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Molecular detection and characterization of *Giardia* spp., *Cryptosporidium* spp., and *Blastocystis* in captive wild animals rescued from central Colombia

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ABSTRACT

Cryptosporidium, *Giardia*, and *Blastocystis* are significant causes of diarrhea worldwide. However, studies on their prevalence in wild animals are limited, compared to humans and domestic animals. In this study, we collected 23 stool samples from captive wild rescued animals in Boyacá, Colombia. Using conventional PCR, we detected *Cryptosporidium* spp., *Giardia* spp., and *Blastocystis* in over half of the samples (69.6%). *Cryptosporidium* spp. (43.5%) were the most commonly found, followed by *Giardia* spp. (39.1%) and *Blastocystis* (13.0%). Co-infections involving these parasites were also observed. Subsequent genotyping revealed *Cryptosporidium canis* and *Cryptosporidium ryanae* as the predominant species. These findings contribute valuable information about the ecoepidemiology of intestinal parasites in Colombian wild animals.

1. Introduction

Intestinal parasites affect nearly 3.5 billion people, and more than 200,000 deaths are reported per year (Hajare et al., 2021; Kamel and Abdel-Latef, 2021). Infections caused primarily by *Cryptosporidium* spp., *Giardia duodenalis* and *Blastocystis* are of significant importance due to their potential for zoonotic transmission (Kamel and Abdel-Latef, 2021; Widmer et al., 2020). *Cryptosporidium* spp. invades the epithelial cells of the host's digestive tract, causing diarrheal disease (cryptosporidiosis) (CDC, 2019; Coklin et al., 2007). In 2021, the number of validated species of *Cryptosporidium* spp. was updated to 44, with 29 species identified in mammalian hosts, 20 of them can infect humans, and 15 species found in other animals such as fish, birds, amphibians, and reptiles. This suggests the potential for zoonotic transmission of these species (Garcia-R et al., 2017; Ryan et al., 2021). *Giardia duodenalis* is the most common intestinal parasite, with eight recognized assemblages (A-H), and it has been reported in humans, domestic animals, and wildlife. It is primarily transmitted through waterborne and foodborne routes, but potential zoonotic and reverse zoonotic transmission has also been proposed (Dixon, 2021). However, it is important to note that 200 million human infection cases are reported annually, resulting in 500,000 deaths per year (Hajare et al., 2022).

Furthermore, *Blastocystis*, another intestinal parasite, exhibits a global distribution. Through sequence analysis of the small-subunit ribosomal RNA (SSU-rRNA), researchers have identified 38 subtypes of *Blastocystis* obtained from animals and human samples (Maloney et al., 2022). Among these subtypes, ST1-ST12 have been found in both humans and animals. These subtypes are suspected to have zoonotic potential (Maloney et al., 2019). Zoonotic transmission between captive animals and their caretakers has been supported by evidence (Stensvold et al., 2009; Köster et al., 2022a; Maloney et al., 2019).

Several studies conducted worldwide have estimated the prevalence and genotypes of *Cryptosporidium* spp., *Giardia* spp., and *Blastocystis* in free-ranging and captive wild animals, including animals in zoos or rescued animal settlements. These studies have revealed varying prevalence rates and associations between protozoan genotypes and mammal species, providing evidence of zoonotic transmission and suggesting that animals may serve as potential sources of transmission to humans, including zookeepers (Geurden et al., 2009; Kamel and Abdel-Latef, 2021; Karim et al., 2021; Li et al., 2020; Zou et al., 2022). For instance, assemblages A, B, D, and E of *G. duodenalis* have been reported in zoo animals in China, and a study involving non-human

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primates and their handlers in European zoos demonstrated zoonotic transmission of *Blastocystis* ST1-ST4 (Köster et al., 2022b; Zou et al., 2022). In Australia and the United Kingdom, ST1 and ST8 were found in zoo primates and their keepers (Stensvold et al., 2009), while ST5 was reported in pigs and their caretakers in China and Australia (Maloney et al., 2019).

We have included comprehensive information regarding the protozoa infection and genotyping in both captured and zoo animals. Supplementary Table 1 provides detailed data on the identified species and their respective genotypes. This valuable information adds depth to our study, shedding light on the diversity and transmission dynamics of these protozoa. By examining the genetic profiles of the identified strains, we gain insights into their origin, potential sources of infection, and possible reservoir hosts. This comprehensive analysis enriches our understanding of the epidemiology and implications of protozoa infections in both wildlife and captive animal populations.

Wild animal trafficking is a significant health and social issue that contributes to the emergence and re-emergence of zoonotic diseases (Tazerji et al., 2022). Although limited studies have focused on protozoan infections in wild animal trafficking, infections have been reported in domestic animals such as dogs and cats. The risk of gastrointestinal parasite infections increases in shelter environments, facilitating their spread among animals, keepers, and visitors (Raza et al., 2018; Ruaux and Stang, 2014). In Colombia, high prevalence of intestinal parasites has been observed in both humans and animals through human surveys (Higuera et al., 2020; Peña-Quistial et al., 2020; Pinilla León et al., 2019; Villamizar et al., 2019). Additionally, due to Colombia's high biodiversity, approximately 58,000 animals are seized by Colombian authorities annually due to illegal wildlife trafficking (Goyes and Sollund, 2016). According to the most recent National Survey of Intestinal Parasitism, *Blastocystis*, and *Giardia* spp., were identified as the predominant pathogenic protozoa (Hernández et al., 2019). However, most studies in the region have focused on humans, domestic animals, and water sources, with a lack of reports on wild animals, particularly rescued wild animals (Higuera et al., 2020; Sánchez et al., 2017, 2018).

Considering the potential significance of wild animals in transmitting intestinal pathogens to humans and domestic animals, and the relatively unexplored nature of this field, there is a general lack of information on the subject. There is a growing interest in studying the zoonotic potential of other hosts/reservoirs, understanding the transmission routes of parasites, and investigating the interaction between humans, wild or domestic animals, and even rescued animals (Thompson et al., 2009). Therefore, the objective of this study is to detect and genotype intestinal parasites, specifically *G. duodenalis*, *Cryptosporidium* spp., and *Blastocystis*, in rescued wild mammals kept in captivity using PCR.

2. Materials and methods

2.1. Study area and population

Stool samples were collected from wild rescued animals in captivity in Central Colombia on a single day in November 2019. These animals were seized by Colombian authorities from various regions of Colombia due to illegal trafficking. The animals were provided with a specific diet as instructed by the veterinarian and were given cistern water. They did not show any symptoms of intestinal infection. These animals were rescued from different regions of Colombia due to illegal trafficking and had a background of living with humans and other domestic or wild animal species. They were provided with a balanced diet sourced from the local market to fulfil their nutritional needs, and a veterinarian supervised their care. The water for the animals was obtained from a reservoir supplied by a stream. Each enclosure was equipped with dedicated feeders and drinkers exclusive to the residents. None of the animals exhibited any signs of intestinal infection. A deworming protocol had not been implemented. A total of 23 different wild mammal species were included in the study, Samples were taken both individually (from animals in separate enclosures) and in pools (from animals of the same species housed together in the same enclosure) (Table 1). Approximately 200 mg of stool were collected from the ground using Eppendorf tubes containing ethanol, ensuring minimal contact between the sylvatic animals and the keepers. The ground was cleaned daily. The collected samples were then stored at −30 °C.

2.2. DNA extraction

DNA was extracted from stool samples using the stool DNA Isolation Kit from Norgen Biotek, following the manufacturer's recommendations.

2.3. Detection and genotyping of intestinal protozoa

Intestinal protozoa were detected using a previously described protocol by Mejia et al. (2013), with modifications as reported by Higuera et al. (2020) (Higuera et al., 2020; Mejia et al., 2013). In brief, we employed a conventional PCR targeting the 18S rRNA to detect *G. duodenalis*, *Cryptosporidium* spp., and *Blastocystis*. Negative controls for the PCR consisted of molecular grade water, while positive controls included DNA extracted from cultures of *G. duodenalis* reference strain WB, *Cryptosporidium parvum* strain [IOWA], and *Blastocystis* subtype 5 (isolated from a colonized patient). If any of the controls do not yield expected and reliable results, the PCR is not validated, and the entire experiment is repeated. The thermal cycling parameters for the PCR were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 1 min, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Samples that tested positive for any of the three mentioned protozoa underwent genotyping PCR. The triose phosphate isomerase (*tpi*) gene was amplified to determine the assemblages of *G. duodenalis*, while SSU-rRNA was amplified for *Cryptosporidium* spp. and *Blastocystis* genotyping and subtype detection (Feng and Xiao, 2011; Higuera et al., 2020; Scicluna et al., 2006). The primer sequences used in these PCR protocols are specified in Supplementary Table 2.

The genotyping-PCR products were subjected to electrophoresis, purified using ExoSAP-IT®, and then sequenced in both forward and reverse directions using the Sanger method by Macrogen (Seoul, South Korea).

The sequenced products were validated and aligned using UGENE software. A consensus sequence was generated by combining the forward and reverse strands. To identify *Cryptosporidium* species, *G. duodenalis* assemblages, and *Blastocystis* subtypes, an in-house database containing species, assemblages, and subtypes of the protozoa was utilized. The database was indexed using the makeblastdb command and queried using the BLASTN tool. The assignment in the BLAST analysis considered criteria such as identity higher than 80%, coverage higher than 90% of the amplicon size, and an e-value of 0.

3. Results

3.1. Detection of Giardia spp., Cryptosporidium spp. and Blastocystis in wild animals kept in captivity in central Colombia

We collected 23 stool samples from individual or pooled wild animals kept in captivity in Boyacá, Colombia, and performed PCR to detect *Blastocystis*, *G. duodenalis*, and *Cryptosporidium* spp. Out of the total samples, 69.6% (16/23) tested positive for one or more of the evaluated parasites. Three out of 23 samples (13.0%) were positive for *Blastocystis*, and 39.1% (9/23) were positive for *G. duodenalis*. *Cryptosporidium* spp. had the highest frequency of occurrence in the samples, with 43.5% (10/23) testing positive. Co-infection between *G. duodenalis* and *Cryptosporidium* spp. was observed in 21.7% (5/23) of the samples, co-infection between *Blastocystis* and *Cryptosporidium* spp. in 4.3% (1/23) of the

Table 1
Detection of intestinal protozoa in wild animals kept in captivity in Central Colombia. Samples with *Blastocystis, Cryptosporidium* and *Giardia duodenalis* discriminated by order, family, and species. * Animals in individual enclosures.

Order	Family	Species	Common name	Parasite infection			Type of sample	Number of individuals
				Giardia	*Cryptosporidium*	*Blastocystis*		
Artiodactyla	Bovidae	*Bison bison*	Bison				Pool	1*
	Cervidae	*Odocoileus virginianus*	White-tailed deer		+	+	Pool	2
	Suidae	*Sus scrofa domesticus*	Domestic pig	+	+		Pool	2
Carnivora	Canidae	*Urocyon cinereoargenteus*	Grey fox				Individual	2
		Cerdocyon thous	Crab-eating fox/ Forest fox	+			Pool	1*
	Felidae	*Puma concolor*	Cougar	+			Pool	3
		Panthera onca	Jaguar	+			Pool	2
		Panthera tigris	Tiger				Individual	2
		Panthera leo	Lioness				Individual	1*
		Panthera leo	Lion				Pool	1*
		Panthera tigris	Tiger				Individual	2
	Mustelidae	*Galictis vittata*	Greater grison		+		Individual	1*
		Eira barbara	Tayra	+	+		Pool	3
	Procyonidae	*Potos flavus*	Kinkajou				Pool	4
		Nasua nasua	South America coati				Pool	4
	Ursidae	*Ursus arctos*	Brown bear	+	+		Pool	2
Primates	Atelidae	*Lagothrix lagotrichia*	Brown woolly monkey				Pool	3
		Ateles hybridus	Brown spider monkey			+	Pool	4
	Cabidae	*Sapajus apella*	Tufted capuchin		+		Pool	3
		Saimiri sciureus	Guianan squirrel monkey		+		Pool	4
Rodentia	Cuniculidae	*Cuniculus paca*	Lowland/Spotted paca		+		Pool	2
	Dasyproctidae	*Dasyprocta fuliginosa*	Black agouti	+	+	+	Pool	2
	Caviidae	*Hydrochoerus hydrochaeris*	Capybara	+	+		Pool	3

* Animals in individual enclosures.

samples, and co-infection among all three parasites in 4.3% (1/23) of the samples (Table 1).

All samples from the Rodentia order (n = 3) tested positive for *Cryptosporidium* spp., with two of them also testing positive for *G. duodenalis*, and one testing positive for *Blastocystis* (Table 1). Co-infections between *G. duodenalis* and *Cryptosporidium* spp. were observed in the Suidae (*Sus scrofa domesticus*), Ursidae (*Ursus arctos*), Dasyproctidae (*Dasyprocta fuliginosa*), and Caviidae (*Hydrochoerus hydrochaeris*) families. Co-infection between *Cryptosporidium* spp. and *Blastocystis* was found in the Cervidae (*Odocoileus virginianus*) and Dasyproctidae (*Dasyprocta fuliginosa*) families. The Cabidae (*Sapajus apella, Saimiri sciureus*) family was only infected with *Cryptosporidium* spp. No infections were found in the Bovidae and Procyonidae families (Table 1). Finally, *Ateles hybridus* was solely infected with *Blastocystis* and was the only infected member of the Atelidae family. Similarly, *Cerdocyon thous*, the only member of Canidae, was infected with *Cryptosporidium* spp. (Table 1).

3.2. Genotyping in wild animals' stools samples

Ten samples positive for *Cryptosporidium* spp. were subjected to a genotyping PCR (Supplementary Table 3). The species found were *Cryptosporidium ryanae and Cryptosporidium cani*. We were unable to identify the *Cryptosporidium* species for 8 positive samples (NI). A relationship between the animals order and *Cryptosporidium* species was observed (Fig. 1). For instance, the Rodentia order was infected with *C. ryanae* and non-identified (NI) species and the Primate order with NI. Conversely, Carnivora and Artiodactyla showed a species-specific association: *Galictis vittata* with NI, and *Sus scrofa domesticus* with *C. canis*. The species of *Cryptosporidium* infecting *Eira barbara* could not be determined (Fig. 1). We found the stool sample of *Ateles hybridus* infected with subtype 3 of *Blastocystis*, but unfortunately, we were not able to evaluate the other two positive samples of this parasite. On the other hand, we could not determine the assemblages of *G. duodenalis* in our positive samples, and the chromatogram showed a double signal that needs to be clarified. Also, the double peaks in the chromatograms may suggest mixed infections by different *Giardia* species and/or

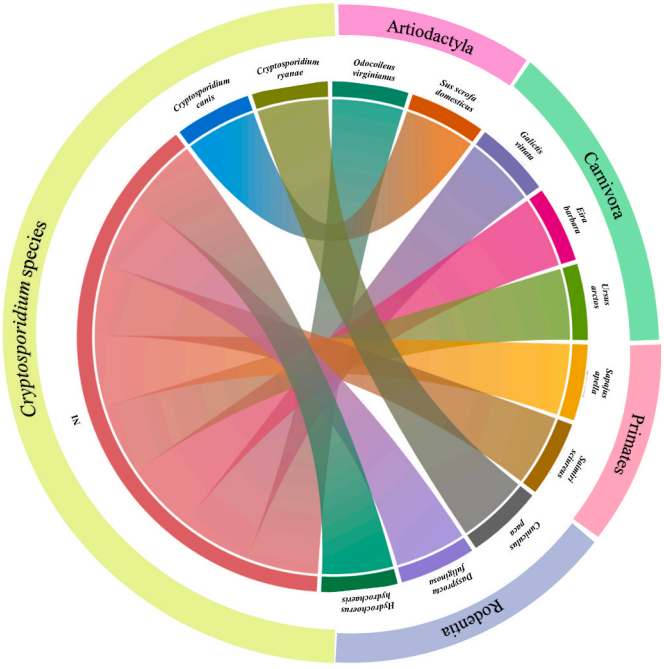

Fig. 1. *Cryptosporidium* species in wild animals kept in captivity. The chord diagram shows the relation between *Cryptosporidium* species and the order and species of infected animals.

assemblages.

4. Discussion

Here, we report a high frequency of *Giardia* spp. and *Cryptosporidium* spp. infections in wild rescued animals in central Colombia, with a prevalence of 69.6%. Results from zoos in China, Belgium, and Bangladesh zoos found a lower frequency of *Giardia* spp. and *Cryptosporidium* spp. infection between 3.5% and 10.6% (Geurden et al., 2009; Karim et al., 2021; Zou et al., 2022). However, an Egyptian zoo showed similar rates of protozoan infection, indicating global variation in prevalence (Kamel and Abdel-Latef, 2021).

Since our study was conducted on wild animals kept in captivity within a zoo, the potential for zoonotic transmission is significant, as some of these protozoan species are known to cause severe diarrhea and other clinical manifestations in humans (Widmer et al., 2020). Our study confirms the ability of these protozoan species to cause multiple species infections, highlighting the need for better control programs to prevent the transmission and spread of these parasites (Widmer et al., 2020).

Co-infections between *Cryptosporidium* spp. and *Giardia* spp., as well as between *Giardia* spp. and *Blastocystis*, were observed in our study (Higuera et al., 2020). In Colombian subjects, the percentage of co-infection between *Cryptosporidium* spp. and *Giardia* spp., as well as between *Giardia* spp. and *Blastocystis*, corresponds to 3.4% in both cases (Higuera et al., 2020). While co-infections are rare in cattle, they are more common in cats and have been associated with diarrheal outbreaks and infection intensity (Delling and Daugschies, 2022; Enemark et al., 2020; Santin, 2020). In our study, the percentage of *Cryptosporidium* spp.-*Giardia* spp. and *Giardia* spp.-*Blastocystis* co-infections increased to 21.7% and 8.7%, respectively, suggesting that co-infections in wild animals may be frequent and pose a risk for animal handlers.

We did not detect *C. hominis* and *C. parvum* in our study, which are the main species responsible for cryptosporidiosis in humans. This finding is not consistent with recent studies conducted on Colombian subjects (Higuera et al., 2020; Villamizar et al., 2019). However, it is possible that using other genes or markers could enable the detection of these species in our samples. Further investigations using alternative molecular targets may provide valuable insights into the presence of *C. hominis* and *C. parvum* in our study population.

C. canis was found in our study, This species has previously been detected in zoonotic infections involving diarrheal samples from humans and dogs (Iwashita et al., 2021). In our study, we identified a case of *Sus scrofa domesticus* infected with this species. It is worth noting that these pigs are in direct contact with zookeepers, raising concerns about the potential for zoonotic transmission. *C. ryanae* was found to infect *Cuniculus paca* in this study, which, to the best of our knowledge, represents the first report of such an infection. Although *C. ryanae* is primarily associated with cattle, some studies have demonstrated and reported its ability to infect other rodents such as mice (Fayer et al., 2008).

These results suggest the influence of environmental factors in *Cryptosporidium* infection (Chalmers et al., 2019). One possible explanation for these unexpected findings is the acquisition of these infections within the rescue institution from other sheltered animal species. Given the proximity and potential for interaction between different species in a rescue institution, cross-contamination and cross-species transmission may have occurred. Further investigation is warranted to explore the possible role of other sheltered animal species as a source of *Cryptosporidium* infections in these non-primary host species. Understanding the dynamics of *Cryptosporidium* transmission in a mixed-species environment is crucial for implementing effective control measures and preventing further spread.

To our knowledge, this is the first study to implement molecular detection for evaluating stool samples in a wild rescue population of animals in Colombia. However, future research should acknowledge some limitations. Firstly, a higher number of individual samples per species, family, and order should be included to corroborate some of the associations found here, and it would be beneficial to include a more representative sample of individuals from each taxonomic group. Secondly, the detection of other intestinal parasites, such as Platyhelminthes and Nematoda, would provide additional valuable information. Despite these limitations, our results provide valuable information about the ecoepidemiology of *Giardia* spp., *Cryptosporidium* spp., and *Blastocystis* in wild animals in Colombia.

Ethics declarations

This study was reviewed and received the ethics committee approval by the ethical committee of Universidad Pedalógica y Tecnológica de Colombia.

Authorship contribution

DG, MP, MO collected the samples. VA made the DNA extraction. LCS and PJ performed the PCRs. LCS analyzed the data and wrote the manuscript. LV reviewed and edited the manuscript. JDR conceived the manuscript, supervised the project, wrote, reviewed and edited the manuscript. All authors approved the final version of the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT, a language model trained by OpenAI, in order to review writing and assist in editing of the text. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

- All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
- The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.
- The following authors have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript:

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.07.005.

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