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Molecular detection and public health risk assessment of *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Blastocystis* sp. of animals in a tropical wildlife park of Hainan Island, China

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ABSTRACT

Objective: To detect the prevalence and characterize of *Cryptosporidium* spp., *Giardia* (*G.*) *duodenalis*, *Enterocytozoon* (*E.*) *bieneusi* and *Blastocystis* sp. of animals at a tropical wildlife park in Hainan Province, China, and to assess zoonotic risks and the potential threat of public health.

Methods: Fecal specimens were collected from animals of 27 species at the wildlife park in Hainan Province, China, and detected these pathogens using polymerase chain reaction (PCR) amplifications and sequencing of gene fragments based on small subunit ribosomal RNA (SSU rRNA) gene, glutamate dehydrogenase (GDH), internal transcribed spacer (ITS) and SSU rRNA gene, respectively.

Results: A total of 99 animals were studied, and 33 (33.3%) animals were found to harbor intestinal parasite, and the prevalence of *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Blastocystis* sp. were 9.1%, 2.0%, 5.1%, and 25.3%, respectively. Five *Cryptosporidium* species (*C. parvum*, *C. ubiquitum*, *C. scrofarum*, *C. xiaoi* and *C. parvum–like*), one *G. duodenalis* assemblages (E), four *E. bieneusi* ITS genotypes (CM1, HLJD-I, HNR-III and 1 novel genotype HNED-III) were detected, and seven subtypes of *Blastocystis* sp. (ST1, ST2, ST3, ST5, ST10, ST14 and ST15) were also identified.

Conclusions: This is the first molecular detection and public health risk assessment of four intestinal protozoa species in Hainan Tropical Wildlife Park and Botanical Garden, China. Almost all species/genotypes/subtypes of four intestinal protozoa identified in this study have the potential of zoonosis and may cause public health risks.

KEYWORDS: Tropical wildlife park; *Cryptosporidium* spp.; *Giardia duodenalis*; *Enterocytozoon bieneusi*; *Blastocystis* sp.; zoonotic risks

Significance

This is the first molecular detection and public health risk assessment of four intestinal protozoa in a tropical wildlife park of Hainan Island, China. The results suggest that the animals at tropical wildlife park could serve as reservoirs for potentially zoonotic parasites and transmit those parasites to humans and other animals.

1. Introduction

Cryptosporidium spp., *Giardia* (*G.*) *duodenalis*, *Enterocytozoon* (*E.*) *bieneusi* and *Blastocystis* sp. are important zoonotic protists in humans and animals around the world, especially in tropical and subtropical regions[1-4]. They can cause diarrhea or other gastrointestinal sufferings in human and animals[5, 6].

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The fecal-oral route is the main route of transmission for these pathogens, and infections can also be caused by contaminated food or water[7, 8].

Based polymerase chain reaction (PCR) and sequence analysis techniques, at least 45 valid species and 120 genotypes of *Cryptosporidium* spp. have been described, and at least 25 species/genotypes have been detected in humans[9]. Eight distinct assemblages (A-H) of *G. duodenalis* have been reported, and assemblages A and B are infectious to humans and other vertebrate hosts[2]. More than 560 *E. bieneusi* genotypes clustered into 13 major groups in phylogenetic analysis were identified and most zoonotic genotypes were clustered into group 1 and group 2[3]. Overall, 28 *Blastocystis* sp. subtypes (ST1-ST17, ST21, ST23-ST32) have been identified, including zoonotic subtypes (ST1-ST10, ST12, ST14, ST16, ST23) and host adopted subtypes from specific animals (ST11, ST13, ST15, ST17, ST21, ST24-ST32)[10].

The wildlife park is a special ecological environment where a variety of species of native and imported animals live in the same natural state. Zoonoses are caused by pathogens that are naturally transmitted from animals to humans, especially workers or visitors in the park. The ongoing occurrence of zoonoses pose significant threats to public health. Moreover, pathogens can spread outside the park through environmental pollution especially by water contamination and cause public health risks[11]. The aim of this study was to detect the prevalence and molecular characterization of the four common zoonosis and opportunistic intestinal protozoa including *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Blastocystis* sp. of animals in the only wildlife park located in tropical island province of China, and to assess their zoonosis and potential public health risks.

2. Materials and methods

2.1. Ethics statement

The protocol of this study was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Hainan Medical University (HYLL-2022-405). Only excreted feces of the wildlife were used for this study and during the entire procedure, no animals were injured.

2.2. Sampling area and collection

From June to July 2021, samples were collected from Hainan Tropical Wildlife Park and Botanical Garden, located in Dongshan Town Xiuying District Haikou City (Figure 1). Hainan Tropical Wildlife Park and Botanical Garden is the first tropical wildlife park in China, covering over 100 hectares and collecting at least 2 000 animals including over 100 species, with more than 500 000 visitors from around world every year. The 99 fecal samples, representing 27 species were obtained from the park at the time of sampling. The fresh samples were placed in plastic bags, which were labeled, recorded and transfered to laboratory for storage at 4 ℃ within 24 h.

2.3. DNA extraction

All the fecal samples were washed with distilled water twice and centrifuged at 3000 x *g* for 10 min. Genomic DNA was extracted from 200 mg of faeces per sample by using the QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA samples was stored at -20 ℃ until PCR analysis was performed.

2.4. PCR amplification

All DNA preparations were detected for the presence of *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Blastocystis* sp. by PCR amplifications and sequencing of gene fragments based on SSU rRNA (~850 bp) gene, GDH (~530 bp) gene, ITS ~390 bp) gene and SSU rRNA (~260 bp) gene, respectively, and PCR conditions have been previously described[12-15] (Table 1). PCR reactions (25 μL) included 0.5 μL of Taq DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan), 0.5 μL of each primer (20 mM), 2.5 μL of 10 × PCR buffer (TaKaRa Bio Inc., Tokyo, Japan), 2 μL of dNTPs (TaKaRa Bio Inc., Tokyo, Japan) and 2 μL of template DNA. A negative control without DNA and a positive control (rat-derived *Cryptosporidium* rat genotype IV DNA for *Cryptosporidium* spp., goat-derived assemblage E DNA for *G. duodenalis*, rat-derived genotype Peru 8 DNA for *E. bieneusi* and cattle-derived ST1 DNA for *Blastocystis* sp.) were included in all the PCR tests. Amplified products were electrophoresed on a 1.5% agarose gel stained with GelRed (Biotium, Inc., Hayward, CA) and visualized under UV light.

2.5. Nucleotide sequencing and analysis

All appropriately sized PCR products were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). Two-directional sequencing and additional sequencing were used to confirm the accuracy of

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the sequence. The Basic Local Alignment Search Tool (BLAST) and Clustal X were used to genotype the isolates by comparing the sequenced data published on GenBank.

2.6. Phylogenetic analysis

The phylogenetic tree was constructed using the Mega 11 (http://www.megasoftware.net/) software based on the neighbor-joining method and Kimura-2-parameter model. The reliability of the tree was assessed through bootstrap analysis with 1 000 replicates.

3. Results

3.1. Prevalence of Cryptosporidium spp., G. duodenalis, E. bieneusi and Blastocystis sp.

In this study, 33 (33.3%) animals were found to harbor intestinal parasite, and the prevalence of *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Blastocystis* sp. were 9.1%, 2.0%, 5.1%, and 25.3%, respectively (Table 2). Co-infection with *Cryptosporidium* spp. and *Blastocystis sp*. were observed in white buffalo (Hainan) (*n*=1), sika deer (*n*=1), red-bellied squirrel (*n*=1), beaver rat (*n*=1). While co-infection with *E. bieneusi* and *Blastocystis* sp. were observed in macaques (*n*=2), brown bear (*n*=1) and Hainan eld's deer (*n*=1).

3.2. Molecular characterization of Cryptosporidium spp., G. duodenalis, E. bieneusi and Blastocystis sp.

Five *Cryptosporidium* species (*C. parvum, C. ubiquitum, C. scrofarum, C. xiaoi and C. parvum–like*) were identified in David's deer (*C. xiaoi*), white buffalo (Hainan) (*C. xiaoi, C. scrofarum*), sika deer (*C. ubiquitum*), emu (*C. ubiquitum*) and Africa lion (*C. parvum–like*), red-bellied squirrel (*C. parvum*) and beaver rat (*C. ubiquitum*) in this study (Table 2). Comparison with the sequenced data published on GenBank showed that the sequences of *C. parvum* (MW769926), *C. scrofarum* (MT071828), and *C. xiaoi* (LC414392) were identical to previous sequences. While the sequence of the *C. parvum–like* isolate (OQ993217) had 98.6% similarity with a sequence (MK956936) which was isolated from a bamboo rat from Guangxi, China. The sequence of the *C. xiaoi* (OQ993214) had 99.8% similarity with a sequence (LC414388) which was isolated from a capra hircus from Algeria. In this study, *C. ubiquitum* were isolated from a sika deer, two beaver rats and an emu, of which the emu was identified as the host for the first time. The *C. ubiquitum* sequences in this study were 99.8% to 100% identical to known *C. ubiquitum* isolates in the GenBank database. Phylogenetic reconstructions of the *Cryptosporidium* spp. based on SSU rRNA gene sequences from zoo animals can be seen in Figure 2.

Nucleotide sequence and phylogenetic analysis based on GDH gene identified one *G. duodenalis* assemblages as assemblage E in a sika deer and a Hainan eld's deer was identified in present study (Figure 3). The isolates shared 99.6%-99.8% identity with the sequence (MK645786) which isolated from Tan sheep in Ningxia, China.

Four *E. bieneusi* genotypes were identified that including three known genotypes named CM1 (*n*=2), HLJD-I (*n*=1) and HNR-III (*n*=1), and one novel genotypes (HNED-III). The novel genotypes HNED-III (OQ674806) from Hainan eld's deer had the largest similarity with genotype CGC2 (MK559495) with one base difference at positions 45 (A→G). Phylogenetic analysis of *E. bieneusi* showed that genotypes CM1 and HNR-III were clustered in group 1, whereas genotype HLJD-I and HNED-III were clustered into group 2 (Figure 4).

A total of 7 *Blastocystis* sp. subtypes were identified as ST1, ST2, ST3, ST5, ST10, ST14 and ST15. The most prevalent *Blastocystis* sp. subtype was ST10 (36%) followed by ST5 (28%). Phylogenetic analyses showed that the sequences of the 7 subtypes obtained in this study clustered with their reference subtypes which obtained from other animals or humans into one branch, with high bootstrap values (Figure 5).

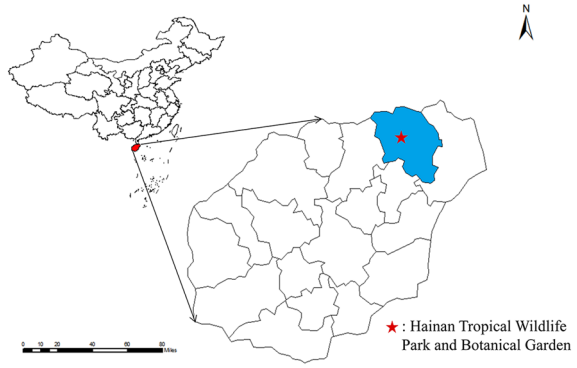

Figure 1. The location of Hainan Tropical Wildlife Park and Botanical Garden, Hainan, China.

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Table 1. Primers used in the characterization of the *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi*, and *Blastocystis* sp.

Target organism	Amplicon size (bp)	Primer name	Nucleotide sequences (5'-3')	Reference
Cryptosporidium spp.	SSU rRNA (851 bp)	18SiCF2	GACATATCATTCAAGTTTCTGACC	[12]
		18SiCR2	CTGAAGGAGTAAGGAACAACC	
		18SiCF1	CCTATCAGCTTTAGACGGTAGG	
		18SiCR1	TCTAAGAATTTCACCTCTGACTG	
G. duodenalis	GDH (530 bp)	GDH-F1	TTCCGTRTYCAGTACAACTC	[13]
		GDH-R1	ACCTCGTTCTGRGTGGCGCA	
		GDH-F2	ATGACYGAGCTYCAGAGGCACGT	
		GDH-R2	GTGGCGCARGGCATGATGCA	
E. bieneusi	ITS (392 bp)	ITS-F1	GGTCATAGGGATGAAGAG	[14]
		ITS-R1	TTCGAGTTCTTTCGCGCTC	
		ITS-F2	GCTCTGAATATCTATGGCT	
		ITS-R2	ATCGCCGACGGATCCAAGTG	
Blastocystis sp.	SSU rRNA (260 bp)	BLF	CGAATGGCTCATTATATCAGTT	[15]
		BLR	TCTTCGTTACCCGTTACTGC	

Note: SSU rRNA: small subunit ribosomal RNA; GDH: glutamate dehydrogenase; ITS: internal transcribed spacer.

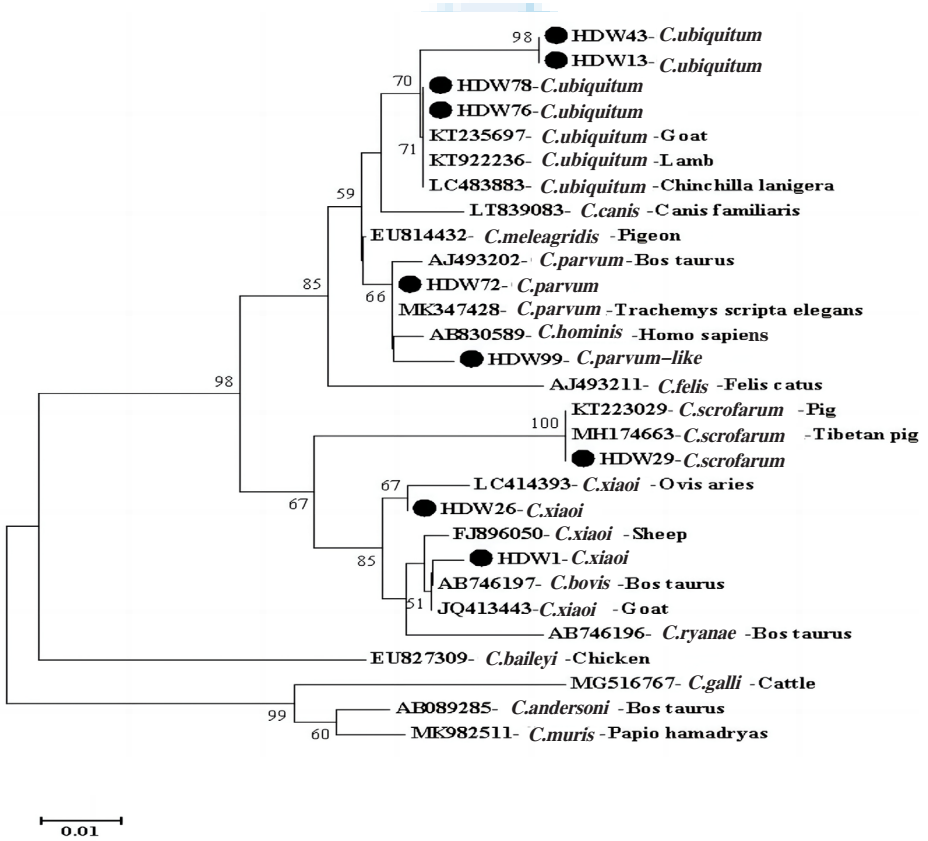

Figure 2. Phylogenetic relationships among *Cryptosporidium* spp. based on the SSU rRNA gene. Neighbor-Joining method and genetic distances calculated with the Kimura 2-parameter model using MEGA 11. Bootstrapping with 1000 replicates was used to support the clades. The bootstrap number is shown on each node. ● Known species detected.

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Table 2. Occurrence of *Cryptosporidium* spp., *G. duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis* sp.

Group	Common name	Scientific name	No.	*Cryptosporidium* spp.	*E. bieneusi*	*G. duodenalis*	*Blastocystis* sp.
Herbivore	David's deer	*Elaphurus davidianus*	9	*C. xiaoi* (1)	0	0	0
	Donkey	*Equus asinus*	3	0	0	0	0
	Pony	*Equus caballus*	5	0	0	0	0
	White buffalo (Hainan)		4	*C. xiaoi* (1), *C. scrofarum* (1)	0	0	ST10 (1)
	Alpaca	*Lama alpacos*	4	0	0	0	ST5 (2), ST14 (1)
	Sika deer	*Cervus nippon*	7	*C. ubiquitum* (1)	CM1 (1)	E (1)	ST10 (5)
	Hainan eld's deer	*Cervus eldi hainanus*	7		HNED-III (1)	E (1)	ST10 (2), ST5 (2)
	Giraffe	*Giraffa camelopardalis*	4	0	0	0	0
	Elephant	*Loxodonta*	1	0	0	0	0
	Camel	*Camelus bactrianus*	3	0	0	0	0
Subtotal			47	8.5% (4/47); *C. xiaoi* (2), *C. scrofarum* (1), *C. ubiquitu*m (1)	4.3% (2/47); CM1 (1), HNED-III (1)	4.3% (2/47); E (2)	27.7% (13/47); ST10 (8), ST5 (4), ST14 (1)
Avian	African ostrich	*Struthio camelus*	2	0	0	0	0
	Emu	*Dromaius novaehollandia*	5	*C. ubiquitum* (1)			ST5 (1)
	Peafowl		1	0	0	0	0
	Ardea cineria rectirostris	*Ardea cinerea rectirostris Gould*	1	0	0	0	0
Subtotal			9	11.1% (1/9); *C. ubiquitum* (1)	0	0	11.1% (1/9); ST5 (1)
Non-human primates	Macaque	*Macaca mulatta*	8	0	CM1 (1), HLJD-I (1)		ST3 (1), ST2 (1), ST10 (1)
	Black Hat Hanging Monkey	*Cebus apella*	1	0	0	0	0
	Crab-eating monkeys	*Macaca fascicularis*	1	0	0	0	0
	Hylobates leucogenys	*Nomascus leucogenys*	1	0	0	0	0
	Pig tailed macaque	*Macaca nemestrina*	1	0	0	0	0
Subtotal			12	0	16.7 (2/12); CM1 (1), HLJD-I (1)	0	25.0% (3/12); ST3 (1), ST2 (1), ST10 (1)
Carnivore	Black Leopard	*Panthera pardus fusca*	1	0	0	0	0
	Brown bear	*Ursus arctos*	1	0	HNR-III (1)	0	ST2 (1)
	Black bear	*Ursus thibetanus*; *Himalayan Black Bear*	6	0	0	0	0
	Tiger	*Panthera tigris ssp.altaica*; *Panthera tigris tigris*	4	0	0	0	0
	Africa lion	*Panthera leo*	4	*C. parvum–like* (1)	0	0	0
Subtotal			16	6.3% (1/16); *C. parvum–li*ke (1)	6.3% (1/16); HNR-III (1)	0	6.3% (1/16); ST2 (1)
Rodents	Red-bellied squirrel	*Callosciurus erythraeus*	3	*C.parvum* (1)	0	0	ST1 (1), ST2 (1), ST15 (1)
	Beaver Rat	*Myocastor coypus*	5	*C. ubiquitum* (2)	0	0	ST15 (1), ST2 (1), ST5 (1)
	Guinea pig	*Cavia porcellus*	7	0	0	0	ST5 (1)
Subtotal			15	20.0% (3/15); *C. parvum* (1), *C. ubiquitu*m (2)	0	0	46.7% (7/15); ST1 (1), ST2 (2), ST5 (2), ST15 (2)
Total			99	9.1% (9/99); *C. ubiquitum* (4), *C. xiaoi* (2), *C. scrofarum* (1), *C. parvum* (1), *C. parvum–like* (1)	5.1% (5/99); CM1 (2), HNR-III (1), HLJD-1 (1), HNED-III (1)	2.0% (2/99); E (2)	25.3% (25/99); ST1 (1), ST2 (4), ST3 (1), ST5 (7), ST10 (9), ST14 (1), ST15 (2)

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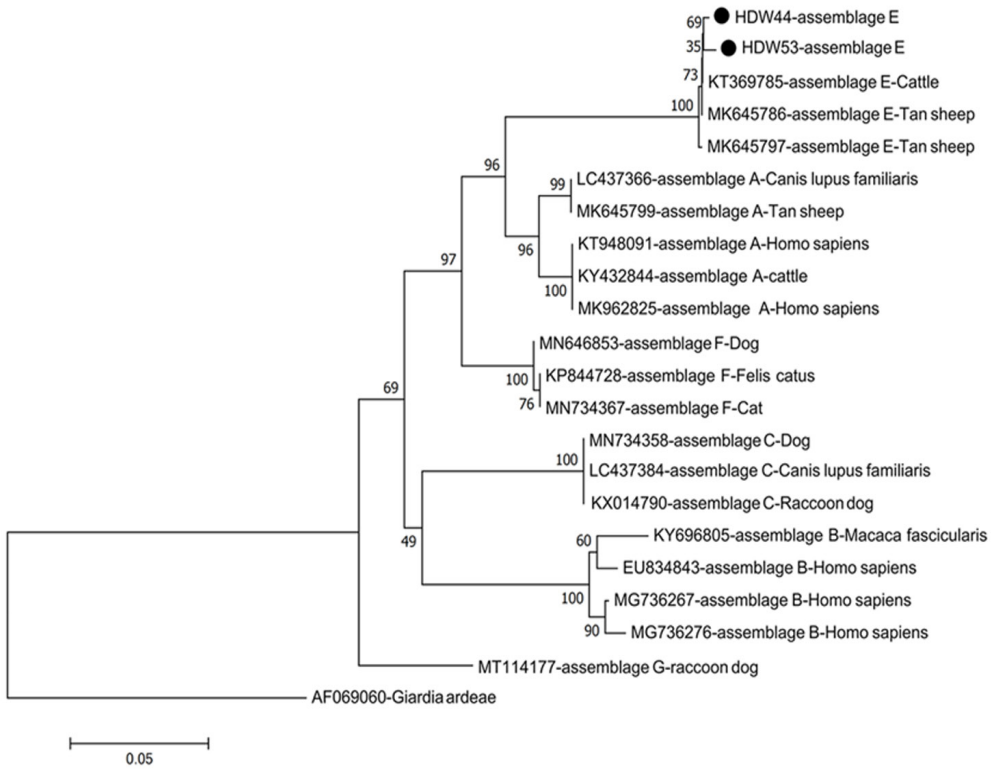

Figure 3. Phylogenetic relationships among *G. duodenalis* based on GDH gene. Neighbor-Joining method and genetic distances calculated with the Kimura 2-parameter model using MEGA 11. Bootstrapping with 1 000 replicates was used to support the clades. The bootstrap number is shown on each node. ● Known assemblages detected.



Figure 4. Phylogenetic relationships among *E. bieneusi* based on ITS gene. Neighbor-Joining method and genetic distances calculated with the Kimura 2-parameter model using MEGA 11. Bootstrapping with 1 000 replicates was used to support the clades. The bootstrap number is shown on each node. ● Known genotypes detected; △Novel genotype detected.

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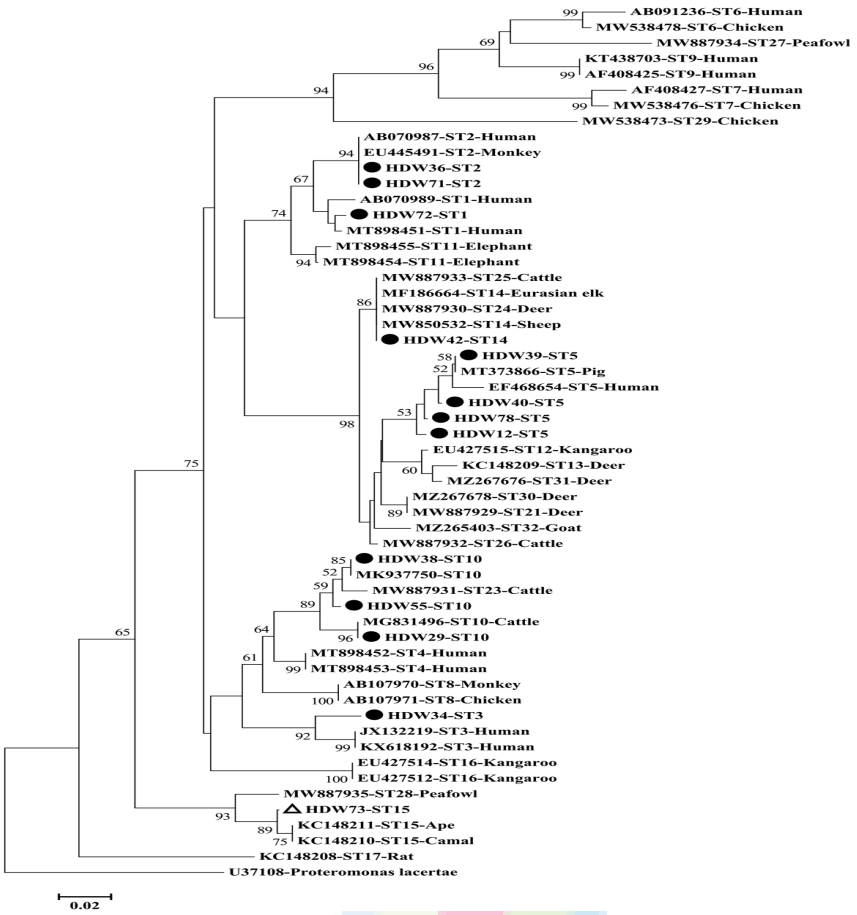

Figure 5. Phylogenetic relationships among *Blastocystis* based on the SSU rRNA gene. Neighbor-Joining method and genetic distances calculated with the Kimura 2-parameter model using MEGA 11. Bootstrapping with 1000 replicates was used to support the clades. The bootstrap number is shown on each node. ● Known sequence detected, △ Novel sequence detected.

4. Discussion

In the present study, the infection rate of *Cryptosporidium* spp. in the wildlife park was 9.1%, which was higher than the rates (2.3%) reported in petting zoos in Canada[16], zoos in Henan, China (2.8%)[17], zoo mammals in Bangladesh (3.5%)[18], birds in Beijing and Harbin, China (1.9%)[19], non-human primates in France, Germany, and Spain (0.9%)[20], but lower than zoo felines in Harbin, China (30.4%)[21].

The infection rate of *G. duodenalis* was 2.0%, which was similar to those in Zhengzhou zoo, China (2.5%)[22], lower than petting zoos in Canada (14.8%)[16], zoo mammals in Bangladesh (5.5%)[18], non-human primates in France, Germany, and Spain (18.1%)[20], while higher than zoos in Henan, China (0.5%)[17].

The *E. bieneusi* prevalence was 5.1%, which was higher than non-human primates in France, Germany, and Spain (0.9%)[20], lower than in previous studies conducted in the great apes in Senegal, the Republic of the Congo and France (7.9%)[23], in Zhengzhou zoo, China (15.8%)[22], in zoos in Henan, China (20.8%)[17].

Concerning *Blastocystis* sp., the prevalence was 25.3% which was similar to the studies conducted in Poland (26.9%)[24], non-human primates in France, Germany, and Spain (20.3%)[20], in a zoo in Slovakia (20.8%)[25], higher than the captive wildlife species in Sichuan, China (15.7%)[26], zoo animals in Hangzhou, Dalian, and Suzhou cities, China (6.0%)[27], non-human primates in Harbin (7.0%)[28], mammalian wildlife species in Bangladesh (15.5%)[29], zoo animals in Henan, China (19.1%)[17], while lower than golden monkeys in Shijiazhuang, China (48.7%)[30], wild mammals in Brazil (51.7%)[31], zoo animals in Australia (42.1%)[32], non-human primates in Spain (45.1%)[33], mammalian and non-mammalian species in French (32.3%)[34], great apes in Senegal, the Republic of the Congo, and France (97.4%)[23].

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According to the literature, all five *Cryptosporidium* species (*C. parvum*, *C. ubiquitum*, *C. scrofarum*, *C. xiaoi* and *C. parvum–like*) identified in present study had zoonotic potential. *C. parvum* was one of the two *Cryptosporidium* responsible for most cases of cryptosporidiosis in humans[35]. *C. ubiquitum*, previously identified as the *Cryptosporidium* cervine genotype, were commonly detected in domestic and wild ruminants, rodents, carnivores, and primates[36]. It has been found in humans worldwide, primarily in industrialized nations[37]. *C. scrofarum* appeared to be the main pig-adapted species[38], but human infection was reported in an immunocompetent man[39]. *C. xiaoi* was most common species in sheep but it was identified in two children with HIV/AIDS in Ethiopia and had zoomotic potential[40]. *C. parvum–like* was described in immunocompetent and immunosuppressed individuals[41]. Therefore, the results above indicate that the animals in the wildlife with those *Cryptosporidium* species may transmit it to other animals and humans.

For *G. duodenalis*, assemblages A and B were considered to be zoonotic and have been found in various mammals including humans. Assemblage E was mostly identified in hoofed mammals (such as cattle, sheep, goats, and pigs), but it has been reported in at least 57 people in several countries, indicating the zoonotic potencial[2].

Four *E. bieneusi* genotypes including CM1, HNR-III, HLJD-1 and HNED-III were detected in this study. Genotype CM1 was only found in NHPs and dog at present[42]. HNR-III was first reported in wild rats in Hainan, China[43]. HLJD-1 was first reported in sika deer[44]. HNED-III is a novel genotype. Although no human infection of these four genotypes was reported, phylogenetic analysis showed they were clustered into group 1 and group 2, which was composed of most zoonotic genotypes of *E. bieneusi*, indicating the zoonotic potential.

Blastocystis ST1–ST9 and ST12 have been detected in humans, and ST1-ST4 are the most common, comprising over 90% of all STs identified in humans globally[45].

In this study, seven *Blastocystis* sp. subtypes were detected from 10 animal species including ST1 (n=1), ST2 (n=4), ST3 (n=1), ST5 (n=7), ST10 (n=9), ST14 (n=1), and ST15 (n=2), all subtypes have zoonotic potential except ST15 from two rodents. The presence of zoonotic *Blastocystis* subtypes indicative those animals represent a potential threat to other animals and humans.

5. Conclusions

This is the first molecular detection and public health risk assessment on four intestinal protozoa in a tropical wildlife park of Hainan Island, China. Almost all species/genotypes/subtypes of four intestinal protozoa identified in this study have zoonotic potential and may cause public health risks. These findings extend the host range for four intestinal protozoa and also provide important data support for prevention and control of zoonoses. Further studies should be made to reveal the genetic characteristics and assess the zoonotic risks of these parasites.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The representative nucleotide sequences obtained in this study were deposited in the GenBank database under the following accession numbers: OQ993214 to OQ993217 for *Cryptosporidium* spp.; OQ999329 and OQ999330 for *G. duodenalis*, OQ674806 for *E. bieneusi*; OQ992504 for *Blastocystis* sp.

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Authors' contributions

Ren GX was responsible for literature collection and writing the draft of this article and performed experiments, software and formal analysis. Li JQ and Xiong JY contributed to acquisition of samples and performed experiments. Lai XY, Wang Y, Lei S, Lu X, He TY and Zhou YF provided administrative, technical support and constructive discussion. As the corresponding authors, Lv G and Zhang Y controlled the content of the whole review. All authors contributed to the article and approved the submitted version.

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