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

Jie Xu
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Hua Liu
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Yanyan Jiang
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Huaiqi Jing
National Institute of Infectious Diseases, Chinese Center for Disease Control and Prevention
Jianping Cao
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Jianhai Yin
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Teng Li
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Yeting Sun
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)
Xin Wang
National Institute of Infectious Diseases, Chinese Center for Disease Control and Prevention
Yujuan Shen (shenyj@nipd.chinacdc.cn)
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention(Chinese Center for Tropical Diseases Research)

Research Article

Keywords: Cryptosporidium spp., Giardia duodenalis, Himalayan marmots, Alashan ground squirrels, Genotyping, Subtyping

Posted Date: October 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-992850/v1

Abstract

Cryptosporidium spp. and *Giardia duodenalis* are common detected intestinal protozoa species in humans and animals, contributing to global gastroenteritis spread. In the present study, we examined the prevalence and zoonotic potential of *Cryptosporidium* spp. and *G. duodenalis* in two rodent species in Qinghai-Tibetan Plateau area (QTPA) of China for the first time. A total of 498 intestinal content samples were collected from five counties of QTPA of Gansu province, China. *Cryptosporidium* spp. and *G. duodenalis* were found in 2.5% (10/399) and 1.5% (6/399) of Himalayan marmots, while in 1.0% (1/99) and 2.0% (2/99) of Alashan ground squirrels, respectively. Four *Cryptosporidium* genotypes were identified, including one known horse genotype (n=1) and three novel genotypes designated as marmot genotype I (n = 7), marmot genotype II (n = 2) and marmot genotype III (n = 1). The horse genotype was further subtyped as novel subtype VIbA10. *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 the two wild rodent species worldwide, suggesting the potential zoonotic transmission of the two pathogens in QTPA.

Introduction

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 main vehicle for *Cryptosporidium* spp. and *G. duodenalis* transmission [1, 2]. Infection can also be acquired following contact with infected persons or animals directly [2, 3].

Currently, at least 45 valid *Cryptosporidium* spp. species and over 100 genotypes have been identified. Over 22 *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 cryptosporidiosis [4–11]. *G. duodenalis* is a complex protozoan species, and it has been divided into at least eight genetically different assemblages (A–H) on the basis of genetic characterization. Among them, assemblage A and B are considered to be the important zoonotic pathogens. Assemblages (C–H) are more 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 seals [12]. However, assemblages C, D, E and F have been also found in humans [13].

Rodents can play an important role in the transmission of a large number of zoonotic pathogens, including bacteria, parasites and viruses. These animals may act as important sources of infection in humans, animals and the environment. Himalayan marmots (*Marmota himalayan*a) and Alashan ground squirrels (*Spermophilus alashanicus*) are two common wild rodent species distributed widely in the Qinghai-Tibetan Plateau area (QTPA) of China. They typically reside near livestock, water sources and human environments, infected hosts can play an important role in environmental contamination by excreting oocysts/cysts via faeces [14]. 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, sheep [15–19]. 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,

slaughter house water and vegetables from street markets [14, 20]. However, no study about the prevalence and transmission of *Cryptosporidium* spp. and *G. duodenalis* in Himalayan marmots and Alashan ground squirrels in China was reported before. In the present study, a cross-sectional investigation of the two protozoa was carried out in two wild rodent species to understand the prevalence of *Cryptosporidium* spp. and *G. duodenalis* and to assess the zoonotic potential at the genotype and subtype levels.

Materials And Methods

Ethics Statement

All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by the Laboratory Animal Welfare & Ethics Committee of National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, China, (Permit No: IPD-2016-15). In addition, all methods were carried out in accordance with relevant guidelines, and all authors complied with the ARRIVE guidelines.

Sample Collection

During a period of three months from June to September 2017, 399 marmots and 99 ground squirrels were captured live by mousetraps from QTPA of western China's Gansu Province, 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 in accordance with security measures. Intestinal content materials were directly collected from each animal in the laboratory of the local Center for Disease Control and Prevention (CDC) 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.

Table 1 Prevalence and molecular identification of *Cryptosporidium* spp. and *G. duodenalis* by rodent species and collection site.

Rodent species	Collection site	No. examined	*Cryptosporidium* spp.			*G. duodenalis*		
			No. positive (%)	Genotype (n)	Subtype (n)	No. positive (%)	Assemblage (n)	
				SSU rRNA	gp60		gdh	bg
Himalayan marmot (*Marmota himalayana*)	Luqu	98	0	-	-	0	-	-
	Sunan	100	7 (7.0)	marmot genotype I [a] (5); marmot genotype II [a] (1); marmot genotype III [a] (1)	-	0	-	-
	Xiahe	102	2 (2.0)	marmot genotype I [a] (2)	-	3 (2.9)	B (1), E (1)	B (2), E [a] (1)
	Zhangye	99	1 (1.0)	marmot genotype II [a] (1)	-	3 (3.0)	B (1)	A (1), B (1)
Subtotal		399	10 (2.5)		-	6 (1.5)	B (2), E (1)	A (1), B (3), E [a] (1)
Alashan ground squirrel (*Spermophilus alaschanicus*)	Huining	99	1 (1.0)	horse genotype (1)	VIbA10 [b] (1)	2 (2.0)	B (2)	B (2)
Total		498	11 (2.2)	marmot genotype I [a] (7); marmot genotype II [a] (2); marmot genotype III [a] (1); horse genotype (1)	VIbA10 [b] (1)	8 (1.6)	B (4), E (1)	A (1), B (5), E [a] (1)

a: novel genotype

b: novel subtype

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. were detected by nested PCR amplification of the fragment (approximately 830bp) of the small subunit (SSU) rRNA gene [21]. Subtyping of *Cryptosporidium* spp. was performed by a sequence analysis of the 60 kDa glycoprotein (*gp60*) gene [22]. The assemblages of *G. duodenalis* were identified and subtyped by amplifying the β-giardin (*bg*), glutamate dehydrogenase (*gdh*) [23-25]. Positive and negative controls were used for the primary and secondary PCR tests to ensure accuracy of PCR tests. The secondary PCR products were visualized under UV light after electrophoresis on a 1.5% agarose gel containing GelRed (Biotium Inc., Hayward, CA, USA).

Nucleotide Analysis

All secondary PCR amplicons of the expected size were sequenced on ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence accuracy was confirmed by bi-directional sequencing of all the PCR-positive products. Obtained DNA sequences were aligned with reference sequences deposited in GenBank databases (http://www.ncbi.nlm.nih.gov) using Clustal X (http://www.clustal.org/) to determine the species/subtypes of *Cryptosporidium* spp. and assemblages of *G. duodenalis*.

Results

Prevalence of Cryptosporidium spp. and G. duodenalis

By PCR amplification and sequence analysis, *Cryptosporidium* spp. and *G. duodenalis* were both found in two wild rodent species. In total, 2.2% (11/498) and 1.6% (8/498) of intestinal content DNA samples were positive for *Cryptosporidium* spp. and *G. duodenalis* in these two animals, respectively, with 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 (Table 1). *Cryptosporidium* spp. and *G. duodenalis* were detected in four and three sites, respectively. Different prevalences of both parasites were observed in two rodent species from different collection sites (Table 1).

Cryptosporidium Genotypes and Subtypes

Based on sequence analysis of the SSU rRNA gene, four *Cryptosporidium* genotypes were identified out of 11 isolates, including one known horse genotype (n=1) in Alashan ground squirrels and three novel genotypes named as marmot genotype I (GenBank: MZ478131, n = 7), marmot genotype II (GenBank:

MZ478132, n = 2) and marmot genotype III (GenBank: MZ478133, n = 1) in Himalayan marmot samples. *Cryptosporidium* marmot genotype I was absolutely dominant in Himalayan marmots, which constituted the largest share (70.0%;7/10) of *Cryptosporidium* isolates. At the SSU rRNA locus, the sequence of the horse genotype obtained in the present study had 100% homology with a sequence (MK775040) from a horse in China. Seven sequences of the marmot genotype I were identical to each other and shared the largest similarity (98.29%) to that (MF411075) of the skunk genotype in an eastern gray squirrel from Italy, with 17 base differences. The two same sequences of the marmot genotype II shared the largest identity (98.46%) with that of *C. rubeyi* (DQ295012) from California ground squirrels in the USA, with 13 base differences. The sequence of the marmot genotype III had 99.00% homology with that (MH940289) of the chipmunk genotype III from a red squirrel in China, with 13 base differences.

The *Cryptosporidium* horse genotype isolate was further subtyped by sequence analysis of gp60 gene. This subtype belonged to VIb subtype family, and were identified as VIbA10 (GenBank: MW531716).

G. duodenalis **Assemblages**

A total of eight *G. duodenalis* isolates were amplified and sequenced successfully in two rodent species. Assemblages A, B and E were identified in one, four and one Himalayan marmots, respectively. Assemblage B was found in two Alashan ground squirrels. Meanwhile, assemblage B was observed to showed a predominance (75.0%, 6/8) in the detected animals. The *gdh* and *bg* loci 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).

At the *gdh* locus, two assemblage B sequences had 100% homology with beaver-derived assemblage B isolate (KM977648) of China, another two different assemblage B sequences were 100% identical to golden monkey-derived assemblage B isolate (MK952602) in China, 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 (GQ329671) from Sweden and chipmunk-derived isolate (MF671918) from China, one assemblage E sequence (GenBank: MZ494459) shared the largest similarity (99.79%) to that (KY633473) from a Tibetan sheep in China.

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. 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)[19, 26–31]. The prevalence in this study was also lower than that in some pet rodent species, including in bamboo rats (3.3%, 3/92),

Siberian hamsters (7.8%, 4/51), red squirrels (8.6%, 27/314 and 26.3%, 5/19), Chichillas (9.3%, 26/280), chinchillas (10.0%, 14/140), campbell hamsters (10.0%, 3/30 and 22.2%, 6/27), Siberian chipmunks (30.0%, 6/20), gold hamsters (32.0%, 16/50), chipmunks (50.0%, 1/2 and 75.0%, 3/4), guinea pigs (52.3%, 162/310 and 85.0%, 34/40), Roborovski dwarf hamsters(100.0%,1/1), and higher than that in pet red-bellied tree squirrels (1.4%, 4/287) [28, 32–37]. In addition, there was difference between prevalence in different farmed and laboratory rodent species, including farmed bamboo rats (2.1%, 9/435 and 29.5%, 209/709), farmed brown rat (7.1%, 12/168), experimental brown rats (0.6%, 2/355), laboratory mouse (1.7%, 4/229), laboratory rat (4.0%, 1/25) [26, 28, 38–40]. These variations in the prevalence of *Cryptosporidium* spp. in different studies may be explained by many factors, including the population densities, health status of hosts, management systems, experimental method and source region [41].

Altogether, four *Cryptosporidium* genotypes were identified in this study. One known horse genotype was originally isolated from a Prezewalski wild horse at the Prague Zoo in Czech Republic, and commonly detected in horses and donkeys, occasionally found in neonatal calves and hedgehogs [42, 43]. It has also been found in human patients with diarrhea in the UK and USA, suggesting its zoonotic potential [44–46]. In the present study, horse genotype was identified in rodents for the first time, indicating it has a broader range of host range 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 gp60 gene: VIa subtype family in animals (horses, donkeys and a calf, etc.) and VIb subtype family in humans and hedgehogs.

In addition, three novel *Cryptosporidium* genotypes (marmot genotype I, marmot genotype II and marmot genotype III) were also detected. To date, a total of 13 *Cryptosporidium* spp. species and 19 genotypes have been detected in 16 studies of various rodents in China, including those obtained in this study (Table 2) [19, 26–36, 38–40]. Among them, 11 species have been detected in humans: *C. parvum*, *C. muris*, *C. ubiquitum*, *C. andersoni*, *C. occultus*, *C. viatorum*, *C. canis*, *C. suis*, *C. erinaceid*, *C. tyzzeri* and horse genotype 4. Indicating rodent species may play an important role in the transmission of zoonotic cryptosporidiosis.

Table 2
Cryptosporidium species/genotypes in rodents in China.

Host species (Latin name)	No. positive (%)	Species/genotype (n)	Sample source	Ref
Alashan ground squirrel (*Spermophilus alaschanicus*)	1/99 (1.0)	horse genotype (1)	wild	this study
Asian house rat (*Rattus tanezumi*)	6/33 (18.2)	*C. parvum* (3), *C. muris* (3)	wild	26
Asian house rat (*Rattus tanezumi*)	6/33 (18.2)	*C. tyzzer* (1), rat genotype II (1), rat genotype III (1), *C. tyzzer* + rat genotype II (1), *C. tyzzer* + rat genotype III (1)	wild	28
Asian house rat (*Rattus tanezumi*)	34/46 (73.9)	rat genotype IV (24), rat genotype III (8), *C. occultus* (1), *C. erinacei* (1)	wild	31
bamboo rats (*Rhizomys sinensis*)	9/435 (2.1)	bamboo rat genotype I (5), *C. parvum* (2), *C. occultus* (1), bamboo rat genotype II (1)	farmed	39
bamboo rats (*Rhizomys sinensis*)	3/92(3.3)	*C. parvum* (3)	pet	33
bamboo rats (*Rhizomys sinensis*)	209/709 (29.5)	*C. ubiquitum*-like (85), *C. parvum* (78), *C. parvum*-like (45), *C. occultus* (1),	farmed	40
Brandt's vole (*Lasiopodomys brandtii*)	127/678 (18.7)	*C. suis*, muskrat genotype II, Brandt's voles genotype I	wild	30
brown rats (*Rattus norvegicus*)	4/64 (6.3)	*C. tyzzer* (3), *C. tyzzer* + rat genotype III (1)	wild	28
brown rat (*Rattus norvegicus*)	12/168 (7.1)	*C. parvum* (9), *C. muris* (3)	farmed	26
brown rats (*Rattus norvegicus*)	22/242 (9.1)	*C. ratti* (14), rat genotype IV (6), *C. occultus* (1)	wild	29
brown rat (*Rattus norvegicus*)	16/56 (28.6)	rat genotype IV (13), *C. muris* (1), *C. occultus* (1), rat genotype III (1)	wild	31
Campbell hamster (*Phodopus campbelli*)	3/30 (10.0)	*C. parvum* (1), *C. andersoni* (1), *C. muris* + *C. parvum* (1)	pet	28
Campbell hamster (*Phodopus campbelli*)	6/27 (22.2)	hamster genotype (4), *C. andersoni* (2)	pet	37
Chichillas (*Chinchilla lanigera*)	26/280 (9.3)	*C. ubiquitum* (23), *C. parvum* (2), chipmunk genotype V (1)	pet	37

Note: Plus signs indicate that the sample was co-infected with different *Cryptosporidium* species/genotypes.

Host species (Latin name)	No. positive (%)	Species/genotype (n)	Sample source	Ref
Chipmunk (*Eutamias asiaticus*)	1/2 (50.0)	ferret genotype (1)	pet	36
Chipmunk (*Eutamias asiaticus*)	3/4 (75.0)	ferret genotype (2), chipmunk genotype V (1)	pet	37
Edward's long-tailed rat (*Leopoldamys edwardsi*)	21/38 (55.3)	rat genotype IV (13), rat genotype III (1), *C. muris* (1), *C. occultus* (1)	wild	31
experimental brown rats (*Ruttus norvegicus*)	2/355 (0.6)	*C. ubiquitum* (1), undetermined *Cryptosporidium* genotype (1)	laboratory	38
gold hamster (*Mesocricetu auratus*)	16/50(32.0)	*C. muris* (6), *C. andersoni* (5), *C. parvum* (2), *C. muris* + *C. parvum* (1), *C. andersoni* + *C. parvum* (1)	et	28
guinea pig (*Cavia porcellus*)	162/310 (52.3)	*C. wrairi* (129), *C. homai* (32), *C. muris* (1)	pet	37
guinea pig (*Cavia porcellus*)	34/40 (85.0)	*C. wrairi* (30)	pet	28
Himalayan marmot (*Marmota himalayana*)	10/399 (2.5)	Himalayan marmot genotype I (7), Himalayan marmot genotype II (2), Himalayan marmot genotype III (1)	wild	this study
house mouse (*Mus musculus*)	1/31 (3.2)	*C. muris* (1)	wild	26
laboratory mouse (*Mus musculus*)	4/229 (1.7)	*C. tyzzer* (4)	laboratory	28
laboratory rat (*Rattus norvegicus*)	1/25 (4.0)	*C. tyzzer* (1)	laboratory	28
long-tailed rats (*Leopoldamys edwardsi*)	4/111 (3.6)	*C. viatorum* (4)	wild	27
Muridae (*Niviventer fulvescens*)	4/10 (40.0)	rat genotype III (2), rat genotype IV (2)	wild	31
pet chinchillas (*Chinchilla lanigera*)	14/140 (10.0)	*C. ubiquitum* (13), *C. parvum* (1)	pet	35
Qinghai vole (*Microtus fuscus*)	8/90 (8.9)	*C. parvum* (3), Qinghai vole genotype (3), *C. canis* (1), *C. ubiquitum* (1)	wild	19
red-bellied tree squirrels (*Callosciurus erythraeus*)	4/287 (1.4)	rat genotype II (2), *C. parvum* (1), *C. wrairi* (1)	pet	32

Note: Plus signs indicate that the sample was co-infected with different *Cryptosporidium* species/genotypes.

Host species (Latin name)	No. positive (%)	Species/genotype (n)	Sample source	Ref
red squirrels (*Sciurus vulgaris*)	27/314 (8.6)	rat genotype II (8), ferret genotype (8), chipmunk genotype III (5), *C. ratti* (4), C. parvum (2)	pet	34
red squirrel (*Sciurus vulgaris*)	5/19 (26.3)	ferret genotype (5)	pet	28
Roborovski dwarf hamster (*Phodopus roborovskii*)	1/1 (100)	*C. muris (1)*	pet	37
Siberian chipmunk (*Tamias sibiricus*)	6/20 (30.0)	ferret genotype (3), ferret genotype + *C. parvum* (1), *C. muris* + *C. parvum* + chipmunk genotype III (1)	pet	28
Siberian flying squirrel (*Pteromys volans*)	1/1 (100)	*C. ubiquitum* (1)	pet	37
Siberian hamster (*Phodopus sungorus*)	4/51 (7.8)	*C. muris* (1), *C. parvum* (1), *C. andersoni* + *C. parvum* (1), hamster genotype (1)	pet	28
Siberian hamster (*Phodopus sungorus*)	32/37 (86.5)	hamster genotype (26),*C. andersoni* (6)	pet	37
Syrian hamster (*Mesocricetus auratus)*	26/30 (86.7)	*C. andersoni* (26)	pet	37
white-toothed rat *(Berylmys bowersi)*	21/117 (18.0)	*C. viatorum* (21)	wild	27
wild plateau pika (*Ochotona curzoniae*)	4/64 (6.3)	*C. parvum* (2), pika genotype (2)	wild	19
Note: Plus signs indicate that the sample was co-infected with different *Cryptosporidium* species/genotypes.				

The present study detected the infection of two pathogens in two 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 woodchuck (*Marmota monax*) in the USA [47, 48]; *C. parvum* in yellow-bellied marmot (*Marmota flaviventris*) in the USA[49]; *C. andersoni* in Bobak marmot (*Marmota bobac*) in the Czech Republic[42]; *C. rubeyi* in California ground squirrels (*Spermophilus beecheyi*) in the USA, Belding's ground squirrels (*Spermophilus beldingi*) and golden-mantled ground squirrels (*Spermophilus lateralis*) in the USA [50–52]; ground squirrel genotype I and ground squirrel genotype III in thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) in USA [53].

In this study, the overall prevalence of *G. duodenalis* was 1.6% (8/498), with 1.5% (6/399) for Himalayan marmots and 2.0% (2/99) for Alashan ground squirrels, which are lower than that reported in rodents 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)[26, 38, 54–57] (Table 3).

Table 3
G. duodenalis assemblages in rodents in China.

Host species (Latin name)	No. positive (%)	Assemblage (n)			Sample source	Ref
		bg	*gdh*	*tpi*		
Alashan ground squirrels (*Spermophilus alashanicus*)	2/99 (2.0)	B (2)	B (2)		wild	this study
Asian house rat (*Rattus tanezumi*)	2/33 (6.1)	G (2)	G (1)	G (1)	wild	26
bamboo rat (*Rhizomys sinensis*)	52/480 (10.8)	B (52)	B (27)	B (12)	farmed	55
brown rat (*Rattus norvegicus*)	11/168 (6·6)	G (11)	G (9)	G (10)	wild	26
brown rat (*Ruttus norvegicus*)	33/355 (9.3)	G (19)	G (20)	G (21)	laboratory	20
coypus (*Myocastor coypus*)	38/308 (12.3)	B (11), A (1)	B (10), A (1)	B (22), A (3)	farm	57
Himalayan marmots (*Marmota himalayan*a)	6/399 (1.5)	A (1), B (3), E (1)	B (2), E (1)	-	wild	this study
house mouse (*Mus musculus*)	1/31 (3.2)	G (1)	-	G (1)	wild	26
pet chinchillas (*Chinchilla lanigera*)	38/140 (27.1)	A (4), B (8)	A (4), B (16)	A (3), B (3)	pet	35
pet chipmunks (*Eutamias asiaticus*)	24/279 (8.6)	G (11), A (13)	G (7), A (10)	G (4), A (13)	pet	54

In this study, the sequences of amplicons from *G. duodenalis*-positive samples were determined to be assemblages A, B, and E, with assemblage B being more prevalent. 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 known, *G. duodenalis* infections in Chinese rodents were reported to be caused by assemblage A, B and G in previous studies [26, 38, 54–57]. Among them, assemblages A and B have a broad host range and commonly found in humans [55]. In fact, some recent studies in China also reported the occurrence of assemblage Ain pet chipmunks, coypus and pet chinchillas, while assemblage Bin bamboo rat, coypus and pet chinchillas [54–57]. These two assemblages detected in this study, suggesting that these two rodent species can play a role in the zoonotic dissemination of *G. duodenalis.* Assemblage E is commonly found in a range of hoofed livestock, it has also been found in human cases, indicating that this assemblage is of zoonotic significance [58].

And one recent study describes the occurrence of assemblage E in a rodent species long-tailed chinchillas in Romania [59]. And in the present study, we characterized the appearances of assemblages E in rodents in China for the first time, and identified a novel assemblage E sequence (GenBank: MZ494459), sequence comparison showed that this isolate had high homology with a known assemblages E sequence available on GenBank (GenBank: KY633473), with only 1 base differences.

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 the two wild rodent species may play an important role in the transmission of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts among humans, animals, water sources and fresh produce in QTPA grassland ecosystem, and pose a threat to grassland ecosystem and public health.

Conclusion

To the best of our knowledge, this study examined the prevalence and zoonotic potential of *Cryptosporidium* spp. and *G. duodenalis* in two rodent species in Qinghai-Tibetan Plateau area (QTPA) of China for the first time. Four *Cryptosporidium* genotypes were identified, including one known horse genotype (novel subtype VIbA10), which was reported in rodent species firstly. And three novel genotypes: marmot genotype I, marmot genotype II and marmot genotype III. *G. duodenalis* zoonotic assemblages A, B, E were identified in this two rodent species. The results expanded the host range of *Cryptosporidium* spp. and *G. duodenalis*, providing more information on 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, provide control and prevention strategies to reduce the risk to human and animals in the investigated area.

Declarations

Acknowledgments

We are grateful to Dr Daqin Xu at Gansu Provincial Centre for Disease Control and Prevention for help in collecting the samples.

Author Contributions

YS and XW designed the study. JX, HL, YJ, LT and YS participated in the sample collection and methodology. JX, HL, HJ and JY contributed to data analysis. YS and JC contributed reagents and materials. JX wrote the manuscript. YS and XW revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Data Availability

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

Ethics statment

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. The protocol was approved by the Laboratory Animal Welfare & Ethics Committee (LAWEC), National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Permit Number: NIPD-2016-15).

Funding

This work was supported by the National Science and Technology Major Program of China (No. 2018ZX10713001-004 to YS), the National Natural Science Foundation of China(Nos. 82072307 and 81772224 to YS), and the Fifth Round of Three-Year Public Health Action Plan of Shanghai, China(No. GWV-10.1-XK13 to JC). The funders had no role in the study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

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