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Molecular characterization and zoonotic risk assessment of *Cryptosporidium* spp. in Philippine bats

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ABSTRACT

Cryptosporidium is a genus of parasitic protozoa known to cause diarrheal disease that impacts both humans and animals through infection of various vertebrate species. Bats are recognized as reservoirs for zoonotic pathogens, including *Cryptosporidium*. The Philippines, renowned for its rich biodiversity, is home to diverse bat species, providing a unique ecological setting to investigate *Cryptosporidium* infection dynamics. Understanding the prevalence and genetic diversity of *Cryptosporidium* in Philippine bats is crucial for assessing their potential role in zoonotic disease transmission and associated public health risks.

We investigated the prevalence and genotypic diversity of *Cryptosporidium* in bats in the Philippines. From January 2019 to March 2024, a total of 569 bats were captured and analyzed, with 14 of the bat samples testing positive for the 18 s rRNA gene of *Cryptosporidium*, yielding an overall infection rate of 2.46 %. One sample exhibited co-infection, with 18 s rRNA sequence analysis indicating mixed infection with a species closely related to *Cryptosporidium parvum* (intestinal *Cryptosporidium*) and *Cryptosporidium* sp. (gastric *Cryptosporidium*). Phylogenetic analysis of the 18S rRNA gene revealed that intestinal and gastric *Cryptosporidium* spp. form two distinct clades. Intestinal *Cryptosporidium* includes *C. parvum*, *C. hominis*, and most bat genotypes, while gastric *Cryptosporidium*, such as *C. andersoni* and *C. serpentis*, is typically found in reptiles and cattle. An unidentified *Cryptosporidium* species was also detected in one sample, whose sequence matched that of *Cryptosporidium* previously isolated from a human patient with diarrhea. Nine other samples exhibited genotypes related to *C. parvum*, indicating a potential for transmission to humans. The remaining three samples exhibited *Cryptosporidium* bat genotypes II and VI, which have previously been detected in Philippine bats. Our findings underscore the role of bats in the Philippines as potential reservoirs for *Cryptosporidium* and highlight the diversity of *Cryptosporidium* species in Philippine bats.

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1. Introduction

Cryptosporidium, a protozoan parasite classified within the phylum Apicomplexa, infects a wide range of vertebrate hosts (Huang et al., 2023). Cryptosporidiosis typically presents with profuse and prolonged watery diarrhea. In immunocompetent individuals, this illness is usually self-limiting; however, it can be severe and life-threatening in immunocompromised patients. Currently, effective treatment options for cryptosporidiosis are limited. More than 90 % of human cases of cryptosporidiosis are caused by *Cryptosporidium hominis* and *Cryptosporidium parvum*. However, several other species within the *Cryptosporidium* genus can infect humans, including *C. muris*, *C. meleagridis*, *C. cuniculus*, *C. felis*, *C. andersoni*, *C. canis*, *C. pestis*, *C. ubiquitum*, and *C. viatorum*. Rare cases of human infection with *C. suis*, *C. fayeri*, or *C. scrofarum* have also been reported (Bouzid et al., 2013; Šlapeta, 2013).

Bats, of the order Chiroptera, constitute a quarter of all mammalian species diversity, ranking second only to rodents. They are distributed worldwide, with records of their presence on every continent except Antarctica. They are divided into two sub-orders, the Mega- and Micro-chiroptera, commonly referred to as the megabats and microbats (Altringham, 1996). Bats are recognized as major reservoirs of pathogens in nature, hosting a wide range of viruses, bacteria, fungi, and parasites, including *Cryptosporidium*, that pose potential health risks to humans and other animals Calisher et al., 2006; Mühldorfer, 2013). Studying bats as reservoirs of pathogens is essential for understanding and mitigating the risk of relevant zoonotic disease transmission. The Philippine archipelago is home to over 70 bat species across seven families. Approximately 32 % of bat species in the Philippines are frugivorous or nectarivorous, whereas the majority are predominantly insectivorous. Twenty-seven species (35 %) are endemic to the region (Ingle and Heaney, 1992; Tanalgo and Hughes, 2018). Therefore, the Philippines presents an ideal location to investigate the risk of zoonotic diseases transmitted by bats.

The prevalence of *Cryptosporidium* infection in the Philippines ranges from 1.8 % to 19.3 % among children (≤10 years old) and from 0.6 % to 21.1 % among adults (Labana, 2019). Previous epidemiological studies (Murakoshi et al., 2016) have identified multiple bat genotypes of *Cryptosporidium* in Philippine bats. *C. parvum* has also been detected in bats in the USA (Kváč et al., 2015). Bat-specific *Cryptosporidium* genotypes account for 91.5 % of *Cryptosporidium*-positive samples genotyped from bats worldwide, with *C. parvum* and *C. hominis* each representing 3.4 % of the typed positive samples (Barbosa et al., 2023). Bat *Cryptosporidium* was initially diagnosed in the big brown bat (*Eptesicus fuscus*) in the USA (Dubey et al., 1998). Bat-associated *Cryptosporidium* has been identified globally, with the number of recognized bat genotypes reaching 22 as of April 2024 (Zhao et al., 2024). The diversity of *Cryptosporidium* species detected in bats has thus been continuously expanding. This study investigated bats in various locations in the Philippines, with the aim of assessing their role as reservoirs of *Cryptosporidium* and evaluating the risk they pose to humans.

2. Materials and methods

2.1. Sample collection

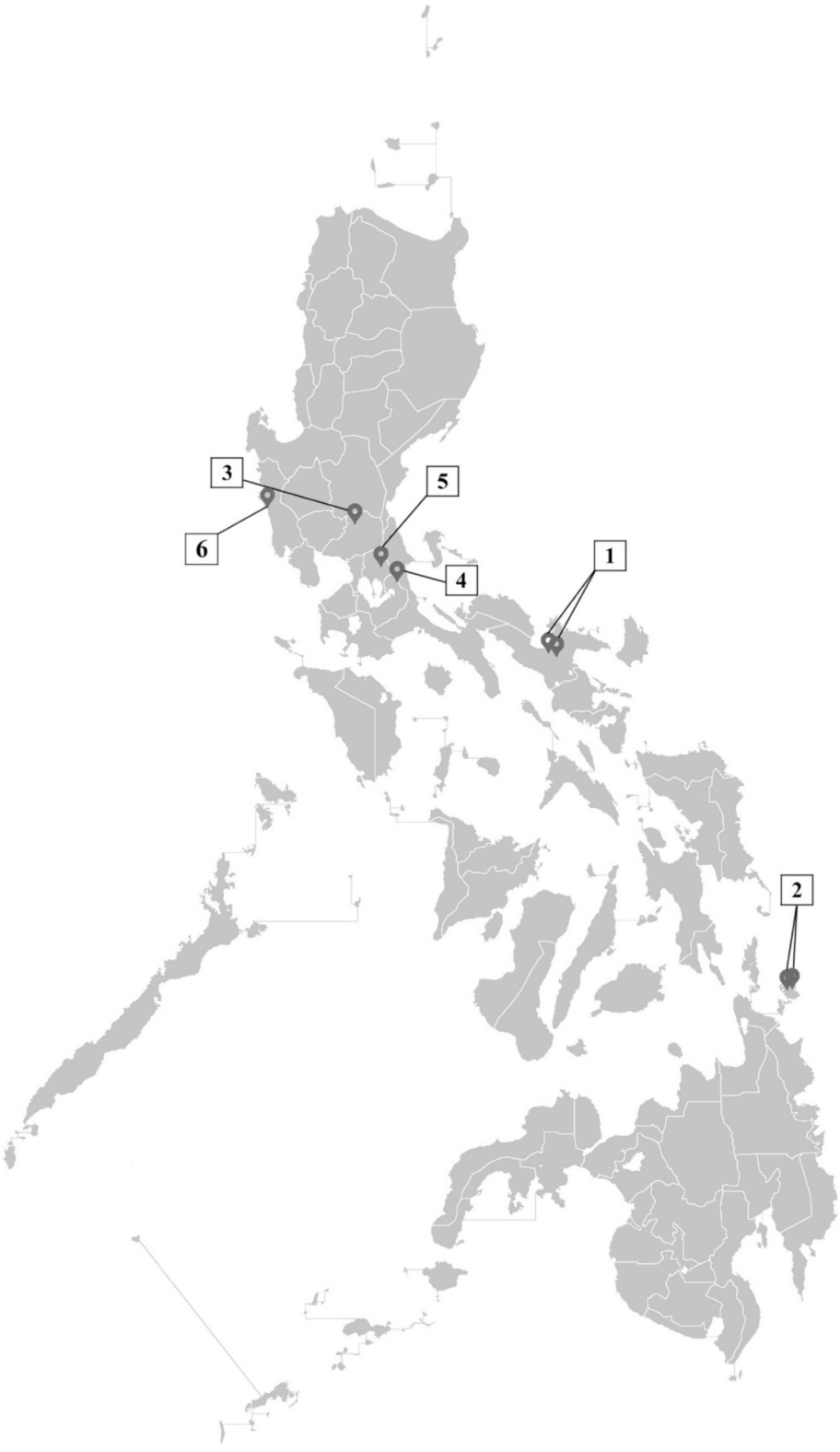
Written permission from the regional director of the Department of Environment and Natural Resources (DENR) was obtained to capture bats. Collection and handling of the bats were done according with the guidelines and protocol of the Museum of Natural History, UP Los Baños.

From January 2019 to March 2024, a total of 569 bats were captured at various locations in the Philippines through six sampling events each conducted over several days (Fig. 1; Table 1). All captured bats were identified by researchers from the University of the Philippines Los Baños based on their morphology and given a four-digit number for subsequent verification of individual information. Bats were euthanized by intraperitoneal injection with tiletamine-zolazepam anesthetic (15 mg/kg, Zoletil®,Virbac, Carros, France) followed by cardiac exsanguination. Subsequently, a small segment (0.5–1 cm) of the small intestine was collected from each bat and immediately immersed in RNAlater Stabilization Solution (Sigma) before being frozen at −80 °C until DNA extraction. The extracted intestinal DNA was analyzed for the presence of *Cryptosporidium*. And the captured bats were identified into 28 species (Table 2).

2.2. Sample analyses

Cryptosporidium was detected in small intestine tissue samples by using molecular methods. The tips of each sample were cut for further analyses. DNA extraction from the tissues was performed using the NucleoSpin® Tissue kit (MACHEREY-NAGEL). First, fragments of 830–850 bp from the small subunit (SSU) ribosomal RNA (rRNA) gene were amplified using KOD FX (TOYOBO, Japan) with primers described previously (Feng et al., 2007). Genomic DNA of *Cryptosporidium* isolated from positive calf feces was used as positive control, and double distilled water for negative control. Samples from which an amplified product was obtained and determined to be positive for *Cryptosporidium* were renumbered. Nested PCR of the actin gene and heat shock protein (HSP70) gene was also performed with the positive samples and primers described previously (Sulaiman et al., 2002; Sulaiman et al., 2000). Positive samples were sequenced using the BigDye Terminator v3.1 (Thermo Fisher Scientific K.K., Tokyo Japan) and SupreDye v3.1 (EdgeBio, CA. USA).

For TA cloning, the insert DNA was prepared by amplifying the target gene using KOD DNA polymerase (TOYOBO, Japan) according to the manufacturer's instructions. The technique involves adding single dA-overhangs to the 3′ ends of the PCR products using a dA-overhang reaction mix (Nippon Gene). These dA overhangs complement the single dT overhangs of the pGEM T-Vector (T-Vector pMD19, TaKaRa), which was used for the ligation step. The ligation reaction, facilitated by the TA Cloning Kit (Nippon Gene), allowed the prepared insert DNA to anneal to the T-vector via complementary base pairing. The ligation mixture was then transformed into



(caption on next page)

Fig. 1. Locations showing where six samplings were conducted in the Philippines between 2019 and 2024. Each sampling was conducted at a different location, sometimes two locations close to each other. Sorted by sampling date:
1) 2019.01.24–01.26 Penafrancia Resort, Barangay, Carolina, Naga City Camarines Sur 4400, & Mt. Isarog (13°39′46.5"N 123°20′07.8″E).
2) 2019.06.22–06.23 Bgy. Katipunan, & Mahayahay Cave, Del Carmen, 8418 Siargao Island, Surigao Del Norte.
3) 2020.02.28–03.02 Biak na Bato National Park, San Miguel, Bulacan 3011.
4) 2022.10.30–11.02 Land Grant (UPLB), Quezon Prov., Real, Laguna 4335.
5) 2023.03.08–03.11 Upper Marikina River Basin Protected Landscape, Rizal Province & Masungi Georeserve, Tanay, Rizal Province 1980.
6) 2024.02.29–03.02 Near sea resort, Locloc, Palauig, Zambales & Sitio Dampay, Salaza, palauig, Zambales 2210.

competent *E. coli* DH5α cells using standard protocols. Positive transformants were screened by performing insert-check PCR with primers specific to the insert DNA sequence. Colonies containing the desired plasmid were selected, incubated, and the plasmid DNA was purified from the bacterial cultures using standard methods.

Fragments of the SSU rRNA nucleotide sequences of *Cryptosporidium* acquired in this study were deposited in GenBank (Sample No. 1: LC844807; No. 2: LC844810 & LC844811; No. 3–11: LC844812; No. 12: LC844813; No. 13: LC844815; No. 14: LC844817). Fragments of the actin and HSP70 gene nucleotide sequences were also deposited in GenBank (Sample No. 1: LC844808 & LC844809; No. 12: LC844814; No. 13: LC844816).

2.3. Phylogenetic analyses

The sequences were aligned using ClustalW (Tamura et al., 2021), and the resulting sequences were manually edited using GENETYX (Ver12). All gaps were removed, and SSU rRNA genes were used for the phylogenetic analysis. Maximum likelihood (ML) analyses were conducted using MEGA 11 (Nei and Kumar, 2000; Tamura et al., 2021). Substitution models and optional parameter sets were assessed by using MEGA 11; the most appropriate sets were chosen based on the Akaike information criterion (AIC). To calculate the bootstrap value, 300 ML trees were generated from the same datasets. Phylogenetic trees were constructed (Fig. 1), employing the general time-reversible model (Tavaré, 1986). The same method was used to analyze the actin and HSP70 genes.

3. Results

3.1. Sample analyses

In total, 569 small intestine samples were examined, with 14 (2.46 %) of them testing positive for the 18 s rRNA gene of *Cryptosporidium* (Table 1). Table 2 summarizes the results for the presence of *Cryptosporidium* spp. among 6 of the 28 bat species tested.

Positive samples were renumbered No.1–14 (Table 3). Based on BLAST search results, all sequences detected in this study were identified as *Cryptosporidium*. Specifically, Sample No. 1 exhibited 100 % identity with a human-derived *Cryptosporidium* sequence (KJ506854.1 *Cryptosporidium* sp. OTUi AVK-2014). Samples No. 3 through No. 11 showed 100 % identity with *C. parvum,* marking the first such finding of *C. parvum* in bats in the Philippines. Sample No. 13 matched 100 % with *Cryptosporidium* sp. bat genotype II (LC089978), No. 12 matched 100 % with *Cryptosporidium* sp. bat genotype VI (LC089976). Sample No. 14 showed 97.68 % identity with bat genotype II found in *Rousettus leschenaultii* from Yunnan, China (KC445655.1).

Unambiguous sequence results were not obtained for sample No. 2, where overlapping multiple peaks suggested possible co-infection with multiple species. Consequently, cloning and sequencing were performed. The sequencing revealed that sample No. 2 exhibited mixed infection with both intestinal *Cryptosporidium* and gastric *Cryptosporidium*. Based on the phylogenetic tree of the 18S rRNA gene, intestinal *Cryptosporidium* and gastric *Cryptosporidium* form two distinct clades. The intestinal *Cryptosporidium* clade typically includes species such as *C. parvum* and *C. hominis*, which are the primary species infecting humans, as well as most of the currently identified bat genotypes. This clade also encompasses other species such as *C. felis*, *C. suis*, and *C. canis*. In contrast, gastric *Cryptosporidium* species, such as *C. andersoni* and *C. serpentis*, are generally found in hosts like cattle, snakes, and other reptiles such as turtles. One species showed 100 % similarity to *C. parvum* (intestinal *Cryptosporidium*) based on BLAST analysis. Another showed 100 % similarity with a gastric *Cryptosporidium* species from bat (OR807540.1).

Despite attempts to amplify the actin and HSP70 genes from all of the *Cryptosporidium*-positive samples, we were only able to

Table 1
The number of samples tested and the number of positive samples for the 18S rRNA gene of *Cryptosporidium* at each sampling location.

Location	No. bats tested	Positive	Detection Rate
Penafrancia Resort, Barangay, Carolina, Naga City Camarines Sur 4400, & Mt. Isarog (13°39′46.5"N 123°20′07.8″E)	68	0	0
Bgy. Katipunan, & Mahayahay Cave, Del Carmen, 8418 Siargao Island, Surigao Del Norte	101	1	1.0 %
Biak na Bato National Park, San Miguel, Bulacan 3011	120	10	8.3 %
UPLB Laguna-Quezon Land Grant (LQLG), Siniloan, Laguna 4019 and Real, Quezon Province 4335	119	1	0.8 %
Upper Marikina River Basin Protected Landscape, Rizal Province & Masungi Georeserve, Tanay, Rizal Province 1980	95	1	1.1 %
Near sea resort, Locloc, Palauig, Zambales & Sitio Dampay, Salaza, Palauig, Zambales 2210	66	1	1.5 %
The Philippines	569	14	2.5 %

successfully obtain sequences from samples No. 1 and No. 13 for the actin gene, and from No. 1 and No. 12 for the HSP70 gene. The *Cryptosporidium* detected in sample No. 1 was 100 % identical to *Cryptosporidium* sp. OTUi AVK-2014 in terms of the 18S rRNA gene, the actin gene, and the HSP70 gene. The actin gene sequence from sample No. 13 was consistent with the analysis of the 18S rRNA gene, showing 100 % identity with the bat genotype II previously detected in Philippine bats. As no sequence information for the HSP70 gene of bat genotype VI had been reported, sample No. 12 cannot be matched.

3.2. Phylogenetic analyses

Fig. 2 shows the phylogenetic relationships based on partial sequences of SSU rRNA among the different *Cryptosporidium* genotypes. The *Cryptosporidium* sequences from Sample Nos. 12, 13, and 14 each formed distinct clades with known bat genotypes (II and VI). Specifically, Sample No. 12 clustered with bat genotype VI, whereas Sample Nos. 13 and 14 formed a separate clade with bat genotype II.

For sample Nos. 1 and 13, phylogenetic analyses inferred from the actin and HSP70 sequences were consistent with the phylogenetic relationships based on the partial sequences of the SSU rRNA (Supplementary 1 & 2). For sample No. 12, due to the lack of HSP70 sequence information for bat genotype II, it formed a distinct branch alongside *C. parvum* (AF221532.1) in the phylogenetic tree for the HSP70 gene.

4. Discussion

4.1. Detection rates compared

In this study conducted from 2019 to 2024, among 569 samples collected from Philippine bats, the *Cryptosporidium* positivity rate was 2.46 % compared to reported rates of 3.2 % (9/281) in Australia, (6/63) 9.5 % in Colombia, 5 %(3/60) in Nepal, and 16.3 % (23/141) in Brazil (Schiller et al., 2016; Silva-Ramos et al., 2023; Adhikari et al., 2020; Batista et al., 2019). Previous research on Philippines bats reported a higher positivity rate of approximately 8.89 % (4/45) (Murakoshi et al., 2016). Due to the significant discrepancy in the positive rates observed in this study, we analyzed other studies with the most comparable and the most divergent results. Research from Australia and Brazil both amplified ~830 bp of the 18S rRNA gene using fecal samples. In the Australian study, the positive rate at urban and rural locations (3/179, 1.7 %) was noticeably lower than at captive facilities (6/102, 5.9 %), although the difference was not statistically significant. Regarding the Brazilian study, the sampling area (the city of Araçatuba, State of São Paulo, Brazil) was found to have numerous reports of *Cryptosporidium* presence. *Cryptosporidium* was detected in domestic captive cockatiels,

Table 2
The species of captured bats and their detection rates for the 18S rRNA gene of *Cryptosporidium*. Bats are categorized into two groups: fruit bats and insectivorous bats.

	Bat species	No. of samples	Detection Rate
Fruit bats	*Cynopterus brachyotis*	128	4 (3.1 %)
	Cynopterus luzoniensis	30	
	Desmalopex leucopterus	2	
	Eonycteris spelaea	6	
	Haplonycteris fisheri	1	
	Macroglossus minimus	22	
	Pteropus hypomelanus	2	
	Ptenochirus jagori	154	3 (2.0 %)
	Rousettus amplexicaudatus	62	1 (1.6 %)
Total		407/569 (71.5 %)	8/407 (2.0 %)
Insectivorous bats	*Chaerophon plicata*	30	2 (6.7 %)
	Hipposideros diadema	31	
	Hipposideros coronatus	1	
	Hipposideros pygmaeus	17	
	Hipposideros obscurus	2	1 (50 %)
	Hipposideros	1	
	Hipposideros lekaguli	2	
	Miniopterus schreibersii	10	
	Myotis horsfieldii	13	
	Miniopterus australis	19	3 (15.8 %)
	Myotis muricola	2	
	Rhinolophus arcusty	1	
	Rhinolophus macrotis	1	
	Rhinolophus araratus	1	
	Rhinolophus philippinensis	1	
	Rhinolophus arcuatus	7	
	Scotophilus kuhlii	14	
	Tylonycteris pachypus	8	
	Tylonycteris jagori	1	
Total		162/569 (28.5 %)	6/162 (3.7 %)

Table 3
Detailed information of positive samples. Including sampling time, location, amplified genes, bats' species and *Cryptosporidium* species/genotypes detected.

No. of positive sample	Sampling time	Location	Species of bats	18 s rRNA gene	Actin gene	HSP 70 gene	Species/ genotypes of *Cryptosporidium*
1	23-06-2019	Bgy. Katipunan, & Mahayahay Cave, Del Carmen, 8418 Siargao Island, Surigao Del Norte	*Hipposideros obscurus*	+	+	+	*Cryptosporidium* sp. OTUi AVK-2014
2	29-02-2020	Biak na Bato National Park, San Miguel, Bulacan 3011	*Chaerophon plicata*	+			*C. parvum* & gastric *Cryptosporidium*
3			*Chaerophon plicata*	+			
4			*Cynopterus brachyotis*	+			
5			*Cynopterus brachyotis*	+			
6			*Cynopterus brachyotis*	+			
7			*Ptenochirus jagori*	+			*C. parvum*
8			*Ptenochirus jagori*	+			
9			*Miniopterus australis*	+			
10			*Miniopterus australis*	+			
11			*Miniopterus australis*	+			
12	02–11–2022	UPLB Laguna-Quezon Land Grant (LQLG), Siniloan, Laguna 4019 and Real, Quezon Province 4335	*Cyropterus brachyotis*	+		+	*Cryptosporidium* sp. bat genotype VI
13	07-03-2023	Upper Marikina River Basin Protected Landscape, Rizal Province & Masungi Georeserve, Tanay, Rizal Province 1980	*Ptenochirus jagori*	+	+		*Cryptosporidium* sp. bat genotype II
14	02-03-2024	Near sea resort, Locloc, Palauig, Zambales & Sitio Dampay, Salaza, Palauig, Zambales 2210	*Rousettus amplexicaudatus*	+			*Cryptosporidium* sp. bat genotype II

wild captive psittacines, pigeons, and foals, suggesting that this area might be a hotspot for *Cryptosporidium* (Inácio et al., 2017; Oliveira et al., 2017a; Oliveira et al., 2017b; Panegossi et al., 2023). These studies also discussed the potential for waterborne transmission of *Cryptosporidium* to animals via rivers and irrigated crops used as animal feed. These findings suggest that detection rates are influenced by the local prevalence of *Cryptosporidium*. Despite the lower detection rate observed in our study, the infection rate may also be influenced by multiple factors including bat population dynamics, sampling methods and timing, as well as the detection techniques and materials employed. Even within the same country, variations in sampling locations can lead to substantial differences in prevalence rates.

The significance of Philippine bats as hosts for *Cryptosporidium* should not be underestimated because their detection of *C. parvum*, a zoonotic species with high potential for cross-species transmission, highlights the role of bats as reservoirs of human-infecting *Cryptosporidium*. Moreover, the observed diversity of *Cryptosporidium* genotypes in Philippine bats suggests that they harbor a broader range of species than previously recognized, possibly including unknown or novel genotypes yet to be discovered. This diversity underscores the need for further research to fully understand the epidemiological and ecological implications of bats in the transmission and evolution of *Cryptosporidium*.

4.2. Unidentified transmission routes of Cryptosporidium in Southeast Asia

The 18S rRNA, actin, and HSP70 genes of *Cryptosporidium* detected in sample No. 1 were identical to those of *Cryptosporidium* genotype OTUi AVK-2014, a strain previously identified in a traveler who visited Bali, Indonesia, and experienced cryptosporidiosis-related diarrhea (Koehler et al., 2014). The detection of this same *Cryptosporidium* sequence in bats from the Philippines may suggest that an unidentified strain capable of infecting humans occur across the region, with bats potentially serving as a reservoir for this genotype (Fig. 2). Interestingly, *Hipposideros obscurus*, a species of bat endemic to the Philippines, typically residing in caves and forests, has a stable habitat and is generally non-migratory. However, other closely related bat species are widely distributed across Southeast Asia, such as *H. galeritus* and *H. cervinus* in Indonesia (Murray et al., 2012). Because genotype OTUi AVK-2014 was detected in human feces, it is not known whether the infection was bat-mediated.

In addition, one of the *Cryptosporidium* species detected in sample No. 2 (*Chaerophon plicata*) showed high similarity to *Cryptosporidium* from *Rousettus leschenaultii*, found in a study in Hainan, China (Zhao et al., 2024). While *Rousettus leschenaultii* is absent from the Philippines and has different dietary habits than those of *Chaerophon plicata*, both bat species are known to migrate to some extent. Nevertheless, their limited migration range is unlikely to allow them to naturally cross the ocean.

The detection of these *Cryptosporidium* sequences, which have only been reported twice and differ significantly from other known

Fig. 2. Phylogenetic tree constructed by ML for *Cryptosporidium* spp. Using the the SSU rRNA gene without gaps. The *Plasmodium falciparum* sequence (JQ627152) was used as the out-group (substitution model and optional parameters = GTR + Γ + I). Only bootstrap values >50 % from 500 pseudo-replicates are shown.

genotypes, raises questions about how these parasites may have spread to geographically isolated regions. Perhaps, there are unknown transmission pathways for *Cryptosporidium* across Southeast Asia. International travelers could possibly serve as carriers. Further research into these unknown transmission routes is necessary to fully understand the distribution of *Cryptosporidium* in this region.

4.3. Infection risks posed by habitat overlap

Several of the sampling sites were located near villages, such as near the seaside resort Locloc and Sitio Dampay, Salaza (Palauig, Zambales 2210, sampling location No. 6), where local residents, including children playing, and free-roaming livestock (such as chickens and dogs) were commonly observed. These villages are adjacent to rivers of varying sizes, which serve as water sources, and the riverbanks are lined with dense forests, the natural habitat of bats and the locations of our sampling. The overlap between bat

habitats and human and livestock living areas suggests a potential risk of environmental contamination. If feces from infected bats containing *Cryptosporidium* oocysts were to enter the shared water sources, it could lead to contamination, posing an infection risk to both people and animals relying on the water.

The detection of *Cryptosporidium* species capable of infecting humans in sample No. 1, obtained from *Hipposideros obscurus*, a species endemic to the Philippines and primarily inhabiting caves and forests, also raises concerns about zoonotic transmission. During a study of bats in a karst landscape in the central Philippines, *Hipposideros obscurus* was captured (Sedlock et al., 2014). This study revealed that 88 % of the caves surveyed had experienced either current or past human disturbances, including the collection of edible bird's nests, hunting of bats for food, and harvesting guano for use as fertilizer. Human activities in these bat-dense areas, particularly contact with guano potentially containing parasite oocysts, increase the risk of pathogen transmission. When guano is transported back to villages for fertilization, the parasite oocysts could spread further, elevating the risk of *Cryptosporidium* infection among local populations and livestock.

4.4. Diversity of Cryptosporidium species in Philippine bats

Samples Nos. 3 to No. 11 were closely related to *C. parvum*, a species known to cause cryptosporidiosis in humans. Samples Nos.13 and 14 were closely related to bat genotype II, whereas sample No. 12 was related to bat genotype VI. As no sequence information for the HSP70 gene of bat genotype VI had been reported previously, this study provides the first documented data. Both genotypes have also been previously detected in Philippine bats (Murakoshi et al., 2016). Bat genotype II has so far only been found in bats from the Philippines and may be endemic to the region. In this study, samples collected from six locations revealed the presence of five *Cryptosporidium* species and genotypes, including *C. parvum*, bat genotype II, bat genotype VI, gastric *Cryptosporidium* and a unidentified *Cryptosporidium* sp.. Meanwhile previous study detected four different bat genotypes from a single location. This highlights the diversity of the *Cryptosporidium* species present in Philippine bats.

4.5. Detection rates among bats with different food habits

In this study, fruit bats accounted for approximately 70 % of the total samples, whereas insectivorous bats comprised the remaining approximately 30 %. Among the positive samples, eight were from fruit bats and six from insectivorous bats. The infection rate in fruit bats was 1.97 %, whereas in insectivorous bats, it was 3.70 %, nearly double that of fruit bats. Although correlation analysis revealed no significant difference in infection rates related to bat feeding habits, another study(Adhikari et al., 2020) has reported a significantly higher prevalence of protozoa infections in insectivorous bats compared to fruit bats (93 % versus 46.7 %, $p < 0.0001$). Specifically, the *Cryptosporidium* infection rate was 10 % (3/30) in insectivorous bats versus 0 % (0/30) in fruit bats. The authors analyzed this difference and attributed it to varying feeding habits and habitats. Insects consumed by insectivorous bats may serve as intermediate hosts or vectors for various parasites. Also, insectivorous bats predominantly inhabit caves with high moisture content, which probably favors the survival and development of gastrointestinal parasites. These bats regularly visit water sources that may be shared with livestock and humans to replenish fluids, potentially facilitating cross-transmission of waterborne parasites during this process. In contrast, fruit bats obtain moisture primarily from plants and fruits and have less exposure to water sources, resulting in lower probabilities of parasite exposure.

4.6. Gastric Cryptosporidium

Gastric *Cryptosporidium* spp. are typically detected in cattle and reptiles (Lindsay et al., 2000; Kváč et al., 2016), with cases in bats being rare, further illustrating the species diversity of *Cryptosporidium* in bats. The mixed infection involving intestinal *Cryptosporidium* (*C. parvum*) in Sample No. 2 is rare. The gastric *Cryptosporidium* identified in No. 2 exhibited 100 % sequence similarity with a gastric *Cryptosporidium* previously detected in bats from Hainan, China (OR807540.1) (Zhao et al., 2024). However, the sequence registered for this fragment is short, and similarity in the terminal region remains unconfirmed. While co-infections of *Cryptosporidium* with other pathogens are well-documented, to the best of our knowledge, this is the first reported case of mixed infection involving multiple *Cryptosporidium* species in bats.

5. Conclusions

In conclusion, our molecular characterization of *Cryptosporidium* spp. in bats from the Philippines revealed the presence of diverse *Cryptosporidium* spp., some of which may pose zoonotic risks to humans and livestock. Despite a relatively low detection rate of 2.46 %, particularly compared to other regions, the potential risks associated with these pathogens may be underestimated. The observed diversity of *Cryptosporidium* species indicates that bats could serve as important reservoirs due to their ability to harbor multiple genotypes and species, facilitating their transmission through fecal contamination of water sources, agricultural areas, or direct contact with humans and livestock. Our findings highlight the importance of continued surveillance, particularly in regions with frequent human-bat interactions, to address potential public health and veterinary risks.

We acknowledge the limitations of our sampling process. Bat dissection and sample collection were conducted by multiple researchers with varying degrees of experience, which may have introduced inconsistencies in sample handling. Additionally, the randomness inherent in sampling and DNA extraction could have resulted in the inadvertent exclusion of infected segments of the small intestine. Given the limitations of our current dataset, further studies with more extensive sampling and molecular analyses are

needed to better understand the *Cryptosporidium* populations carried by bats. Such investigations should allow for a more thorough assessment of the potential role bats play in the transmission of *Cryptosporidium* to other vertebrates, deepening our understanding of the associated zoonotic risks.

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CRediT authorship contribution statement

Lin Xu: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Yasuhiro Fukuda:** Writing – review & editing, Validation. **Fumi Murakoshi:** Writing – review & editing, Validation. **Phillip Alviola:** Investigation. **Joseph Masangkay:** Project administration, Investigation. **Frances Cagayat Recuenco:** Investigation. **Ayman Shehata:** Formal analysis. **Tsutomu Omatsu:** Project administration, Investigation, Funding acquisition. **Hironori Bando:** Investigation. **Hikaru Fujii:** Investigation. **Yumi Une:** Investigation. **Kentaro Kato:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

All authors declare no competing financial interests.

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