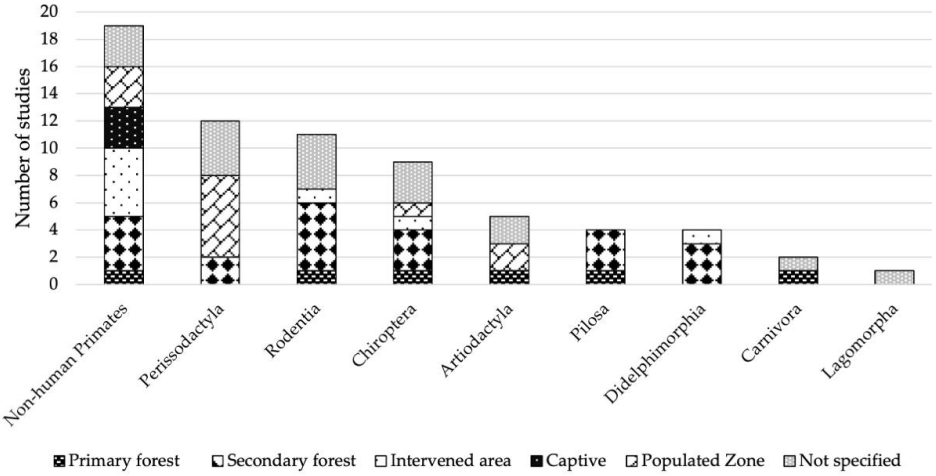


Figure 7. Habitat types with positive records of arboviruses in mammalian orders in the Neotropics.

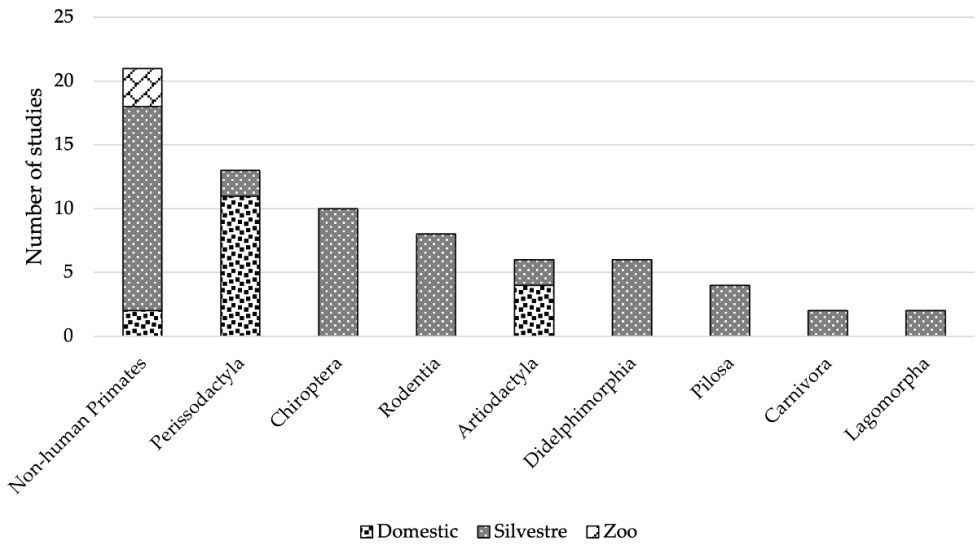


Figure 8. Sampling conditions by mammalian order in the Neotropics.

3.4. Risk Factors

In the Neotropical region, vegetation cover and deforested fronts were identified, as well as the arbovirus positive and negative records that were identified in the nine orders of mammals. The map obtained showed that the arbovirus-positive and negative records for each publication analyzed were mostly found outside the areas with vegetation cover and others were recorded within deforested fronts (Figure 9 and Table A2). The risk factor map includes positive and negative records. All but four studies (which had only positive records) had both negative and positive records. No studies were found with only negative records. The odds ratio of a mammal becoming being exposed to an arbovirus is 1.46 higher when its habitat is located in deforested fronts (95% confidence interval: 1.34–1.59) than if its habitat is with vegetation cover ($p < 0.0001$) (Table 6).

Table 6. Mammals positive to arbovirus in Neotropical countries and occurrence according to habitat.

	Positives Mammals to Arbovirus	Negatives Mammals to Arbovirus	Total
Mammals in deforested fronts	1312	3268	4580
Mammals in vegetation cover	1549	5634	7183
Total	2861	8902	11,763

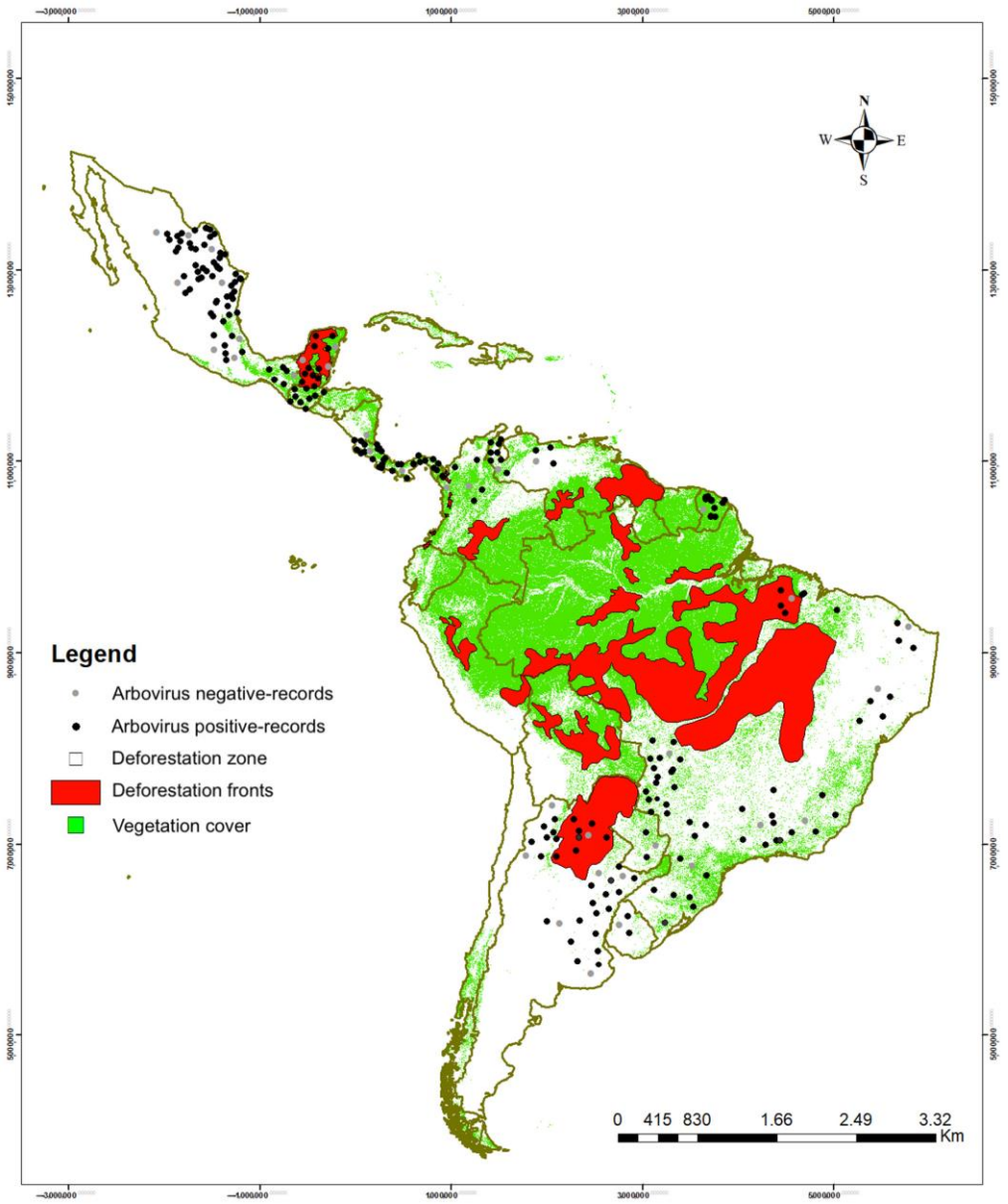


Figure 9. Representation of arbovirus positive and negative records, vegetation cover, and deforested fronts in the Neotropics.

4. Discussion

4.1. Richness of Arboviruses

The results of the literature review reported that monitoring efforts have been concentrated in Argentina, Brazil, Mexico, and Panama, which may be associated with the diversity of arboviruses and vectors they harbor [142,143]. These countries share the occurrence of three arboviruses, i.e., VEEV, SLEV, and WNV. SLEV and VEEV are neotropical viruses, and WNV is introduced from Africa. They are viruses with complex transmission

cycles, a variety of hosts, and a variety of vectors. In addition, in the Neotropics, they are distributed in rural areas, jungle areas, and a few cities on the periphery. SLEV and WNV are Flaviviruses and in humans they exist as cross-protections with the antibodies of the population against dengue and yellow fever, which is why they do not occur in the Neotropics. Dengue and Chikungunya are introduced from Africa and transmitted by the vector *Aedes aegypti*; they are part of an exact transmission cycle in urban areas in the Neotropics [110,144].

Among the countries with arbovirus records are Guatemala, Costa Rica, French Guiana, Paraguay, and Uruguay. In most cases, the pathways for the introduction of arboviruses into new regions are unknown [145]. However, the emergence and spread of arboviruses rapidly and geographically may be due to the growth of global transportation systems [146–151] and the adaptation of humans and arthropods due to increasing urbanization [52]. The countries that obtained the highest number of arbovirus similarity are geographically close, such as Colombia and Panama, which share the biogeographic region of El Choco, and Mexico and Guatemala with shared ecosystems, thus creating the possibility of harboring similar arbovirus species in the same ecozone [152,153].

Recently, arbovirus diseases have been reported with increased frequency worldwide [154]. In the Neotropics, Brazil is characterized by encompassing a large land area covered by tropical forests and densely populated areas [155,156]. These characteristics are closely linked to the strengthening of research efforts according to the number of resources invested for research and development in arbovirus research areas [157]. Similarly, surveillance preferences for specific species and the ease of sampling sites favor the detection of arbovirus prevalence due to the high rates of the infected population [158]. These conditions coincide with the results obtained from the systematic review carried out, which reflect that Brazil is the country with the highest arbovirus richness in the region (n = 27). Due to the high biodiversity of environments and components, a large number of arboviruses have been isolated in Brazil [67,134,135], especially those involved in human diseases such as the Western equine encephalitis virus [98], Saint Louis encephalitis virus [125], Mucambo virus [159], Guaroa virus [99], Tacaiuma virus [102], and Guama virus [106].

The ability of transmission vectors to spread is a determining factor for arbovirus outbreaks worldwide. A previous systematic review of arboviruses in Western Europe reflected that current outbreaks are due to the spread of *Aedes albopictus* and *Aedes japonicus* [160]. Dengue is the most important emerging arboviral disease globally [161,162] due to the wide variety of ecosystems in which it is found and the ease of its spread. Studies have also focused on other arboviruses [163]. An example of this is the VEEV [164], whose natural and most efficient vector in Latin America [165] is *Culex* sp. *Melanoconion* [166,167]. The VEEV is recorded in human [168], equine, and bovine serology studies [126,169] and wild animals [101,170], so it tends to replicate in livestock animals and results in higher levels of contagion in rural environments [171].

Changes in ecological conditions favor the creation of new habitats for arbovirus vectors [132,172,173] and may cause arthropods to adapt to new mammalian hosts [52,174], leading to the emergence of new pathogens in the domestic environment, which is sometimes the main reservoir [175]. Infection in domestic animals can increase circulation and human exposure in peridomestic habitats [168,176].

In the Neotropics, the SLEV is found in a wide distribution from Mexico to Argentina via the mosquito vector *Culex* sp. [177]. Initially, outbreaks were localized in the United States with high human case fatality rates [178]. In Argentina, these records are attributed to the expansion of agricultural and urban habitats [179]. Based on the records of this study, the occurrence of SLEV infection was evidenced in countries of the region such as Costa Rica [107], Guatemala [120], Mexico [104], and Uruguay [125,177].

West Nile virus has the ability to infect a wide variety of wild and captive mammals in all regions of the world [180]. Historically, WNV has one of the broadest host ranges [181–183], and mosquitoes act as vectors, e.g., *Culex* sp. [184] of arboviruses in humans and equines [185]. However, very few studies associated with infection in mammalian

hosts have been conducted during the last decades [181]. Due to the wide distribution of WNV and the association of records of increased prevalence in mammals in urban areas [186], there is a possibility of new outbreaks in most continents [187–191].

4.2. Prevalence and Detection Methodologies

The variability in the number of individuals monitored in this review may be linked to the objectives of each study, the sampling effort, the order of mammals sampled, the capture techniques [192], the type of sample analysis [193], and the characteristics of the site [194].

Different types of methodologies were used to capture the mammals studied. For rodents and marsupials, Sherman traps were used [113] and mist nets were used to capture bats [109,120]. In relation to NHP, dart immobilization [103,115] and manual capture [107] were used. These techniques were used to obtain blood, serum, or tissue samples from the vertebrate host, which in some cases studies involved the sacrifice of species, such as rodents and marsupials [101,113,121,132].

In the three arboviruses with the highest number of records, both serological virus isolation and molecular techniques were used. This allows a better confirmation of the presence of arboviruses in individuals [101,118,124,141] due to the materials and equipment necessary for the correct execution of the analysis protocols for each sample, either under culture, molecular, or serological techniques. The most applied detection methodologies for arbovirus detection were hemagglutination inhibition by its capacity or facility and plaque reduction neutralization test. The combined use of serological and molecular techniques facilitates the indirect detection of arboviruses. For example, the simultaneous application of techniques, such as ELISA and RT-PCR, allowed the identification of co-infection of dengue with other arboviruses, such as Chikungunya and Zika, respectively [195].

In the last decade with the significant development of new molecular detection technologies in epidemiological surveillance, there are still few studies using PCRs or qPCRs for arbovirus species determination (16/46) [100,101,196]. However, according to Mendoza-Ponce, Corona-Núnez, Galicia, and Kraxner [16] worldwide, qPCR is highly effective for diagnosing arboviruses in humans, even with low viremias. The accuracy of this technique [65] is important in wildlife, as samples cannot always be repeated or individuals have already died. This methodology is sensitive and specific and should be used in wildlife. Phylogenetic studies that would complement detection studies are not widely present [108,113]. Currently, with the COVID-19 pandemic, the importance of having more specific tools, such as sequencing and metagenomics, at hand for proper species detection and determination of outbreak origins were observed [197,198]. Additionally, advances in equipment mean that samples can be taken and analyzed in the field, reducing data loss [199].

The results of the literature review showed that most of the records were identified in secondary forest mammals, which is associated with previous research showing that areas with greater intervention have greater potential for zoonotic diseases, which could act as a potential danger to surrounding communities [55]. For example, in Singapore, it was reported mosquitoes of the genus *Aedes* could be both in the forest and in urban open areas that are highly frequented by people [200]. Similarly, in Brazil, it was reported there were a high abundance of vectors in urban forests and a dominance of vector species according to habitat [172].

The use of non-invasive samples is an opportunity that could facilitate the diagnosis and detection of arboviruses in animals using urine and saliva samples [201–203]. Detection using fecal samples is another cost-effective and non-invasive option to monitor wild populations that could be potential reservoirs of arboviruses [204]. The use of this type of sample is effective for arbovirus detection and is an option to avoid stress on monitored species. Similarly, research focused on this sampling protocol could increase the number of orders that currently have not been monitored and contribute to conservation genetics, as well as behavioral ecology and infectious and parasitic diseases [205–207].

It is important to note that, although this study reflects the reality of arbovirus monitoring in the neotropics, there is a lack of study of negative records (zero prevalence). Unfortunately, few studies on infectious and parasitic diseases publish negative results [208]. This study confirms what others have already suggested regarding the publication of positive results [198,209]. There are more publications with positive results than negative ones. In this study, we found no publications with completely negative results. This is called publication bias [210]. Negative results are important because they may have an ecological, behavioral, or management explanation [211], which can directly contribute to our knowledge of infectious and parasitic diseases and contribute to epidemiological monitoring plans or policies. Additionally, in some neotropical countries, most research remains unpublish for economic reasons or publication rejections [212], which favors the perception that it is better not to publish negative results. Publication of negative results is highly recommended.

4.3. Risk Factors

Climate change implies impacts on human health and vector-borne infectious disease burden [213]. Fluctuating climatic conditions such as precipitation [214], temperature [215], and humidity [216] impact infection rates in tropical regions [217,218] that facilitate arbovirus reproduction and transmission in a wide diversity of habitats [156]. Patterns of vector population distribution, reproduction, and competition [219] can be influenced by temperature [220], precipitation [221], abundance, and the affinity of the vector for a mammalian host species [222]. Variability among hematophagous arthropod species [75], mammalian hosts [223], and the environments or niches they occupy [224] impacts arbovirus transmission dynamics [37,192] and are subject to changes in temperature and precipitation [186,225].

The current co-circulation of three arboviruses, Zika, dengue, and Chikungunya, spreading globally in the Americas suggests the need for more integrative studies and the use of new approaches to identify the cause and risk posed by these combinations [226]. González-Salazar, Stephens, and Sánchez-Cordero [221] even created a model of possible mammalian hosts of Zika for the Neotropical region where seven of the nine species identified were bats. As for Chikungunya, this was not recorded in any order of non-human mammals in the records of the Neotropics, although a study in Brazil carried out serological and molecular analyses of bats without any detection [227]. Based on this background, the question now arises whether Chikungunya and Zika have been found in non-human hosts in America for their maintenance cycle, similar to what occurred with the yellow fever virus and the Mayaro virus, which originated in Africa. Currently, incorrect diagnoses are made based on known symptoms in areas where several arboviruses are circulating simultaneously [228].

On the other hand, in countries in other regions of the world there have already been records of this arbovirus in NHP [229], rats [230], bats [230], and horses [231]. Monitoring and detection should be continued in mammals such as primates, rodents, and bats that are susceptible to infection [232], even without the intervention of a vector [233].

Arbovirus circulation can occur in sylvatic or urban cycles [234]. Thus, altering the balance of natural systems can increase vector abundance, create new reservoirs, or induce arboviruses to adapt to new maintenance cycles [235]. Additionally, the relationship that humans maintain with various species of domestic mammals is considered another factor that favors the reproduction cycles of arboviruses and their vectors [100,236].

4.4. Socio-Ecological Aspects

Records of arboviruses in new areas are linked to the distribution and spread patterns of vectors, such as *Aedes aegypti*, from Africa to the Neotropics due to the influence of human trade routes [237]. Social phenomena, such as human migration and species trafficking, are key factors that have favored the spread of arboviruses [60]. Factors that determine vector trajectory conditions are established in various regions [238]. These factors are associated

with climate change [239], deforestation [240], uncontrolled growth of urban areas [241], difficulty in accessing clean water sources [242], and population displacement [243]. In the Brazilian Amazon region, there are a greater number of positive records of sylvatic arboviruses associated with deforestation [155], mining [244], road expansion [245], and urbanization with the emergence and/or re-emergence of relevant arboviruses [172].

4.5. Ecological Aspects

Environmental factors and environments are determinants in the life cycles of arboviruses as they determine their distribution and dispersal patterns, as well as their transmission to mammalian hosts [40]. The interaction between vectors and mammalian hosts conditions the dynamics and impact of arboviruses in human and domestic animal communities [32].

Arboviruses have a high host-specific association, so changes in range or distribution significantly influence their adaptations in new areas [176]. For example, as shown in Figure 7, NHP is the order identified in the greatest variability of habitats in primary forests, secondary forests, disturbed areas, captivity, and populated areas. Non-human primates are reservoirs for a large number of blood-borne pathogens and ecological factors such as host density, climate change, and activities facilitate the transmission of these pathogens [207].

As a survival mechanism, arboviruses have the ability to develop adaptive mutations when they reach new territories new hosts, vectors, and environments to adapt quickly and improve transmission. Thus, positive records of arboviruses are reflected in variability of sampling conditions, including domestic, wild, and zoo, which can be seen in Figure 8, with the wild condition being the most frequent in all orders. However, in the face of changes in the environment, it is difficult to predict the speed of species response [225,246], so research on the ecology of interactions between arboviruses and mosquito vectors is needed to understand the dynamics of invasion and adaptation in new areas [165].

Ecological mechanisms are an important part of every stage and enzootic potential of the Neotropics, and we must consider within them the exposure of wildlife, the pressures of propagation, the enzootic infection that affects the exposed animals, and the persistence of enzootic transmission [63]. The impact of deforestation on the abundance of vectors, which facilitate arbovirus transmission, influences their movement from wild areas to urban or rural areas [247] where they can adapt to new domestic hosts that maintain direct contact with humans. Figure 9 reflects that the effects of deforestation in forested areas of the Neotropics are more evident over time and increase the contact of the wide variety of vectors with humans, and thus the risk of contagion, dispersal, and epizootics [248,249].

4.6. Health Policies

Population growth [250], the expansion of the agricultural frontier [251], the impact of anthropogenic activities [252], and climate change [253] contribute to the spread of arbovirus infections [254]. The 2015 Lancet Commission on Health and Climate Change mentions that the fight against climate change could be the greatest opportunity for global health in the twenty-first century [255].

The levels of epidemiological and entomological surveillance should be potentiated, as well as the joint analysis of the factors that condition the level of vulnerability of a certain area to arboviral diseases [256]. Success in the execution, monitoring, and evaluation of programs focused on the prevention and control of emerging diseases will mitigate the spread of arboviruses in the Neotropics.

At the international level, arthropod vector-borne arboviruses are part of the public health problem that requires cooperation and joint research to establish effective control strategies. All the parameters that are part of the biology of the vectors must be taken into account, as well as the dynamics of reproduction of the arboviruses [257]. Institutions such as the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado FMT-HVD in Brazil work on the detection of arbovirus infections, considering that the country has suffered silent outbreaks due to problems with the identification of arbovirus [156].

The World Organisation for Animal Health (OIE) seeks to promote the importance of animal disease surveillance and communication systems. By generating the necessary knowledge, it will be possible to manage risks, evaluate priorities and generate policies according to the guidelines of each country [258]. Additionally, eradication programs [259,260] consider the social, economic, and even political part of each zone in order to mitigate or control arbovirus infections in the Neotropics.

4.7. Actions to Take

The need to strengthen research, surveillance programs, and public policies is indispensable in the Neotropics. The identification of possible regions and habitats with ecological conditions suitable for the circulation of arboviruses and/or specific areas with a high risk of infection is required [261]. In addition, climate change-based modeling associated with infectious diseases supports early warning systems [262].

There should be an increase in the use of non-invasive techniques. The protocols used for sampling in each publication analyzed in the systematic review do not establish non-invasive methodologies, even though several species of mammals that registered positive arboviruses fall within a threat category on the International Union for Conservation of Nature's (IUCN) red list.

Due to the complexity of transmission and contagion dynamics, as well as the biology of vectors and hosts, it is considered that an integrated solution would allow effective vector control through new technologies, adequate management of space and resources, and control and sanitation policies [263]. For example, in the last decade, the use of geographic information systems (GIS) has become an important tool for the detection, analysis, and prediction of epidemiological patterns that have contributed to the prevention and control plans of diseases caused by arboviruses [264]. In the case of vectors that transmit arboviruses, a biotechnological tool has been generated that is helping to control mosquito vectors by genetically modifying them. It is highly effective, does not harm the environment, is efficient, and mainly has a low production cost [265]. The use of insecticides, on the other hand, has shown that it has created resistance in mosquitoes of the genus *Aedes* [266] that have developed resistance against insecticides. These mosquitoes are transmitters of different arboviruses such as dengue [267], yellow fever [268], Chikungunya [269], and Zika [270].

Long-term monitoring of the interactions of nearby populations is necessary to prevent the spread of arboviruses to uncontaminated locations [271]. It is essential to strengthen epidemiological monitoring in the areas that maintain research initiatives for the control of arboviruses in the Neotropics. Considering that knowledge is the basis for the prevention and control of emerging diseases, in areas that do not yet maintain plans and/or policies of epidemiological monitoring, basic but transcendental measures should be considered, such as training the general population for the elimination of spaces that could serve as potential reservoirs for vectors [272]. Similarly, health education campaigns should be strengthened [273] and encourage research for the early detection of arboviruses, mainly in the areas most likely to be infected [274].

5. Conclusions

This study identified the diversity of arboviruses in the Neotropics region, within the list of the 17 countries that make it up. We recorded a greater sampling effort in 11 countries (Argentina, Brazil, Colombia, Costa Rica, Guatemala, French Guiana, Mexico, Panama, Paraguay, Uruguay, and Venezuela). However, there are countries in which no effort has been made, despite the great biodiversity of the area and the wide range of host mammals.

The Venezuelan equine encephalitis virus (VEEV), St. Louis encephalitis virus (SLEV), and the West Nile virus (WNV) share the most mammalian orders. In addition, it was identified that there are orders with greater sampling effort that can be associated with ease in terms of the sampling technique and the objectives of each study. The prevalence of arboviruses (1–100%) varies due to the sample size of each study, as cases range from 1 to 2214 individuals found in various habitats and conditions.

As the present research highlights the record of the Mayaro virus in mammalian hosts and the influence of climate change that facilitates the creation of new environments and the adaptation of vectors, it would be important to focus the monitoring effort on determining if we can consider the Mayaro virus as the next emerging arbovirus given its phylogenetic closeness to the Chikungunya virus [228,275,276]. Furthermore, in the case of Mayaro, due to environmental changes, contact between peri-urban and urban areas is increasingly possible. Finally, experimentally, competition between three vectors (especially *Ae. aegypti* and *Ae. albopictus*, and to a lesser extent *Cx. quinquefasciatus*) has been observed, which means that these vectors may also play a role in the circulation of Mayaro [277–279].

Deforestation was observed as an important risk factor in terms of the observed records, as a large number of positive records for arbovirus number of publications are found outside forested areas and within deforested fronts of the Neotropics, which is associated with the fact that disturbed habitats increase the risk of infection [73].

The studies analyzed in the review maintain conventional analysis techniques, such as serology. However, the use of molecular detection tools, e.g., PCR and qPCR, is a priority to have an adequate response in the case of outbreaks. Response tools require monitoring in wild areas to be able to relate outbreaks to origins, as well as the application of techniques that provide more specific information, such as sequencing and metagenomics for the detection of arboviruses.

The orders with the greatest monitoring effort are non-human primates, Perissodactyla, Chiroptera, and Rodentia. In addition, research gaps were identified. Future research should focus on the orders Artiodactyla, Carnivora, Chiroptera, Didelphimorphia, Lagomorpha, Perissodactyla, Pilosa, NHP, and Rodentia, which are important sources of information for disease monitoring. In biodiverse areas, such as Amazonian ecosystems, there are information gaps, so research should focus on the dynamics of emerging diseases and local fauna as a monitoring tool for conservation [245,280].

Interactions between factors such as environment, hosts, and vectors are a potential risk to disease prevalence. Long-term and constant monitoring is required, accompanied by monitoring and sampling techniques focused on methodologies with non-invasive techniques that are cost-effective and provide the same results without altering the dynamics of populations or the health of individuals.

Health education through the generation of educational instruments and constant training for the population is a good option on a smaller scale that could complement the monitoring and prevention initiatives of public policies in each country. Anthropic effects have a direct impact on the factors that determine the trajectory of vectors, the distribution ranges of host mammals, and the distribution of arboviruses in the Neotropics.

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Appendix A

Table A1. Mammalian orders with arbovirus-positive records by habitat and environment.

Mammalian Host	Environment	Country	Arbovirus	Habitat	References
Artiodactyla	Domestic	Argentina Brazil Mexico	Apeu virus (APEUV) Guaroa virus (GROV) Venezuelan equine encephalitis virus (VEEV) Maguari virus (MAGV) Murutucu virus (MURV) Oriboca virus (ORIV) Oropouche virus (OROV) Tacaiuma virus (TCMV) West Nile virus (WNV) Xingu virus (XINV)	Primary forest Populated zone Not specified	[98,99,104,129]
	Wild	French Guiana Mexico	Saint Louis encephalitis virus (SLEV) Jamestown Canyon virus (JCV)	Primary fores Not specified	[105,114]
Carnívora	Wild	French Guiana Mexico	Dengue virus (DENV) Cache Valley virus (CVV)	Primary forest Not specified	[105,114]
Chiroptera	Wild	Brazil Colombia Costa Rica Guatemala French Guiana Mexico	Dengue virus (DENV) Eastern equine encephalitis virus (EEEV) Venezuelan Equine Encephalitis virus (VEEV) Saint Louis encephalitis virus (SLEV) Vesicular stomatitis, New Jersey virus (VSNJV) Vesicular stomatitis, Indiana (VSIV) Mojui dos Campos virus (MDCV) West Nile virus (WNV)	Primary forest Secondary forest Intervened area Not specified Populated zone	[108,109,113,116–118,120,133,138,281]
Didelphimorphia	Wild	Brazil Colombia French Guiana Panama Venezuela	Bunyamwera virus (BUNV) Catu virus (CATUV) Dengue virus (DENV) Venezuelan equine encephalitis virus (VEEV) Saint Louis encephalitis virus (SLEV) Vesicular stomatitis, Indiana virus (VSIV)	Primary forest Secondary forest Not specified Intervened area	[67,101,106,113,114,132]
Lagomorpha	Wild	Mexico Panama	Melao virus (MELV) Rio Grande virus (RGV)	Not specified	[105,106]
Perissodactyla	Domestic	Argentina Brazil Mexico Panama Uruguay Venezuela	ALPHA: alphavirus undetermined Bunyamwera virus (BUNV) Bussuquara virus (BSQV) Eastern equine encephalitis virus (EEEV) Weastern equine encephalitis virus (WEEV) Venezuelan equine encephalitis virus (VEEV) Saint Louis encephalitis virus (SLEV) Fort Sherman virus (FSV) Icoaraci virus (ICOV) Itaqui virus (ITQV) Madrid virus (MADV) Maguari virus (MAGV) Mayaro virus (MAYV) Rocio virus (ROCV) Tacaiuma virus (TCMV) West Nile virus (WNV) Xingu virus (XINV)	Secondary forest Not specified Populated zone	[98–100,104,119,122,123,125,126,129–131,135]

Table A1. *Cont.*

Mammalian Host	Environment	Country	Arbovirus	Habitat	References
Pilosa	Wild	Brazil Costa Rica French Guiana	Changuinola virus (CGLV) Venezuelan equine encephalitis virus (VEEV) Saint Louis encephalitis virus (SLEV) Vesicular stomatitis, Indiana virus (VSIV) Yellow fever virus (YFV) Ilheus virus (ILHV) Punta Toro virus (PTV) Rio Grande virus (RGV) Tacaiuma virus (TCMV) West Nile virus (WNV) Zika virus (ZIKV)	Primary forest Secondary forest	[102,107,114,124]
Non-human primates	Domestic or Zoological settings	Brazil Costa Rica	Dengue virus (DENV) Yellow fever virus (YFV) Flavavirus indeterminado Fort Sherman virus (FSV) Oropouche virus (OROV)	Captive Populated zone	[110,111,134,136]
	Wild	Argentina Brazil Costa Rica French Guiana Panama Paraguay Venezuela	Bussuquara virus (BSQV) Cacipacore virus (CPCV) Caraparu virus (CARV) Changuinola virus (CGLV) Dengue virus (DENV) Eastern equine encephalitis virus (EEEV) Saint Louis encephalitis virus (SLEV) Vesicular stomatitis, Indiana virus (VSIV) Yellow fever virus (YFV) Icoaraci virus (ICOV) Ilheus virus (ILHV) Mayaro virus (MAYV) Oropouche virus (OROV) Rocio virus (ROCV) UNA virus (UNAV) Utinga virus (UTIV) West Nile virus (WNV) Zika virus (ZIKV)	Primary forest Secondary forest Intervened area Populated zone	[102,103,106,110–112,114,115,124,127,128,131,137,139–141]
Rodentia	Wild	Brazil Colombia French Guiana Mexico Panama Venezuela	Cache Valley virus (CVV) Changuinola virus (CGLV) Dengue virus (DENV) Eastern equine encephalitis virus (EEEV) Weastern equine encephalitis virus (WEEV)Venezuelan equine encephalitis virus (VEEV) Saint Louis encephalitis virus SLEV) Vesicular stomatitis, New Jersey virus (VSNJV) Vesicular stomatitis, Indiana virus (VSIV) Yellow fever virus (YFV) Gamboa virus (GAMV) Guama virus (GMAV) Ilheus virus (ILHV) Madrid virus (MADV) Melao virus (MELV) Ossa virus (OSSAV) UNA virus (UNAV) West Nile virus (WNV)	Primary forest Secondary forest Captive Not specified Intervened area	[67,101,105,106,113,114,121,132,196]

Appendix B

Table A2. Representation of arbovirus-positive and -negative records in every type of area.

#	Title	Author	Prevalence	Positive	Negative	Type of Area
1	Arbovirus serosurvey Orthobunyavirus, Flavivirus, and Alphavirus) in a draft horse population from Santa Fe, Argentina 2013–2016)	Albrieu-Llinás 2021 [123]	TOTAL 222 = INFECTED prevalence FSV = 76.8%, SLEV = 59.6%, WNV = 27.5%, RNV = 7.4%	FSV: 170 SLEV: 132 WNV: 61 RNV: 16	FSV: 52 SLEV: 90 WNV: 161 RNV: 206	Deforested fronts
2	Prevalence of Flavivirus antibodies in Alouatta caraya primate autochthonous of Argentina	Contigiani et al., 2000 [127]	TOTAL 105 = INFECTED prevalence HI 35.23%), NT 32.38%)	37 34	68 71	Deforested fronts
3	Serological survey on arbovirus detected in animals in the province of Tucuman, Argentina	De Ruiz Holgado et al., 1967 [129]	Total 75 = infected 58	58	17	Deforested fronts
4	Infection by UNA virus Alphavirus; Togaviridae) and risk factor analysis in black howler monkeys Alouatta caraya) from Paraguay and Argentina	Díaz et al., 2007 [137]	TOTAL 90 = INFECTED 67 46 UNAV y 21 UNAV y el MAYV)	67	23	Deforested fronts
5	First isolation of Bunyamwera virus Bunyaviridae family) from horses with neurological disease and an abortion in Argentina	Tauro et al., 2015 [100]	2 HORSES = 2 INFECTED	2	0	Deforested fronts
6	Neutralizing antibodies for orthobunyaviruses in Pantanal, Brazil	Pauvolid-Corrêa et al., 2017 [99]	TOTAL 607 = 373 equidos + 126 bovinos INFECTED TOTAL 499	499	108	Deforested fronts
7	Identification of animal hosts of Fort Sherman virus, a New World zoonotic orthobunyavirus	de Oliveira Filho et al., 2020 [135]	TOTAL 192 = 2 INFECTED	2	190	Deforested fronts
8	Yellow fever surveillance challenge: Investigation of a marmoset non-autochthonous case	Fernandes et al., 2020 [134]	TOTAL 1 = 1 INFECTED	1	0	Vegetation cover
9	Identification of the encephalitis equine virus, Parana, Brazil	Fernández et al., 2000 [119]	TOTAL 22 = INFECTED 12	12	10	Deforested fronts
10	Detection of antibodies to Oropouche virus in non-human primates in Goiânia City, Goiás	Gibrail et al., 2016 [136]	TOTAL 50 = 2 INFECTED	2	48	Vegetation cover
11	Neutralising antibodies for Mayaro virus in Pantanal, Brazil	Pauvolid-Corrêa et al., 2015 [98]	TOTAL 985 = 239 INFECTED	239	746	Vegetation cover
12	A Saint Louis encephalitis and Rocio virus serosurvey in Brazilian horses	Silva et al., 2014 [130]	TOTAL 753 = INFECTED 415	415	338	Vegetation cover
13	Serological evidence for Saint Louis encephalitis virus in free-ranging New World monkeys and horses within the upper Paraná River basin region, Southern Brazil	Svoboda et al., 2014 [131]	TOTAL 133 = 30 INFECTED	30	103	Vegetation cover
14	Ultrastructural, antigenic and physicochemical characterization of the Mojuí dos Campos Bunyavirus) isolated from bat in the Brazilian Amazon region	Wanzeller et al., 2002 [138]	TOTAL 1 = INFECTED 1	1	0	Deforested fronts
15	A survey to assess potential human disease hazards along proposed sea level canal routes in Panamá and Colombia. V. Arbovirus infection in non-human vertebrates	Srihonges et al., 1974 [106]	TOTAL 2214 = INFECTED 28	28	2186	Vegetation cover

Table A2. *Cont.*

#	Title	Author	Prevalence	Positive	Negative	Type of Area
16	Serosurvey of selected arboviral pathogens in free-ranging, two-toed sloths (*Choloepus hoffmanni*) and three-toed sloths (*Bradypus variegatus*) in Costa Rica, 2005-07	Medlin et al., 2016 [107]	TOTAL 109 = 87 INFECTED	87	22	Vegetation cover
17	Serologic survey of neotropical bats in Guatemala for virus antibodies	Ubico et al., 1995 [120]	TOTAL 332 = INFECTED 87	87	245	Vegetation cover
18	Dengue infection in neotropical forest mammals	de Thoisy et al., 2009 [113]	TOTAL 616 = INFECTED 92	92	524	Vegetation cover
19	Health evaluation of translocated free-ranging primates in French Guiana	de Thoisy et al., 2001 [112]	TOTAL 141 = INFECTED	Dengue virus II 20/141, Yellow fever virus 47/141, Saint Louis virus 16/141, Mayaro virus 76/141 Average 40	Dengue virus II 121/141, Yellow fever virus 94/141, Saint Louis virus 125/141, Mayaro virus 65/141 Average 101	Vegetation cover
20	Serologic survey for selected arboviruses and other potential pathogens in wildlife from Mexico	Aguirre et al., 1992 [105]	TOTAL 80 = 1 INFECTED	1	79	Deforested fronts
21	Serologic survey of domestic animals for zoonotic arbovirus infections in the Lacandón Forest region of Chiapas, Mexico	Ulloa et al., 2003 [104]	TOTA 72 = INFECTED 54	54	18	Vegetation cover
22	Human and Equine Infection with Alphaviruses and Flaviviruses in Panamá during 2010: A Cross-Sectional Study of Household Contacts during an Encephalitis Outbreak	Carrera et al., 2018 [126]	TOTAL 194 = INFECTED 126	126	68	Vegetation cover
23	Seroprevalence of St. Louis encephalitis virus and West Nile virus Flavivirus, Flaviviridae) in horses, Uruguay	Burgueño et al., 2013 [125]	TOTAL 425 = INFCTED 205	205	220	Deforested fronts
24	Ecological studies of enzootic Venezuelan equine encephalitis in north-central Venezuela, 1997–1998	Salas et al., 2001 [101]	TOTAL 543 = INFECTED 30	30	513	Deforested fronts
25	Studies of arboviruses in Southwestern Venezuela: I. Isolations of Venezuelan and Eastern Equine Encephalitis viruses from sentinel hamsters in the Catatumbo region	Walder et al., 1976 [121]	TOTAL 95 = INFECTED 30	30	65	Deforested fronts
26	Arbovirus studies in the Guajira region of Venezuela: activities of eastern equine encephalitis and Venezuelan equine encephalitis viruses during an interepizootic period	Walder et al., 1984 [122]	TOTAL 64 = INFECTED 60	60	4	Deforested fronts
27	Detection of dengue virus in bat flies Diptera: Streblidae) of common vampire bats, Desmodus rotundus, in Progreso, Hidalgo, Mexico	Abundes-Gallegos et al., 2018 [108]	TOTAL 16 = INFECTED 8	8	8	Vegetation cover

Table A2. *Cont.*

#	Title	Author	Prevalence	Positive	Negative	Type of Area
28	Detection of antibodies against Icoaraci, Ilhéus, and Saint Louis Encephalitis arboviruses during yellow fever monitoring surveillance in non-human primates Alouatta caraya) in southern Brazil	Almeida et al., 2019 [124]	TOTAL 26 = INFECTED 5	5	21	Deforested fronts
29	Contrasting sylvatic foci of Venezuelan equine encephalitis virus in northern South America	Barrera et al., 2002 [132]	TOTAL 546 = INFECTED 20	20	526	Deforested fronts
30	Study of Arboviruses in Philander opossum, Didelphis marsupialis and Nectomys rattus captured from forest fragments in the municipality of Belém, Pará, Brazil	Bernal et al., 2021 [67]	TOTAL 14 = INFECTED 8	8	6	Deforested fronts
31	Dengue virus infection in neotropical forest mammals: incidental hosts or potential reservoirs?	Lavergne et al., 2009 [281]	TOTAL 464 = INFECTED 92	92	372	Vegetation cover
32	Two Cases of Natural Infection of Dengue-2 Virus in Bats in the Colombian Caribbean	Calderón et al., 2021 [109]	TOTAL 286 = INFECTED 2	2	284	Deforested fronts
33	Surveillance of arboviruses in primates and sloths in the Atlantic Forest, Bahia, Brazil	Catenacci et al., 2018 [102]	TOTAL 139 = INFECTED 25	25	114	Vegetation cover
34	Flaviviruses infections in neotropical primates suggest long-term circulation of Saint Louis Encephalitis and Dengue virus spillback in socioeconomic regions with high numbers of Dengue human cases in Costa Rica	Chaves et al., 2020 [110]	TOTAL 86 = INFECTED 35	35	51	Vegetation cover
35	Serosurvey of Nonhuman Primates in Costa Rica at the Human–Wildlife Interface Reveals High Exposure to Flaviviruses	Chaves et al., 2021 [111]	TOTAL 86 = INFECTED 38	38	48	Vegetation cover
36	Immunity to yellow fever, Oropouche and Saint Louis viruses in a wild howler monkey	de Almeida et al., 2016 [128]	TOTAL 1 = INFECTED 1	1	0	Deforested fronts
37	Detection of a novel African-lineage-like Zika virus naturally infecting free-living neotropical primates in Southern Brazil	de Almeida et al., 2019 [140]	TOAL 50 = INFECTED 9	9	41	Vegetation cover
38	Wild terrestrial rainforest mammals as potential reservoirs for flaviviruses yellow fever, dengue 2 and St Louis encephalitis viruses) in French Guiana	De Thoisy, B Dussart, Philippe Kazanji, M. 2004 [114]	TOTAL 574= INFECTED 50	50	524	Vegetation cover
39	Zika Virus in peridomestic neotropical primates, Northeast Brazil	Favoretto, Silvana et al., 2019 [141]	TOTAL 132 = INFECTED 9	9	123	Deforested fronts
40	Detection of antibodies against flavivirus over time in wild non-human primates from the lowlands of Costa Rica	Dolz, Gaby et al., 2019 [115]	TOTAL 209 = INFECTED 53	53	156	Deforested fronts
41	Serologic evidence of flavivirus infection in bats in the Yucatan Peninsula of Mexico	Machain-Williams, Carlos et al., 2013 [116]	TOTAL 140 = infected 26	26	114	Deforested fronts

Table A2. *Cont.*

#	Title	Author	Prevalence	Positive	Negative	Type of Area
42	Neotropical bats that co-habit with humans function as dead-end hosts for dengue virus	Vicente-Santos, Amanda et al., 2017 [118]	TOTAL 318 = INFECTED 28	28	290	Deforested fronts
43	Dengue virus in bats from southeastern Mexico	Sotomayor-Bonilla, Jesús et al., 2014 [117]	TOTAL 146 = INFECTED 79	79	67	Vegetation cover
44	Isolation of Madre de Dios Virus Orthobunyavirus; Bunyaviridae), an Oropouche virus species reassortant, from a monkey in Venezuela	Navarro et al., 2016 [139]	TOTAL 2 = INFECTED 1	1	1	Deforested fronts
45	Eco-epidemiology of the Venezuelan equine encephalitis virus in bats of Cordoba and Sucre, Colombia	Guzmán, Camilo et al., 2019 [133]	TOTAL 286 = INFECTED 2	2	284	Deforested fronts
46	Detection of the mosquito-borne flaviviruses, West Nile, dengue, Saint Louis encephalitis, Ilheus, Bussuquara, and yellow fever in free-ranging black howlers *Alouatta caraya*) of northeastern Argentina	Morales et al., 2017 [103]	TOTAL 108 = INFECTED 70	70	38	Deforested fronts

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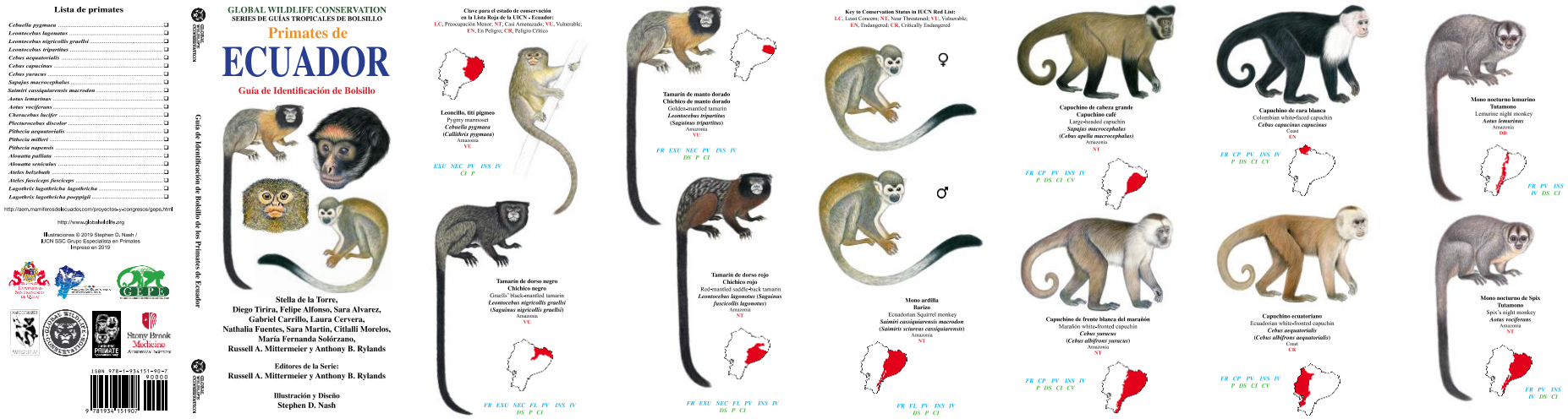
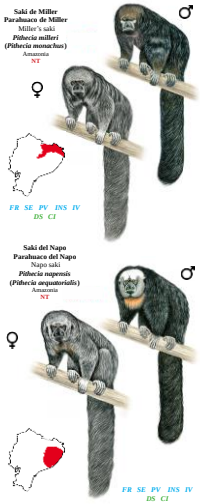
Appendix 4

Global Wildlife Conservation – Series de Guías Tropicales de bolsillo – Primates de Ecuador – Guía de identificación de Bolsillo

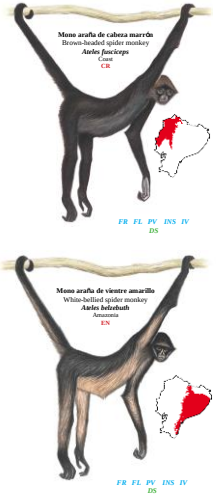


Key to Conservation Status in the Ecuadorian IUCN Red List:
LC, Least Concern; NT, Near Threatened; VU, Vulnerable;
EN, Endangered; CR, Critically Endangered
Titi rojizo
Cotoncillo rojo
Sungo sungo
Red-crowned titi monkey
Plecturocebus discolor
(Callicebus discolor)
Amazonia
NT
FR PV INS IV
DS CI
Titi de manos amarillas
Cotoncillo negro
Yellow-handed titi monkey
Cheracebus lucifer
(Callicebus lucifer)
Amazonia
VU
FR PV INS IV
DS CI

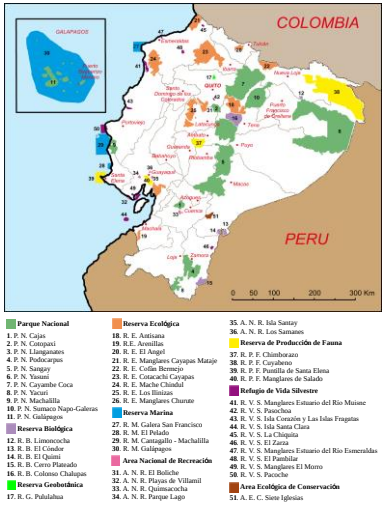
Saki ecuatorial
Parahuaco ecuatorial
Equatorial saki
Pithecia aequatorialis
Amazonia
NE
FR SE PV INS IV
DS CI
Clave para la dieta y funciones ecológicas:
Dieta
EXU = Exudados
HJL = Hojas
NEC = Néctar
FL = Flores
SE = Semillas
PV = Pequeños Vertebrados
INS = Insectos
IV = Invertebrados
FR = Frutas
CP = Corazón de Palmas
Funciones Ecológicas
P = Polinizador
DS = Dispersor de Semillas
CI = Control de Poblaciones de Insectos
CV = Control de Poblaciones de Vertebrados
Key to diet and ecological roles:
Diet
EXU = Exudates
HJL = Leaves
NEC = Nectar
FL = Flowers
SE = Seeds
PV = Small Vertebrates
INS = Insects
IV = Invertebrates
FR = Fruits
CP = Hearts of Palm
Ecological Roles
P = Pollinator
DS = Seed Disperser
CI = Control of Insect Populations
CV = Control of Vertebrate Populations


Saki de Miller
Parahuaco de Miller
Miller's saki
Pithecia milleri
(Pithecia monachus)
Amazonia
NT
FR SE PV INS IV
DS CI
Saki del Napo
Parahuaco del Napo
Napo saki
Pithecia napensis
(Pithecia aequatorialis)
Amazonia
NT
FR SE PV INS IV
DS CI

Mono aullador rojo de Linneo
Coto rojo
Colombian red howler
Alouatta sericulus
Amazonia
NT
FR FL HJL PV INS IV
DS
Mono aullador de manto dorado
Aullador negro
Uve
Ecuadorian mantled howler
Alouatta palliata aequatorialis
Coast
EN
FR FL HJL PV INS IV
DS


Mono araña de cabeza marrón
Brown-headed spider monkey
Ateles fusciceps
Coast
CR
FR FL PV INS IV
DS
Mono araña de vientre amarillo
White-bellied spider monkey
Ateles belzebuth
Amazonia
EN
FR FL PV INS IV
DS

FR HJL PV INS IV
DS
Mono lanudo de Humboldt
Chorongo ceniza
Woolly monkey
Humboldt's woolly monkey
Lagothrix lagotricha lagotricha
Amazonia
EN
FR HJL PV INS IV
DS
Mono lanudo de Poeppig
Macaco barrigudo
Mono barrigudo
Mono chuvo
Chorongo rojizo
Poeppig's woolly monkey
Lagothrix lagotricha poeppigii
Amazonia
EN


COLOMBIA
PERU
GALAPAGOS
0 50 100 200 300 Km
Parque Nacional
1. P. N. Cajas
2. P. N. Cotopaxi
3. P. N. Llanganates
4. P. N. Podocarpus
5. P. N. Sangay
6. P. N. Yasuní
7. P. N. Cayambe Coca
8. P. N. Yacuri
9. P. N. Machalilla
10. P. N. Sumaco Napo-Galeras
11. P. N. Galápagos
Reserva Biológica
12. R. B. Limoncocha
13. R. B. El Cóndor
14. R. B. El Quimi
15. R. B. Cerro Plateado
16. R. B. Colonso Chalupas
Reserva Geobotánica
17. R. G. Pululahua
Reserva Ecológica
18. R. E. Antisana
19. R.E. Aromillas
20. R. E. El Angel
21. R. E. Manglares Cayapas Mataje
22. R. E. Cofán Bermejo
23. R. E. Cotacachi Cayapas
24. R. E. Mache Chindul
25. R. E. Los Ilinizas
26. R. E. Manglares Churute
Reserva Marina
27. R. M. Galera San Francisco
28. R. M. El Pelado
29. R. M. Cantagallo - Machalilla
30. R. M. Galápagos
Area Nacional de Recreación
31. A. N. R. El Boliche
32. A. N. R. Playas de Villamil
33. A. N. R. Quimsacocha
34. A. N. R. Parque Lago
35. A. N. R. Isla Santay
36. A. N. R. Los Samanes
Reserva de Producción de Fauna
37. R. P. F. Chimborazo
38. R. P. F. Cuyabeno
39. R. P. F. Puntilla de Santa Elena
40. R. P. F. Manglares de Salado
Refugio de Vida Silvestre
41. R. V. S. Manglares Estuario del Río Muisne
42. R. V. S. Pasochoa
43. R. V. S. Isla Corazón y Las Islas Fragatas
44. R. V. S. Isla Santa Clara
45. R. V. S. La Chiquita
46. R. V. S. El Zarza
47. R. V. S. Manglares Estuario del Río Esmeraldas
48. R. V. S. El Pambilar
49. R. V. S. Manglares El Morro
50. R. V. S. Pacoche
Area Ecológica de Conservación
51. A. E. C. Siete Iglesias