Genotyping and subtyping of Cryptosporidium spp. and Giardia duodenalis isolates from two wild rodent species in Gansu Province, China

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Cryptosporidium spp. and Giardia duodenalis are commonly detected intestinal protozoa species in humans and animals, contributing to global gastroenteritis spread. The present study examined the prevalence and zoonotic potential of Cryptosporidium spp. and G. duodenalis in Himalayan marmots and Alashan ground squirrels in China’s Qinghai-Tibetan Plateau area (QTPA) for the first time. Four hundred ninety-eight intestinal content samples were collected from five counties of QTPA of Gansu province, China. All samples were examined for Cryptosporidium spp. and G. duodenalis by PCR amplification. The resultant data were statistically analyzed by chi-square, Fisher’s test and Bonferroni correction using SPSS software 25.0. Cryptosporidium positive samples were further subtyped through analysis of the 60-kDa glycoprotein (gp60) gene sequence. A total of 11 and 8 samples were positive for Cryptosporidium spp. and G. duodenalis, respectively. Prevalence of Cryptosporidium spp. and G. duodenalis were 2.5% (10/399) and 1.5% (6/399) in Himalayan marmots, 1.0% (1/99) and 2.0% (2/99) in Alashan ground squirrels, respectively. Sequence analysis confirmed the presence of C. rubeyi (n = 2), ground squirrel genotype II (n = 7), chipmunk genotype V (n = 1) and horse genotype (n = 1). The horse genotype was further subtyped as novel subtype VlbA10. G. duodenalis zoonotic assemblages A (n = 1), B (n = 6), E (n = 1) were identified in the present study. This is the first study to identify Cryptosporidium spp. and G. duodenalis in Himalayan marmots and Alashan ground squirrels, suggesting the potential zoonotic transmission of the two pathogens in QTPA.

Cryptosporidium spp. and Giardia duodenalis are critical protozoan parasites responsible for diarrhea and infect a wide range of hosts including humans worldwide. Typically, contaminated food or water has been identified as the primary vehicle for Cryptosporidium spp. and G. duodenalis transmission1,2. Infection of these pathogens can also be acquired following contact with infected persons or animals directly2,3.

Currently, at least 45 valid Cryptosporidium spp. species and over 120 genotypes have been identified. Over 23 Cryptosporidium species/genotypes have been identified in humans, and C. hominis and C. parvum are the most common species (more than 90%) responsible for human cryptosporidiosis4–12. G. duodenalis is a complex protozoan species, and it has been divided into at least eight genetically different assemblages (A–H) based on genetic characterization. Among them, assemblages A and B are considered as critical zoonotic pathogens. Assemblages (C–H) are host-specific: assemblages C and D in canines, assemblage E in cloven-hoofed mammals, assemblage F in cats, assemblage G in rodents, and assemblage H in seals13. However, assemblages C, D, E and F have also been found in humans14.

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Rodents can act as reservoirs or carriers for numerous zoonotic pathogens, including bacteria, parasites and viruses. Himalayan marmots (Marmota himalayana) and Alashan ground squirrels (Spermophilus alashanicus) are two common wild rodent species distributed widely in Qinghai-Tibetan Plateau area (QTPA) of China. They typically reside near livestock, water sources and human environments. Among them, infected hosts can play essential roles in environmental contamination by excreting oocysts/cysts via feces\(^\text{15}\). Some epidemiological studies also revealed the identity of Cryptosporidium spp. and G. duodenalis in numerous investigated hosts in QTPA, such as wild Qinghai voles, plateau pikas, wild birds, cattle, yaks and sheep\(^\text{16–20}\). Furthermore, the zoonotic species and genotypes of Cryptosporidium spp. and G. duodenalis were also reported in environmental samples in QTPA, including sewage and river water, slaughterhouse water and vegetables from street markets\(^\text{15,21}\). However, no previously study about the prevalence and transmission of Cryptosporidium spp. and G. duodenalis in Himalayan marmots and Alashan ground squirrels in China was reported. In the present study, a cross-sectional investigation was carried out in Himalayan marmots and Alashan ground squirrels to understand the prevalence of Cryptosporidium spp. and G. duodenalis and assess the zoonotic potential at the genotype and subtype levels.

Materials and methods

Sample collection. During a period of three months from June to September 2017, 399 Himalayan marmots and 99 Alashan ground squirrels were captured live by mousetraps from QTPA of western China’s Gansu Province (Fig. 1), with the former from Luqu (n = 98), Sunan (n = 100), Xiahe (n = 102) and Zhangye (n = 99) and latter from Huining County (n = 99) (Table 1). These animals were euthanized with a high dose of CO\(_2\) following security measures. Intestinal content materials were directly collected from each animal in the local Center for Disease Control and Prevention (CDC) laboratory and placed in 2 ml sterile tubes. They were kept in a freezer and then transported in ice packs to our laboratory in Shanghai for further molecular analysis.

DNA extraction. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Cat. #69506; Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Extracted DNA was stored at –20 °C in a freezer until further use.

PCR amplification. Cryptosporidium spp. was detected by nested PCR amplification of the fragment (approximately 830 bp) of the small subunit (SSU) rRNA gene\(^\text{22}\). Subtyping of Cryptosporidium spp. was performed by sequence analysis of the 60 kDa glycoprotein (gp60) gene\(^\text{23}\). All the isolates of Cryptosporidium-positive samples were selected for further sequence characterization via the actin gene and 70-kDa heat shock protein (HSP70) gene\(^\text{40,42}\). The assemblages of G. duodenalis were identified and subtyped by amplifying the β-giardin (bg), glutamate dehydrogenase (gdh) and triosephosphate isomerase (tpi)\(^\text{24–26}\). DNA of human-derived C. parvum and C. viatorum were used as positive controls in PCR tests to amplify the SSU rRNA, gp60, actin and HSP70 genes, respectively. Premiers and reaction conditions were shown in Supplementary Table S1. DNA of human-derived G. duodenalis was used as a positive control in PCR tests to amplify the bg, gdh and tpi genes. DNase-free water was used as a negative control in each PCR test. The secondary PCR products were visual-
Table 1. Prevalence and molecular identification of Cryptosporidium spp. and G. duodenalis by rodent species and collection site. *Novel subtype.

| Rodent species                           | Collection site | No. examined | No. positive (%) | Genotype (n) | Subtype (n) | No. positive (%) | Assemblage (n) |
|------------------------------------------|-----------------|--------------|------------------|-------------|-------------|-----------------|---------------|
| Himalayan marmot (Marmota himalayana)   | Luqu            | 98           | 0                | -           | -           | 0               | -             |
|                                          | Sunan           | 100          | 7 (7.0)          | C. rubeyi (1); ground squirrel genotype II (5); chipmunk genotype V (1) | -           | 0               | -             |
|                                          | Xiahe           | 102          | 2 (2.0)          | Ground squirrel genotype II (2) | -           | 3 (2.9)         | B (1), E (1)   |
|                                          | Zhangye         | 99           | 1 (1.0)          | C. rubeyi (1) | -           | 3 (3.0)         | B (1), A (1), B (1) |
| Subtotal                                 | 399             | 10 (2.5)     | C. rubeyi (2); ground squirrel genotype II (7); chipmunk genotype V (1) | -           | 6 (1.5)     | B (2), E (1)   | A (1), B (3), E (1) |
| Alashan ground squirrel (Spermophilus alaschanicus) | Huining        | 99           | 1 (1.0)          | Horse genotype (1) | VIbA10a(1) | 2 (2.0)         | B (2)         |
| Total                                    | 498             | 11 (2.2)     | C. rubeyi (2); ground squirrel genotype II (7); chipmunk genotype V (1); horse genotype (1) | VIbA10a(1) | 8 (1.6)     | B (4), E (1)   | A (1), B (5), E (1) |

Results

Prevalence of Cryptosporidium spp. and G. duodenalis. Using PCR amplification and sequence analysis, Cryptosporidium spp. and G. duodenalis were found in Himalayan marmots and Alashan ground squirrels. The agarose gel electrophoresis results of PCR amplification products were shown in Supplementary Fig. S1 (partial samples) and Fig. S2 (partial samples). A total of 11 and 8 samples were positive for Cryptosporidium spp. and G. duodenalis, respectively. Prevalence of Cryptosporidium spp. and G. duodenalis were 2.5% (10/399) and 1.5% (6/399) in Himalayan marmots, and 1.0% (1/99) and 2.0% (2/99) in Alashan ground squirrels, respectively (Table 1). The statistical analysis showed no significant difference in the prevalence of Cryptosporidium spp. (P = 0.365) and G. duodenalis (P = 0.714) between Himalayan marmots and Alashan ground squirrels. Different prevalence of Cryptosporidium spp. and G. duodenalis were observed in five different investigated areas (Table 1): Luqu (0.0% and 0.0%), Sunan (7.0% and 0.0%), Xiahe (2.0% and 2.9%), Zhangye (1.0% and 3.0%) and Huining (1.0% and 2.0%). Moreover, there was no significant difference observed in the prevalence of Cryptosporidium spp. and G. duodenalis in each pairwise comparison between investigated areas (P > 0.05). No mixed infection of Cryptosporidium spp. and G. duodenalis identified in this study.

Cryptosporidium genotypes and subtypes. Based on sequence analysis of the SSU rRNA gene, a total four species/genotypes of Cryptosporidium spp. were identified out of 11 isolates, including C. rubeyi (n = 2), ground squirrel genotype II (n = 7), and chipmunk genotype V (n = 1) in Himalayan marmots, and horse genotype (n = 1) in Alashan ground squirrels. Cryptosporidium ground squirrel genotype II was dominant in Hima-
layan marmots, accounting for 70.0% (7/10) of Cryptosporidium isolates. At the SSU rRNA gene locus, the two identical sequences of *C. rubeyi* shared the most significant identity (98.43%) with that of *C. rubeyi* (DQ295012) from California ground squirrels in the USA, with 13 base differences. Seven sequences of ground squirrel genotype II were identical and shared the most-prominent identity (98.28%) to that of the ground squirrel genotype II (KT027480) from black-tailed prairie dogs, with 14 base differences. The sequence of the chipmunk genotype V had 98.90% homology with that (MW521250) of the chipmunk genotype V from chinchillas in China, with nine base differences. The sequence of the horse genotype obtained in the present study had 100% homology with a sequence (MK775040) from a horse in China. The horse genotype isolate was further subtyped by sequence analysis of the *gp60* gene. This subtype belonged to the Vlb subtype family and was identified as VlbA10 (GenBank: MW531716).

None of the two sequences of *C. rubeyi* were successfully amplified at the *HSP70* gene locus but successfully amplified at the *actin* gene locus, and the two sequences were identical to each other, had 100% similarity with that of *C. rubeyi* (GenBank: KT027530) from black-tailed prairie dog. Meanwhile, two of seven isolates of ground squirrel genotype II were successfully amplified at the *actin* gene locus, and the two isolates shared the same sequence which had 97.68% similarity with that of ground squirrel genotype II (GenBank: KT027545) from black-tailed prairie dog in the USA. The *HSP70* sequences have not been reported for ground squirrel genotype II. Three of seven isolates of ground squirrel genotype II were successfully amplified at the *HSP70* gene locus and had 93.50% similarity with that of *C. viatorum* (GenBank: JX978274) from human in Guatemala. The sequence of chipmunk genotype V was only successfully amplified at the *actin* gene locus and shared 99.69% identity with that of chipmunk genotype V (MW521262) from chinchillas in China. Horse genotype was successfully amplified at the *actin* gene locus and shared 100% similarity with horse genotype (KU892571) isolated from humans of Kenya.

Phylogenetic analyses of the SSU rDNA, *actin*, *HSP70* and *gp60* gene sequences were shown in Figs. 2, 3, 4 and 5.

**G. duodenalis** assemblages. A total of eight *G. duodenalis* isolates were amplified and sequenced successfully in Himalayan marmots and Alashan ground squirrels in this study. Assemblages A, B and E were identified in one, four and one Himalayan marmot samples, respectively. Assemble B was found in two Alashan ground squirrel samples. Meanwhile, assemblage B was observed to show a predominance (75.0%, 6/8) in the detected animals. The *gdh* and *bg* genes were successfully amplified in five samples—assemblages B (n = 4) and E (n = 1) and seven samples—assemblages A (n = 1), B (n = 5) and E (n = 1), respectively (Table 1). In this study, PCR amplification failed at the *tpi* locus.

At the *gdh* locus, two assemblage B sequences had 100% homology with beaver-derived assemblage B isolated (KM977648) from China. Another two different assemblage B sequences were 100% identical to golden monkey-derived assemblage B isolate (MK952602) from China, and one assemblage E sequence was 100% identical to a pig-derived assemblage E isolate (MK426742) from South Korea. At the *bg* locus, five assemblage B sequences shared 100% homology with squirrel monkey-derived assemblage B isolate (KJ888974) from China, one assemblage A sequence had 100% homology with human-derived assemblage A isolates (GG329671) from Sweden and chipmunk-derived isolate (MF671918) from China, one assemblage E sequence (GenBank: MZ494459) shared the most considerable similarity (99.79%) to that (KY633473) from a Tibetan sheep in China, with only one base difference.

**Discussion**

In this study, the overall prevalence of *Cryptosporidium* spp. was 2.2% (11/498), with 2.5% in Himalayan marmots, and 1.0% in Alashan ground squirrels. There was no significant difference in the prevalence of *Cryptosporidium* spp. and *G. duodenalis*, and we will enlarge the research sample size for further verification. Other studies reported much higher prevalence of *Cryptosporidium* spp. in wild rodent species in China than this study, including in house mice (3.2%, 1/31), long-tailed rats (3.6%, 4/111 and 55.3%, 21/38), brown rats (6.3%, 4/64; 9.1%, 22/242 and 28.6%, 16/56), wild plateau pikas (6.3%, 4/64), Qinghai voles (8.9%, 8/90), Asian house rats (18.0%, 21/117; 18.2%, 6/33 and 73.9%, 4/46), Brandt’s voles (18.7%, 127/678), Muridae (40.0%, 4/10)30–32. The prevalence in this study was also lower than that in some pet rodent species, including farmed bamboo rats (2.1%, 9/435 and 29.5%, 209/709), farmed brown rats (7.1%, 12/168), species and 17 genotypes have been detected in 16 studies of various rodents in China (Table 2)20,27–37,39–41. Among them, 11 species/genotypes have been detected in humans: *C. parvum*, *C. canis*, *C. occulta*, *C. viatorum*, *C. suis*, *C. erinaceid*, *C. bovis*, and horse genotype4, indicating rodents may play essential roles in the transmission of zoonotic cryptosporidiosis.

Altogether, four *Cryptosporidium* species/genotypes were identified in this study: *C. rubeyi*, ground squirrel genotype II, chipmunk genotype V in Himalayan marmots, and horse genotype in Alashan ground squirrels. *C. rubeyi* was characterized by numerous wild rodent hosts such as golden-mantled ground squirrels, California
Figure 2. Phyllogenetic relationship among Cryptosporidium spp. based on a neighbor-joining tree of the SSU rRNA gene. The numbers on the branches are percent bootstrapping values from 1000 replicates, and the sequences generated in the present study are indicated with the triangles.

ground squirrels, Belding’s ground squirrels, and black-tailed prairie dogs\(^{43,44}\). Previously ground squirrel genotype II and chipmunk genotype V were only identified in black-tailed prairie dogs in the USA\(^{43}\) and chinchillas in China\(^{38}\), respectively. Our identification of ground squirrel genotype II and chipmunk genotype V expanded the host range of the two genotypes. Horse genotype was initially isolated from a Przewalski wild horse at the Prague Zoo in the Czech Republic, and commonly detected in horses and donkeys, occasionally found in neonatal calves and hedgehogs\(^{45,46}\). Horse genotype has also been found in human patients with diarrhea in the UK and the USA, suggesting its zoonotic potential\(^{47–49}\). In the present study, the horse genotype was identified in rodents for the first time, indicating it has a broader range of host than initially anticipated. Horse genotype isolated from Alashan ground squirrels was further identified as novel subtype VIbA10. Currently, two subtype families are recognized within the Cryptosporidium horse genotype by sequence analysis targeting the gp60 gene: the VIa subtype family in animals (horses, donkeys and calves, etc.) and the VIb subtype family in humans and hedgehogs.
The present study detected the infection of Cryptosporidium spp. in wild rodent species of the genus Marmota and genus Spermophilus. Further, eight previous studies have reported the occurrence of Cryptosporidium species/genotypes in other three species of the genus Marmota and other four species of genus Spermophilus: including  
C. ubiquitum in woodchucks (Marmota monax) in the USA; C. parvum in yellow-bellied marmots (Marmota flaviventris) in the USA; C. andersoni in Bobak marmots (Marmota bobac) in the Czech Republic; C. rubeyi in California ground squirrels (Spermophilus beebei) in the USA, Belding’s ground squirrels (Spermophilus beldingi) and golden-mantled ground squirrels (Spermophilus lateralis) in the USA; ground squirrel genotype I and ground squirrel genotype III in thirteen-lined ground squirrels (Spermophilus tridecemlineatus) in USA.

In this study, the overall prevalence of G. duodenalis were 1.6% (8/498), with 1.5% (6/399) for Himalayan marmots and 2.0% (2/99) for Alashan ground squirrels. This study reported much lower prevalence of G. duodenalis than other studies in wild rodent species in China: house mouse (3.2%, 1/31); Asian house rat (6.1%, 2/33); brown rat (6.6%, 11/168 and 9.3%, 33/355); pet chipmunks (8.6%, 24/279); bamboo rat (10.8%, 52/480); coypus (12.3%, 38/308); pet chinchillas (27.1%, 38/140) (Table 3).
Figure 4. Phylogenetic relationship among *Cryptosporidium* spp. based on a neighbor-joining tree of the HSP70 gene. The numbers on the branches are percent bootstrapping values from 1000 replicates, and the sequences generated in the present study are indicated with the triangles.

Figure 5. Phylogenetic relationship of *Cryptosporidium* subtypes based on a neighbor-joining tree of the gp60 gene. The numbers on the branches are percent bootstrapping values from 1000 replicates, and the sequences generated in the present study are indicated with the triangles.
In this study, the sequences of amplicons from *G. duodenalis*-positive samples were determined to be assemblages A, B, and E, with assemblages B showing dominance in the detected animals. Assemblages A, B, and E were identified in Himalayan marmots and assemblage B in Alashan ground squirrels. *G. duodenalis* assemblages in Himalayan marmots were richer than Alashan ground squirrels. As we know, in previous studies, *G. duodenalis* infections of Chinese rodents were reported to be caused by assemblages A, B and G. Among them, assemblages A and B have a broad host range and are commonly found in humans. Some recent studies in China

Table 2. *Cryptosporidium* species/genotypes in rodents in China. Plus signs indicate that the sample was co-infected with different *Cryptosporidium* species/genotypes.
also reported the occurrence of assemblage A in pet chipmunks, coypus and pet chinchillas. These two assemblages were detected in this study suggest that Himalayan marmots and Alashan ground squirrels can play roles in the zoonotic dissemination of G. duodenalis. Assemblage E is commonly found in a range of hoofed livestock and occasionally found in rodent species, and it has also been found in human cases, indicating that this assemblage is of zoonotic significance.

In the investigated areas of QTPA, wild rodent species Himalayan marmots and Alashan ground squirrels have strong migration habits and often share pasture with humans, herbivorous animals and other wild animals. Results of this study suggest that these two wild rodent species may play a role in the transmission cycle of Cryptosporidium spp. oocysts and G. duodenalis cysts among humans, animals, water sources and fresh produce in QTPA grassland ecosystem.

## Conclusion

This study examined the prevalence and zoonotic potential of Cryptosporidium spp. and G. duodenalis in Himalayan marmots and Alashan ground squirrels in the Qinghai-Tibetan Plateau area (QTPA) for China for the first time. Four Cryptosporidium species/genotypes were identified, including C. rubeyi, ground squirrel genotype II, chipmunk genotype V and horse genotype (novel subtype VIbA10). These two rodent species identified G. duodenalis zoonotic assemblages A, B, and E. The results expanded the host range of Cryptosporidium spp. and G. duodenalis, providing more information on the prevalence, epidemiology and genetic characterizations of the two pathogens in Himalayan marmots and Alashan ground squirrels. Further surveys are also required to understand the prevalence and transmission dynamics of the two pathogens.

## Data availability

Nucleotide sequences of this article were deposited in the GenBank database under following accession numbers: MZ478131-MZ478133, ON384432 (SSU rRNA), ON419488-ON419491 (actin), ON456466 (HSP70), MW531716 (gp60) for Cryptosporidium spp.; MZ494459 (bg) for G. duodenalis.

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### Table 3. G. duodenalis assemblages in rodents in China.

| Host species (Latin name) | No. positive (%) | Assemblages (n) | Sample source | References |
|--------------------------|-----------------|----------------|--------------|------------|
| Alaskan ground squirrels (Spermophilus alaskanicus) | 2/99 (2.0) | bg (2) | Wild | This study |
| Asian house rats (Rattus tanezumi) | 2/33 (6.1) | G (2) | G (1) | Wild | 27 |
| Bamboo rats (Rhizomyz sinesis) | 52/480 (10.8) | B (52) | B (27) | Farmed | 26 |
| Brown rats (Rattus norvegicus) | 11/168 (6.6) | G (11) | G (9) | Wild | 27 |
| Brown rats (Rattus norvegicus) | 33/355 (9.3) | G (19) | G (20) | Laboratory | 38 |
| Coypus (Myocastor coypus) | 38/308 (12.3) | B (11), A (1) | B (10), A (1) | Farm | 36 |
| Himalayan marmots (Marmota himalayana) | 6/599 (1.5) | A (1), B (3), E (1) | B (2), E (1) | Wild | This study |
| House mice (Mus musculus) | 1/31 (3.2) | G (1) | – | Wild | 27 |
| Pet chinchillas (Chinchilla lanigera) | 38/140 (27.1) | A (4), B (8) | A (4), B (16) | Pet | 37 |
| Pet chipmunks (Eutamias asiaticus) | 24/279 (8.6) | G (11), A (13) | G (7), A (10) | Pet | 36 |
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**Author contributions**

Y.S. and X.W. designed the study. J.X., H.L., Y.J., L.T. and Y.S. participated in the sample collection and methodology. J.X., H.L., H.J. and J.Y. contributed to data analysis. Y.S. and J.C. contributed reagents and materials. J.X. wrote the manuscript. Y.S. and X.W. revised the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare no competing interests.

**Additional information**

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