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Original Article

The Prevalence of *Cryptosporidium* spp. and *Giardia duodenalis* in *Marmota himalayana* (Rodentia: Sciuridae) in the Qinghai Tibetan Plateau area, China

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Abstract

Background: *Cryptosporidium* and *Giardia* are well-known important intestinal zoonotic pathogens that can infect various hosts and cause diarrhoeal diseases. We aimed to determine the epidemiological prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species in Himalayan marmot (*Marmota himalayana*, class Marmota) in the Qinghai Tibetan Plateau Area of Qinghai Province, Northwest China.

Methods: Overall, 243 Himalayan marmot fecal samples were collected in 2017 and in 2019 and a two-step nested PCR technique was performed to amplify the fragments of the SSU rRNA gene of *Cryptosporidium* and 18S ribosomal RNA gene of *Giardia*. Molecular characterization of *Cryptosporidium* was performed with the primary primers N-DIAGF2 and N-DIAGR2, the secondary primers CPB-DIAGF and CPB-DIAGR. Similarly, molecular characterization of *Giardia* was used the first primers Gia2029 and Gia2150c, the secondary primers RH11 and RH4. The positive PCR products were sequenced and the sequences were processed by Clustal Omega and BLAST. Phylogenetic analysis was achieved by NJ method in MEGA.

Results: The infection rate of *Cryptosporidium* spp. and *G. duodenalis* was 4.9% (12/243) and 0.8% (2/243) in *M. himalayana*, respectively. *Cryptosporidium* spp. are characterized as novel genotypes *Cryptosporidium* marmot genotype I (n=3) and *Cryptosporidium* marmot genotype II (n=9); *G. duodenalis* assemblage A (n=2) was found in *M. himalayana*.

Conclusion: This is the first report of *Cryptosporidium* spp. and *G. duodenalis* infections in *M. himalayana* in Qinghai of Northwest China. The results indicate the existence of *Cryptosporidium* species and *G. duodenalis* infections that may have a potential public health significance.

Introduction

Cryptosporidium spp. and *Giardia duodenalis* are well-known, enteric pathogens that can cause diarrhoeal diseases in various hosts including humans and domestic and wild animals worldwide (1-3). Cryptosporidiosis and giardiasis are foodborne and waterborne diseases, and various hosts are usually infected by ingesting contaminated water and/or food (4-8). *Cryptosporidium* and *Giardia* infections can lead to acute or chronic, even life-threatening diarrhoeal diseases of the infected hosts, especially, in immunocompromised humans. Moreover, these two parasitic protozoa pathogens have attracted increased attention and a series of epidemiological investigations focusing on public and veterinary health. *Cryptosporidium* and *Giardia* are prevalent in livestock and wild animals (9-13). At least 44 *Cryptosporidium* spp. have been identified, and 120 genotypes are recognized (14-18). Eight assemblages, A–H, have been identified for *Giardia* (19-21).

The Himalayan marmot (*Marmota himalayana*) is a marmot species that inhabits alpine grasslands distributed at elevations from 3500 to 5200 m in the Qinghai Tibetan Plateau Area (QTPA) of China. They feed mainly on grasses, such as stem leaves and twigs, the soft and juicy parts of grassy plants. Sometimes, they reside near human dwellings and their water sources. Moreover, the Himalayan marmot is a host for a variety of pathogens, such as tick-borne encephalitis virus, astroviruses, *Yersinia pestis* and *Streptococcus respiraculi* (22-25).

As known, the Himalayan marmot occupies the same habitat of yaks, sheep and humans. We were ultimately interested in the potential for humans, domestic and wild animals' exposure, whether these animals may pose a possible risk of zoonotic infection to both animal and human health.

We aimed to determine the infection rates and prevalence of *Cryptosporidium* spp. and *G. duodenalis* genotypes in the Himalayan marmot of the QTPA, Qinghai Province, China.

Materials and Methods

The study sites

The faecal materials were collected from Himalayan marmots in different locations in the QTPA of Qinghai Province, Northwest China during 2017 and 2019 (Fig. 1). The town of Yushu Xiewu (33° N and 97° E) is located in the beach land and mountainous area on the north side of the Tongtian River, which is the main stream of the source of the Yangtze River. Huzhu Beishan (36° N and 102° E) is a mountain-type natural scenic tourist area, also known as the "Kingdom of Plants" and "Natural Animal Park" in the QTPA. The Hudong area (36° N and 100° E) is the eastern part of Qinghai Lake and is characterized by green meadow beaches and arid desert. There are many rivers, lakes, swamps and glaciers, with hills, valleys and plains on the terrain of the Tianjun area (37° N and 99° E). These areas represent the habitats of the Himalayan marmot with different landforms and altitudes (38).

Specimen collection

A total of 243 unique Himalayan marmot fecal samples were collected from a single cave in Yushu Xiewu town, Huzhu Beishan, the Hudong area and the Tianjun area. When we collected the samples, we usually observed movement of the marmots, and then went to the nearby caves to look for fresh faeces and then collected one sample in each of the caves, thus to guarantee the sample was from one single exact host Himalayan marmot. Each individual faecal sample was placed in a standard Eppendorf tube (50.0 ml), capped and preserved in 2.5% potassium dichromate, then transported to the laboratory and kept at 4 °C until further analysis. In the laboratory, the

sample were washed several times with distilled water, then the total genomic DNA was extracted from each faecal sample with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, with the addition of 10 freeze-thaw cycles.

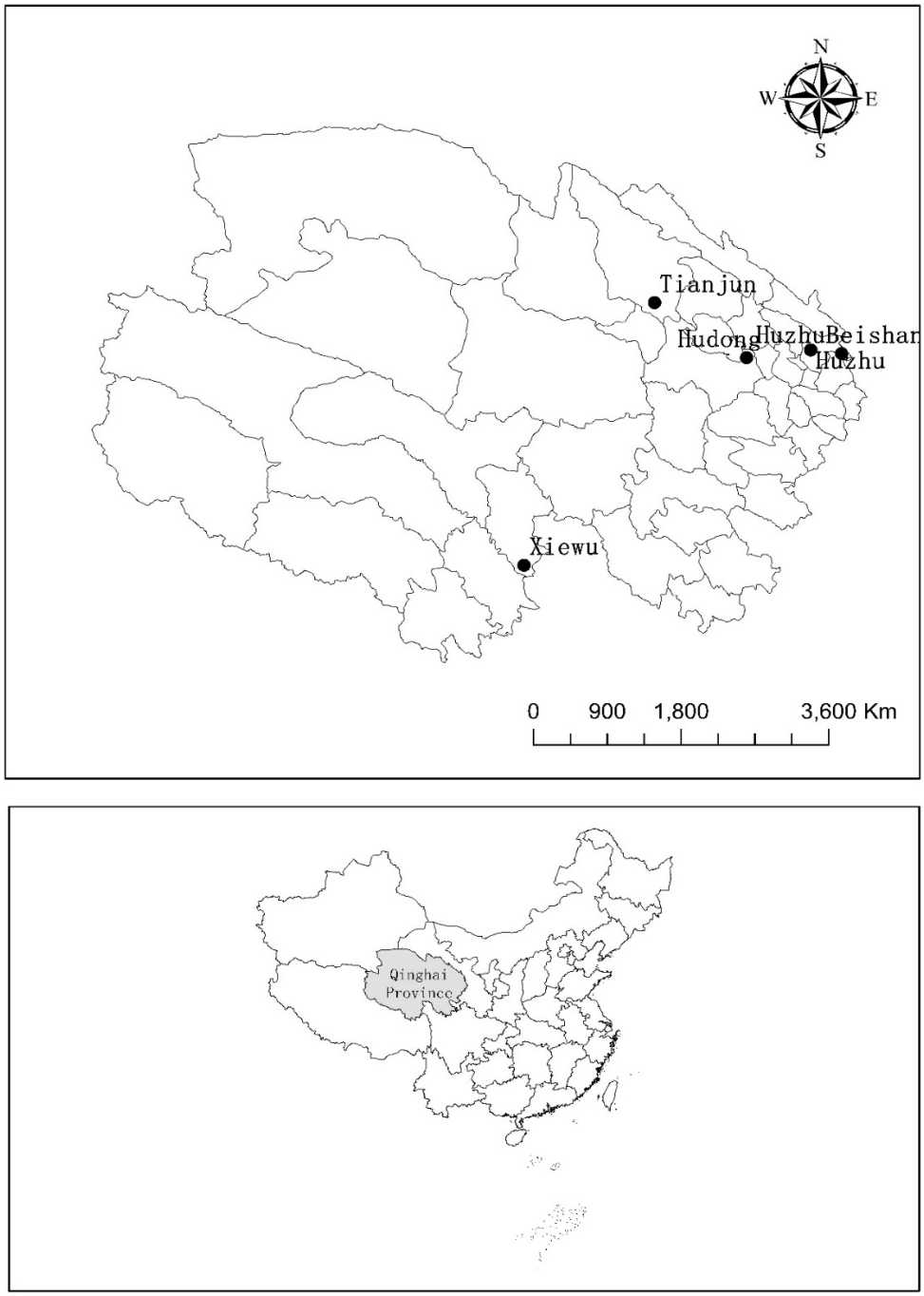

Fig. 1: Distribution of the locations (●) of samples collection in this study

Molecular characterization of Cryptosporidium and Giardia

A two-step nested PCR technique was performed to amplify the fragments of the SSU rRNA to detect *Cryptosporidium* oocysts. The expected lengths were obtained after primary amplification with the primers N-DIAGF2 (CAATTGGAGGG-CAAGTCTGGTGCCAGC) and N-DIAGR2 (CCTTCCTATGTCTGGACCTGGTGAGT), and the products varied from 655 to 667 bp depending on the species of *Cryptosporidium*. The secondary amplification employed the primers CPB-DIAGF (AAGCTCGTAGTT-GGATTTCTG) and CPB-DIAGR (TAAGGTGCTGAAGGAGTAAGG) generated a 435-bp product (27,28). Similarly, for the molecular detection of *Giardia* cysts, nested PCR was also performed using the 18S ribosomal RNA gene (29) for *Giardia* with the first primers Gia2029 (AAGTGTGGTG-CAGACGGACTC) and Gia2150c (CTGCTGCCGTCCT TGGATGT), the secondary primers RH11 (CATCCGGTCGATCCTGCC) and RH4 (AGTCGAACCCTGATTCTCCGCCAGG) (Table 1). The genomic DNA of *C. parvum* and *G. duodenalis* was used as the positive control, water without template DNA was used as the negative control, which were included in each amplification. The amplification PCR products were analyzed using a 1.5% agarose gel containing ethidium bromide (0.6 mg/mL) and were observed under UV light.

Table 1: PCR primers and cycling protocols to amplify target gene sequences from *Cryptosporidium* spp. and *Giardia duodenalis*

Parasite	*PCR target*	*Size (bp)*	*Primer sequence (5'---3')*	*Cycling protocol*
Cryptosporidium spp.	SSU rRNA gene	655 ~ 667	N-DIAGF2: CAATTGGAGGG-CAAGTCTGGTGCCAGC N-DIAGR2: CCTTCCTATGTCTG-GACCTGGTGAGT	Primary PCR were performed in a 25 µl reaction volume containing 10 µM of each primer and we added 2 µl of DNA. Thirty-five PCR cycles (94 °C for 45 s, 68 °C for 1 min, 72 °C for 1 min) were carried out in a thermal cycler (MyCycler, Bio-Rad, Hercules, USA) with an initial hot start (95 °C for 15 min) and a final extension (72 °C for 10 min).
		435	CPB-DIAGF: AAGCTCGTAGTT-GGATTTCTG CPB-DIAGR: TAAGGTGCTGAAGGAGTAAGG	1 µl of primary PCR product as DNA performed in a 25 µl reaction volume containing 10 µM of each primer. The conditions of the secondary PCR were thirty-five PCR cycles (94 °C for 45 s, 60 °C for 1 min, 72 °C for 45 s) that were carried out in a thermal cycler with an initial hot start (95 °C for 15 min) and a final extension (72 °C for 10 min).
Giardia duodenalis	18S ribosomal RNA gene	502	Gia2029: AAGTGTGGTGCAGAC-GGACTC Gia2150c: CTGCTGCCGTCCT TGGATGT	Primary PCR were performed in a 25 µl reaction volume containing 10 µM of each primer and we added 2 µl of DNA. All reactions started with an initial denaturation step at 95 °C for 15 min and then were carried out for 35 cycles, each consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 45 s and a final extension at 72 °C for 10 min.
		292	RH11: CATCCGGTCGATCCTGCC RH4: AGTCGAACCCTGAT-TCTCCGCCAGG	1 µl of primary PCR product as DNA performed in a 25 µl reaction volume containing 10 µM of each primer. A secondary PCR was run using the following conditions: An initial step at 95 °C for 15 min, followed by 35 cycles of 94 °C for 45 s, 59 °C for 30 s, 72 °C for 45 s, followed by 72 °C for 10 min.

Sequencing and phylogenetic analysis

The positive PCR products were sequenced by BEIJING GENEWIZ Company (Beijing, China). To confirm their genotypes, the sequences were processed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) aligning with reference sequences in GenBank. Phylogenetic analysis of *Cryptosporidium* species was achieved by the neighbour-joining (NJ) method, which was calculated by the Jukes-Cantor model with 2000 bootstrap replicates constructed in MEGA version 5.05. The data analysis was performed in Microsoft Excel.

Results

Among these samples (243 fecal samples), 12 specimens were *Cryptosporidium* positive and 2 were *Giardia* positive as confirmed by PCR amplification of the *SSU rRNA* gene, with a prevalence of 4.9% (12/243) and 0.8% (2/243), respectively (Table 2). These positive samples were found in hosts with no obvious symptoms of diarrhea, and were still granular in appearance. *Cryptosporidium* spp. infection of marmots were prevalent only in the Hudong area and Huzhu Beishan surroundings; *Giardia* infection of marmots was also found in Huzhu Beishan. Sequencing and phylogenetic analyses of *Cryptosporidium* spp. identified the following: eight *Cryptosporidium*-positive faecal samples in Huzhu Beishan (8/104) and four at a Hudong sheep farm (4/35). These samples were identified as *Cryptosporidium* marmot genotype I (n=3), *Cryptosporidium* marmot genotype II (n=5) and *Cryptosporidium* genotype II (n=4) (Table 2). We found a prevalence of 0.8% for *G. duodenalis*, and assemblage A was detected in the two infected marmots in Huzhu Beishan.

Table 2: Prevalence and species/genotypes of *Cryptosporidium* spp. and *Giardia duodenalis* in *Marmota himalayana*

Study Sites	*Cryptosporidium-PCR-positive*	*Species*	*Giardia-PCR-positive*	*Species*
Yushu Xiewu Town	0, (n=18)	-	0, (n=18)	-
Huzhu Beishan	5.2%, (8/155, n=155)	*Cryptosporidium* genotypeI(n=3) *Cryptosporidium* genotypeII (n=5)	1.3%, (2/155, n=155)	*Giardia duodenalis* assemblage A
Hudong Area	11.4%, (4/35, n=35)	*Cryptosporidium* genotypeII (n=4)	0, (n=35)	-
Tianjun Area	0, (n=35)	-	0, (n=35)	-
Total	4.9%, (12/243, n=243)	-	0.8%, (2/243, n=243)	-

The novel genotypes from the Himalayan marmot that exhibited close similarity, 97.33%~99.21% with *Cryptosporidium* spp. chipmunk genotype V (MZ478133) submitted were named as *Cryptosporidium* marmot genotype I; those that exhibited a similarity of 95.78%-100.00% with *Cryptosporidium* spp. Sltl05c (DQ295014), Sbey05c (DQ295012), Sbey03c (AY462233), Sbld05c (DQ295013) and *C. rubeyi* (MZ478132) submitted were named *Cryptosporidium* marmot genotype II.

The novel genotypes of *Cryptosporidium* marmot genotype I and *Cryptosporidium* marmot genotype II from Himalayan marmots exhibited 98.82%-92.79% genetic similarity with each other. The identified gene sequences were deposited into NCBI GenBank under accession numbers KY882006-KY882017 for *Cryptosporidium* spp. and KY882004-5 for *Giardia*. The phylogenetic analysis employing the NJ method indicated that all SSU rRNA gene sequences from *Cryptosporidium* spp. generated in the present study formed well-defined clusters with their respective reference sequences (Fig. 2).

Fig. 2: Phylogenetic analysis of *Cryptosporidium* spp. based on sequences of the partial small subunit ribosomal RNA gene. The black markers represented the positive sample in this study

Discussion

This report of prevalence and the molecular characterization of *Cryptosporidium* and *Giardia* spp. in faecal samples collected from Himalayan marmots is first time reported in the QTPA of China. Overall, we detected 2 geno-

types of *Cryptosporidium* (*Cryptosporidium* marmot genotype I and *Cryptosporidium* marmot genotype II) and one assemblage of *G. duodenalis* (Assemblage A) in fresh Himalayan marmota faecal samples. There have been a few reports regarding *Cryptosporidium* and *Giardia* infections in wild rodents, including the identification of infection rates of 8.2% (19/232) and 6.0% (14/232) in commensal rodents on animal farms and farm neighbourhoods in China. The species identified were: *C. parvum* (n=12), *C. muris* (n=7), and *G. duodenalis* assemblage G (n=14) (30); infection rates of 61.7%, 68.3% and 68.1% for *Cryptosporidium* spp. and 41.7%, 24.4% and 38.4% for *Giardia* spp. in *Apodemus agrarius*, *Apodemus flavicollis* and *Myodes glareolus*, respectively, in southwestern Poland (31); infection rates of 30.3% and 25.5% for *Cryptosporidium* and *Giardia*, respectively, in 208 deer mice on the California Central Coast of the United States (32); and infection rates of 10.5% for *Cryptosporidium* in lemurs from the Ranomafana National Park, Madagascar (33); and infection rates of 14.7% for *Cryptosporidium* in the yellow-bellied marmot (*Marmota flaviventris*) in the Sierra Nevada Mountains in California of the United States (34). The prevalence of *Cryptosporidium* and *Giardia* spp. from the above-mentioned wild animals was much higher than the prevalence in Himalayan marmot in the QTPA of China in the present study. The low prevalence of *Cryptosporidium* spp. was similar to the *Cryptosporidium* spp. infection rate in Qinghai voles (8.9%) and in wild plateau pikas (6.25%) in the QTPA of China (18). The unexpected low prevalence of *Cryptosporidium* and *Giardia* spp. can be attributed to the factors including marmot population movement and density, and human and livestock (yaks, cattle and sheep) intervention.

The novel *Cryptosporidium* genotypes (marmot I and II) from Himalayan marmots identified in the present study were most closely related to *Cryptosporidium* spp. genotypes and *Cryptosporidium* spp. Sltl05c from mice (*Peromyscus* spp.) and golden-mantled ground squirrels (*Spermophilus lateralis*). More extensive characterization at multiple loci is required to validate the novel status of these genotypes. Of the eight *G. duodenalis* assemblages, only assemblage A, which is primarily associated with humans, livestock, and wild ruminants, was detected in the present study. Assemblage A is pathogenic to humans and animals and has public health significance.

Himalayan marmots live on the grasslands and alpine meadows of plains and mountains. Importantly, these environments, where the sheep, goats, cattle, and yaks graze grass, are also close to water sources. In addition, marmots infected with *Cryptosporidium* and *Giardia* can spread these parasitic pathogens to other places when the animals migrate, resulting in a potential threat of contamination. Therefore, it is necessary to carry out further epidemiological investigations.

Himalayan marmots were screened for the presence of *Cryptosporidium* and *Giardia* in their fecal samples. *Cryptosporidium* marmot genotypes I and II and *G. duodenalis* assemblage A were detected. This is the first study to report *Cryptosporidium* marmot genotypes as well as zoonotic *G. duodenalis* assemblage A in Himalayan marmots. Travel activities and wild outings increase the potential contact between marmots, and enhanced awareness of animal protection increases the number of animals, which potentially increases the number of shed (oo)-cysts in the environment. Longitudinal studies should investigate marmot densities, seasonal effects (temperature and rainfall) and environmental factors (yaks, cattle, sheep, and humans) to determine and control the transmission dynamics of these pathogens in the QTPA of China.

Conclusion

This is the first report of *Cryptosporidium* spp. and *G. duodenalis* infections in *M. himalayana* in Qinghai of Northwest China. The results indicate the existence of *Cryptosporidium* species

and *G. duodenalis* infections that may have a potential public health significance.

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Conflicts of interest

Authors declare no conflict of interest.

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