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RESEARCH

Molecular evaluation of *Cryptosporidium* spp. in sheep in southern Xinjiang, China

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Abstract

Cryptosporidium spp. are diarrheagenic intestinal parasites with multiple hosts worldwide. A total of 1252 fresh fecal samples of sheep were collected from 10 large-scale farms in southern Xinjiang. Based on the small subunit ribosomal (*SSU* rRNA) gene of *Cryptosporidium*, 100 *Cryptosporidium*-positive samples (8.0%, 100/1252) were detected by PCR. Nine out of 10 farms were positive for *Cryptosporidium*, with the highest infection rate being 18.4% (23/125) on farm 9 in Qira. The infection rates of *Cryptosporidium* in pre-weaned lambs, weaned lambs, fattening sheep, and adult sheep were 20.3% (61/301), 10.3% (34/329), 0.9% (3/327), and 0.7% (2/295), respectively. Three *Cryptosporidium* species were identified, namely, *C. xiaoi* ($n = 61$), *C. parvum* ($n = 22$), and *C. ubiquitum* ($n = 17$). Of them, *C. xiaoi* was detected on all positive farms and in different age groups of sheep. The subtypes of *C. parvum* and *C. ubiquitum* were identified by PCR at the 60 kDa glycoprotein (*gp60*) gene. Two *C. parvum* subtypes were identified: IIdA19G1 ($n = 21$) and IIdA15G1 ($n = 1$). One *C. ubiquitum* subtype was identified with XIIa ($n = 17$). These results indicated the common transmission and genetic diversity of *Cryptosporidium* in sheep in southern Xinjiang, and further investigations are needed on the zoonotic potential of *C. parvum* and *C. ubiquitum* in this region.

Keywords *Cryptosporidium* · Infection · Subtype · Genetic · Sheep

Introduction

Cryptosporidium spp. are apicomplexan parasites commonly found across many host species, including humans, livestock, companion animals, and wild animals (Menon et al. 2022). As an important reservoir and susceptible host of *Cryptosporidium*, sheep may suffer from diarrhea and other symptoms, as well as limited growth and performance (Chen et al. 2022; Yang et al. 2021). Cryptosporidiosis in sheep is worthy of attention because of its zoonotic potential and cross-host transmission ability.

Of the over 44 established *Cryptosporidium* species, the ones that dominantly infect sheep are *C. parvum*, *C. ubiquitum*, and *C. xiaoi* (Chen et al. 2022). *C. parvum* is the dominant species in European and Australian sheep, whereas *C. xiaoi* predominates in Asian and African sheep. However, *C. ubiquitum* appears to be more common in Asia and America (Guo et al. 2021). Subtyping of *C. parvum* from sheep has identified almost exclusively IIa and IId subtypes with differences across geographic locations or host ages. Host adaptation to *C. ubiquitum* subtypes is apparent, with subtypes in XIIa found in sheep (Santin 2020).

Cryptosporidium infection is prevalent in sheep worldwide. In China, studies on the epidemiology of *Cryptosporidium* in sheep were mainly concentrated in the central and eastern regions (Lang et al. 2023; Li et al. 2016; Li et al. 2019b; Mi et al. 2018; Ye et al. 2013; Zhang et al. 2020). Xinjiang Uygur Autonomous Region (hereinafter referred to as Xinjiang) is an important hub connecting the interior of eastern China and Central Asia, with a unique ecological environment, unique climatic conditions, and special dietary

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habits. Sheep farming has always been an important part of the economy and people's livelihood in the region. In comparison with surveys of cattle in Xinjiang, epidemiological data of *Cryptosporidium* from large-scale sheep farms in this area are quite scarce. Therefore, this study aimed to investigate the infection and molecular genetic characteristics of *Cryptosporidium* on concentrated sheep farms in southern Xinjiang.

Materials and methods

Ethics statement

The protocol in this study was not required to be reviewed and approved by the Animal Ethical Committee.

Fecal sample collection

A total of 1252 fresh fecal samples were randomly collected from 10 large-scale sheep farms in southern Xinjiang from May 2021 to August 2022. The sampling locations were distributed in nine cities (Kuqa, Wensu, Wushi, Alaer, Kalpin, Marabishi, Shufu, Qira, and Yutian) in southern Xinjiang (Fig. 1). All the samples were divided into four groups according to host age, including pre-weaned lambs at <3 months old (n = 301), weaned lambs at 3–6 months old (n = 329), fattening sheep at 6–12 months old (n = 327), and adult sheep older than 12 months old (n = 295) (Fig. 1). Most of the stool samples were collected from a single rectum with disposable clean gloves or fresh excreta from individual sheep (especially to avoid contact with the ground) weighing 5–30 g. The marked samples were placed in sealed sampling bags, sent to the laboratory, and stored at a low temperature of 4 °C until detection.

Nucleic acid extraction and PCR amplification

Approximately 200 μg of each fecal sample was selected for nucleic acid extraction using a fecal whole genome extraction kit (E.Z.N.A.® D4015-02, OMEGA Bio-Tek) following the product procedures. The extracted nucleic acid samples were stored at −20 °C.

The small subunit ribosomal (*SSU* rRNA) gene and the glycoprotein 60 (*gp60*) gene were detected in *Cryptosporidium* species and subtypes and were amplified by nested PCR. The primer sequences required for PCR amplification were obtained from others and have been evaluated by peers and were synthesized by Anshengda (Suzhou) Biotechnology Co., Ltd. (Table 1).

For accurate quality control in the assay, positive and negative controls were used for each PCR amplification. The positive controls were derived from the nucleic acids of *C. andersoni* and *C. parvum* (subtype IIdA20G1) from cattle. All the controls were identified and stored at the Veterinary Parasitology Laboratory of Tarim University, and the negative control samples were sterilized in double-distilled water. Five microliters of the second PCR amplification products was placed in a 1.5% agarose gel for electrophoresis and then transferred to a gel imaging system for visualization.

Sequencing identification and phylogenetic analysis

A DNA sequencing instrument (ABI PRISMTM3730 XL DNA Analyzer) was used to identify the positive

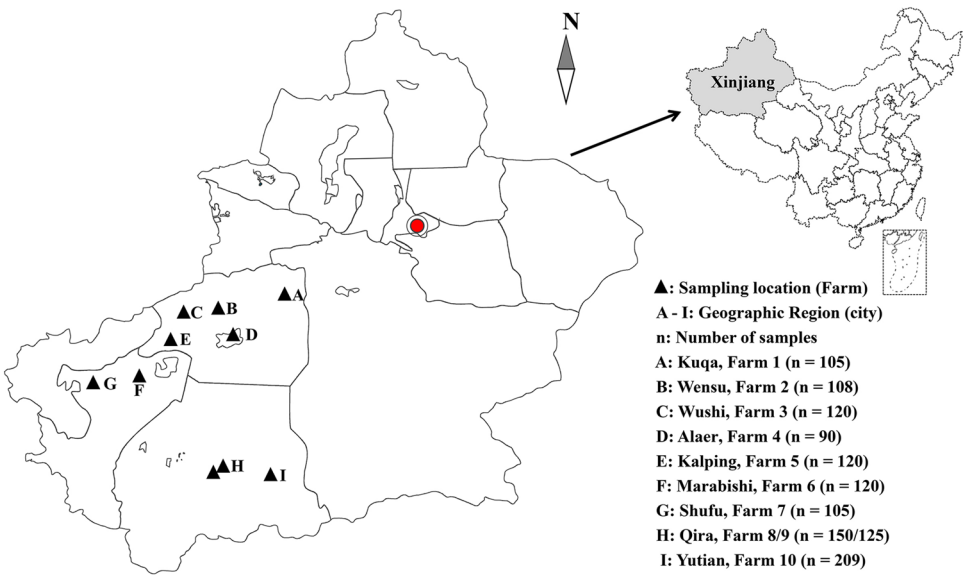

Fig. 1 Distribution of sampling locations in southern Xinjiang, China. Filled triangles indicate sampling farms. The letters A–I represent the cities where the sampling sites were located

Table 1 The primers used in the characterization of *Cryptosporidium* species and subtypes in the present study

Gene	Primer sequence (5′ to 3′)	Fragment length	Annealing temperature	Usage (s)	Reference
SSU rRNA	18SiCF1: GACATATCATTCAAGTTTCTGACC	~763 bp	58 °C	Specific nested PCR of *Cryptosporidium* spp.	(Ryan et al. 2003)
	18SiCR1: CTGAAGGAGTAAGGAACAACC				
	18SiCF2: CCTATCAGCTTTAGACGGTAGG	~587 bp	58 °C		
	18SiCR2: TCTAAGAATTTCACCTCTGACTG				
gp60	AGP-F1: ATAGTCTCCGCTGTATTC	~1280 bp	55 °C	Subtyping of *C. parvum*	(Alves et al. 2003)
	AGP-R1: GGAAGGAACGATGTATCT				
	AGP-F2: TCCGCTGTATTCTCAGCC	~850 bp	58 °C		
	AGP-R2: GCAGAGGAACCAGCATC				
gp60	UGP-F1: TTTACCCACACATCTGTAGCGTCG	~1044 bp	58 °C	Subtyping of *C. ubiquitum*	(Li et al. 2014)
	UGP-R1: ACGGACGGAATGATGTATCTGA				
	UGP-F2: ATAGGTGATAATTAGTCAGTCTTT AAT	~948 bp	55 °C		
	UGP-R1: TCCAAAAGCGGCTGAGTCAGCATC				

samples, and the sequencing process was commissioned by Ansengda (Suzhou) Biotechnology Co., Ltd. To ensure the accuracy of the sequences, amplicons of positive DNA samples were identified by forward and reverse sequencing. The calibrated sequences were assembled and calibrated by ChromasPro version 1.5 (http://technelysium.com.au/wp/chromaspro/). The *SSU* rRNA gene sequences of *Cryptosporidium* obtained in this study were compared with the known sequences in the GenBank database by BLAST (Basic Local Alignment Search Tool) to determine the species or genotype. The *gp60* gene sequences obtained from *C. parvum* and *C. ubiquitum* were also subjected to the same procedure to determine their subtypes.

The obtained sequences and reference sequences were analyzed by ClustalX 2.1 software (http://www.clustal.org), and a molecular phylogenetic tree was constructed by the general time-reversible model based on the maximum likelihood method in MEGA 7.0 (https://megasoftware.net). The reliability of the phylogenetic tree was tested by bootstrap analysis with 1000 replicates.

Statistical analysis

SPSS (Statistical Product Service Solutions) version 26.0 (IBM Corp.) was used for all statistical analyses. The infection rates and their 95% confidence intervals (*CIs*) were calculated with the Wald method. Differences in *Cryptosporidium* infection rates were evaluated with the chi-squared test, and differences were considered significant at $P < 0.05$.

Results

Cryptosporidium infection of sheep at different farms

Cryptosporidium was identified in 9 out of the 10 sheep farms, with an overall infection rate of 8.0% (100/1252). The infection rate of *Cryptosporidium* in different farms ranged from 0% (0/120) to 18.40% (23/125), with the highest infection rate being 18.4% (23/125) on farm 9 in Qira (Table 2). In general, the infection rate of *Cryptosporidium* was significantly different among the farms ($\chi^2 = 45.994$, $df = 9$, $P = 0.000$).

Cryptosporidium infection of sheep of different ages

The highest infection rate of *Cryptosporidium* was found in pre-weaned lambs (20.3%, 61/301), followed by weaned lambs (10.3%, 34/329), fattening sheep (0.92%, 3/327), and adult sheep (0.68%, 2/295). The infection rates of *Cryptosporidium* were significantly different between the four age groups of sheep ($\chi^2 = 107.897$, $df = 3$, $P = 0.000$) (Table 3).

Distribution of *Cryptosporidium* species and subtypes

Sequence alignment analysis based on the *SSU* rRNA gene showed that *C. xiaoi* ($n = 61$), *C. parvum* ($n = 22$), and *C. ubiquitum* ($n = 17$) were identified in the 100 positive samples. Further analysis based on the *gp60* gene revealed that *C. parvum* contained two subtypes, IIdA19G1 ($n = 21$) and

Table 2 Prevalence and distribution of *Cryptosporidium* species/subtypes in sheep by farm in southern Xinjiang, China

Region	Farm	No. samples	No. positive (%)	95% *CI*	Species (*n*)			Subtypes (*n*)	
					C. xiaoi	*C. ubiquitm*	*C. parvum*	*C. ubiquitm*	*C. parvum*
Kuqa	Farm 1	105	6 (5.7)	0.8–10.6	6	0	0	/	/
Wensu	Farm 2	108	5 (4.6)	0.2–9.1	5	0	0	/	
Wushi	Farm 3	120	14 (11.7)	5.5–17.8	9	4	1	XIIa (4)	IIdA19G1 (1)
Alaer	Farm 4	90	3 (3.3)	0.9–7.6	3	0	0	/	/
Kalpin	Farm 5	120	0	/	0	0	0	/	/
Marabishi	Farm 6	120	9 (7.5)	2.4–12.6	3	5	1	XIIa (5)	IIdA15G1 (1)
Shufu	Farm 7	105	5 (4.8)	0.2–9.3	5	0	0	/	/
Qira	Farm 8	150	8 (5.3)	1.4–9.3	8	0	0	/	/
	Farm 9	125	23 (18.4)	11.2–25.6	8	0	15	/	IIdA19G1 (15)
Yutian	Farm 10	209	27 (12.9)	8.1–17.7	14	8	5	XIIa (8)	IIdA19G1 (5)
Total		1252	100 (8)	6.5–9.5	61 (4.9)	17 (1.4)	22 (1.8)	XIIa (17)	IIdA19G1 (21), IIdA15G1 (1)

IIdA15G1 ($n = 1$), while all detected *C. ubiquitum* belonged to subtype XIIa ($n = 17$) (Tables 2 and 3).

C. xiaoi was present on all positive farms, but *C. ubiquitum* and *C. parvum* were found only on three and four farms, respectively. *C. xiaoi* was found in all age groups, especially in pre-weaned ($n = 29$) and weaned ($n = 28$) lambs, with three positives in fattening sheep and one positive in adult sheep. *C. ubiquitum* was entirely concentrated in pre-weaned ($n = 11$) and weaned ($n = 6$) lambs. *C. parvum* mainly infected pre-weaned lambs ($n = 21$), except for one positive occurrence in the adult flock. Notably, the dominant subtype of IIdA19G1 was present in pre-weaned lambs, while one IIdA15G1 was detected in the adult flock (Table 3).

Molecular characterization of *Cryptosporidium* species and subtypes

Cryptosporidium parvum contained two haplotype sequences, which showed 100% identity with the reference sequence from *Camelus dromedarius* (MK491509) from Egypt ($n = 1$) and yak (KP334136) from China ($n = 21$). Similarly, the two haplotypes contained in *C. ubiquitum* showed 100% identity with the reference sequence from a bovine (MT044136) from India ($n = 16$) and 99.79% identity with the reference sequence from a goat (KM199749) from China ($n = 1$). The dominant haplotype of *C. xiaoi* showed 100% identity with that from a sheep in Algeria (LC414392) ($n = 59$), and the other two haplotype sequences were consistent with two goat reference sequences in China (KT235703 and KT235699) (Table 4).

In terms of subtypes, only the *C. parvum* IIdA15G1 subtype shared the same identity with the reference sequence (MH794167) of sheep from Xinjiang, China. In addition, one isolate of subtype IIdA19G1 had 99.62% identity with the reference sequence of sheep from Shaanxi (KT235713), China, and the others shared 99.09–100% identity with the reference sequence of sheep from Anhui (MH049734), China. All *C. ubiquitum* XIIa subtypes maintained high identity with reference sequences (MH049733) from sheep from Jiangsu, China (Table 4).

Phylogenetic analysis based on the *SSU* rRNA gene showed that most isolates of the three *Cryptosporidium* species identified in this study were clustered together with the ruminant isolates (cattle, sheep, and goat) from China in the genetic evolutionary tree, except for one *C. parvum* isolate on the same branch as the Egyptian camelid isolate (Fig. 2). Phylogenetic analysis based on the *gp60* gene revealed high genetic diversity of *C. parvum* isolates. There were a variety of haplotypes based on the single nucleotide polymorphisms (SNPs) within subtype IIdA19G1 in this study, and most of them were on independent branches of the genetic evolutionary tree (Fig. 3).

Discussion

The results of this study confirmed that the overall infection rate of *Cryptosporidium* was 8.0% (100/1252), much higher than that in grazing adult sheep (0.9, 3/318) and lower than that in captive sheep (36.4%, 36/99) in Xinjiang, according to the reports mentioned above (Mi et al. 2018; Qi et al. 2019). It is slightly lower than the total infection rate of sheep in China reported so far (9.6%, 571/5946), and according to the specific analysis of administrative divisions, the infection rate in this study is lower than that reported in East China (11.1%, 134/1215), Northwest China (14.4%, 171/1184), and North China (16.5%, 16.5%). In contrast, it was higher than that reported in South China (4.8%, 82/1701) and Northeast China (4.5%, 25/559) (Guo et al. 2021; Mi et al. 2018; Wang et al. 2022; Yang et al.

Table 3 Prevalence and distribution of *Cryptosporidium* species/subtypes in sheep by age in southern Xinjiang, China

Age groups	No. samples	No. positive	Infection rate (95% *CI*)	χ^2	*P*	Species (*n*)			Subtypes (*n*)	
						C. xiaoi	*C. ubiquitum*	*C. parvum*	*C. ubiquitm*	*C. parvum*
Pre-weaned	301	61	20.3% (15.6–24.9)	Reference		29	11	21	XIIa (11)	IIdA19G1 (21)
Weaned	329	34	10.3% (6.9–13.8)	26.68	0.000	28	6	0	XIIa (6)	/
Fattening	327	3	0.9% (0.3–2.1)	12.11	0.001	3	0	0	/	/
Adults	295	2	0.7% (0.4–1.8)	60.47	0.000	1	0	1	/	IIdA15G1 (1)
Total	1252	100	8% (6.5–9.5)	39.37	0.000	61 (4.9)	17 (1.4)	22 (1.8)	XIIa (17)	IIdA19G1 (21), IIdA15G1 (1)

2021). Globally, the prevalence of *Cryptosporidium* infection in sheep was 18.9% (7836/47585), with some heterogeneity between 6 continents, namely, Asia (14.8%), Europe (20.2%), Africa (21.7%), North America (29.8%), South America (20.3%), and Oceania (19.1%) (Chen et al. 2022; Santin 2020). Several factors, including sampling area, sampling time, age distribution of hosts, and detection methods, may explain the differences between the results of this study and those of other reports.

In the current study, the infection rate of *Cryptosporidium* was negatively and significantly related to sheep age, which was similar to most previous findings for sheep indicating a higher prevalence of *Cryptosporidium* in lambs than in adult sheep worldwide. A relevant global meta-analysis showed that sheep aged <3 months had a significantly higher prevalence (27.8%, 3284/11938) than those aged 3–12 and >12 months (Chen et al. 2022). Domestically, a survey of *Cryptosporidium* in sheep from several provinces in China showed that the infection rate of lambs (31.2%, 224/718) was higher than that of adult sheep (22.4%, 71/317) (Mi et al. 2018). The result was consistent with one report in Inner Mongolia that the prevalence in 15- to 16-week-old lambs (weaned) was higher than that in 3- to 4-week-old lambs (pre-weaned) (Ye et al. 2013). It may be that, compared with newborn lambs, older lambs before and after weaning may be more susceptible to *Cryptosporidium* oocysts secreted by adult sheep or existing in the environment due to lower maternal antibodies, weakened immunity, and weaning stress.

Cryptosporidium xiaoi, *C. ubiquitum*, and *C. parvum* were detected in this study, which have been generally recognized as the dominant species infecting sheep (Guo et al. 2021; Santin 2020). Although the majority of sheep studies worldwide have shown the predominance of *C. ubiquitum*, *C. xiaoi*, and *C. parvum* and reported to be more prevalent in European countries (Dessi et al. 2020; Kaupke et al. 2017; Mammeri et al. 2019). In a small-scale study in Poland, *C. parvum* was found in lambs <4 weeks of age, whereas older hosts were infected with only *C. xiaoi* (Kaupke et al. 2017). In a longitudinal study conducted in Australia, of the four *Cryptosporidium* species identified in sheep, *C. parvum* was detected in lambs of various ages, *C. ubiquitum* was mostly identified in lambs <2 months of age, *C. andersoni* was identified in lambs older than 3 months, and *C. xiaoi* was the only species identified in ewes (Sweeny et al. 2011a). In contrast, the results of this study were somewhat unique and complex, in which *C. xiaoi* was distributed in sheep of all ages, *C. parvum* was mostly restricted to pre-weaned lambs, with only one isolate occurring in adult lambs, and *C. ubiquitum* was found in lambs before and after weaning.

In the current study, *C. xiaoi* was distributed on all nine positive farms, but *C. parvum* and *C. ubiquitum*, which are generally considered to have a wide range of hosts and strong diarrheagenic properties when infecting ruminants

Table 4 Genetic homology of *Cryptosporidium* species and subtypes in this study with reference sequences from GenBank

Cryptosporidium	Sample code	No. samples	The closest BLAST match	
			Identities	Accession numbers (host, country)
Species				
C. *parvum*	XJS1	1	100%	MK491509 (*Camelus dromedarius*, Egypt)
	XJS2 - XJS22	21	100%	KP334136 (Yak, China)
C. *ubiquitum*	XJS23 - XJS38	16	100%	MT044136 (Cattle, India)
	XJS39	1	99.79%	KM199749 (Goat, China)
C. *xiaoi*	XJS40 - XJS98	59	100%	LC414392 (Sheep, Algeria)
	XJS99	1	100%	KT235703 (Goat, China)
	XJS100	1	100%	KT235699 (Goat, China)
Subtypes				
C. *parvum* IIdA15G1	XJS1	1	100%	MH794167 (Sheep, China: Xinjiang)
C. *parvum* IIdA19G1	XJS2 - XJS7	6	100%	MH049734 (Goat, China: Anhui)
	XJS8 - XJS13	6	98.87%	MH049734 (Goat, China: Anhui)
	XJS14 - XJS15	1	99.88%	MH049734 (Goat, China: Anhui)
	XJS16 - XJS17	1	99.88%	MH049734 (Goat, China: Anhui)
	XJS18	1	99.64%	MH049734 (Goat, China: Anhui)
	XJS19	1	99.08%	MH049734 (Goat, China: Anhui)
	XJS20	1	99.62%	KT235713 (Sheep, China: Shaanxi)
	XJS21	1	99.36%	MH049734 (Goat, China: Anhui)
	XJS22	1	99.09%	MH049734 (Goat, China: Anhui)
C. *ubiquitum* XIIa	XJS23 - XJS38	16	99.78%	MH049733 (Sheep, China: Jiangsu)
	XJS39	1	99.89%	MH049733 (Sheep, China: Jiangsu)

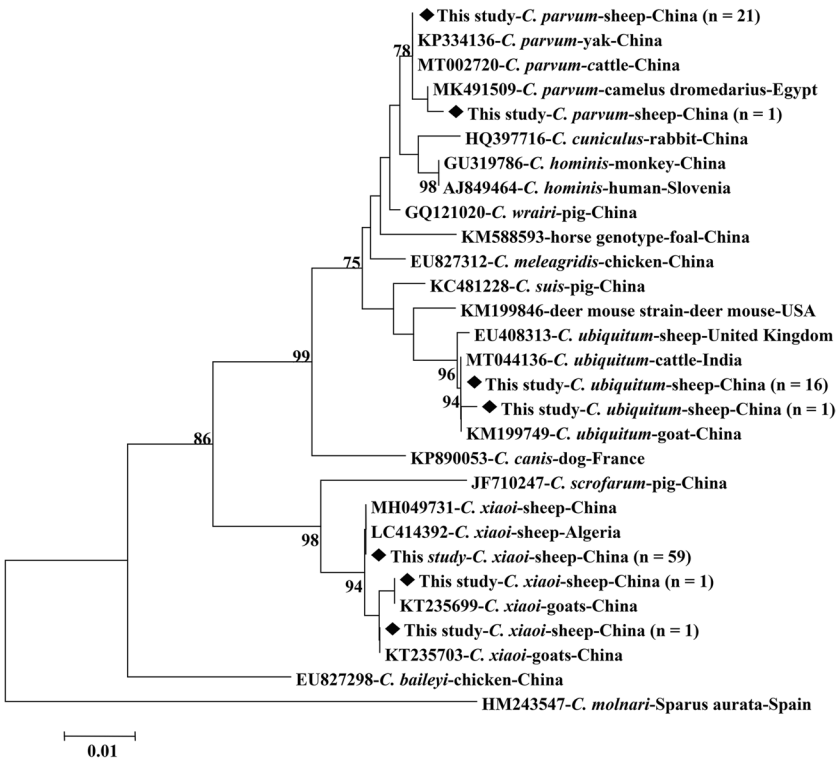

Fig. 2 Phylogenetic relationships of *Cryptosporidium* species from sheep based on the partial sequence of the *SSU* rRNA gene. The host, region of identification, and GenBank accession number of each isolate are shown. Solid black diamonds represent the *Cryptosporidium* isolates in this study

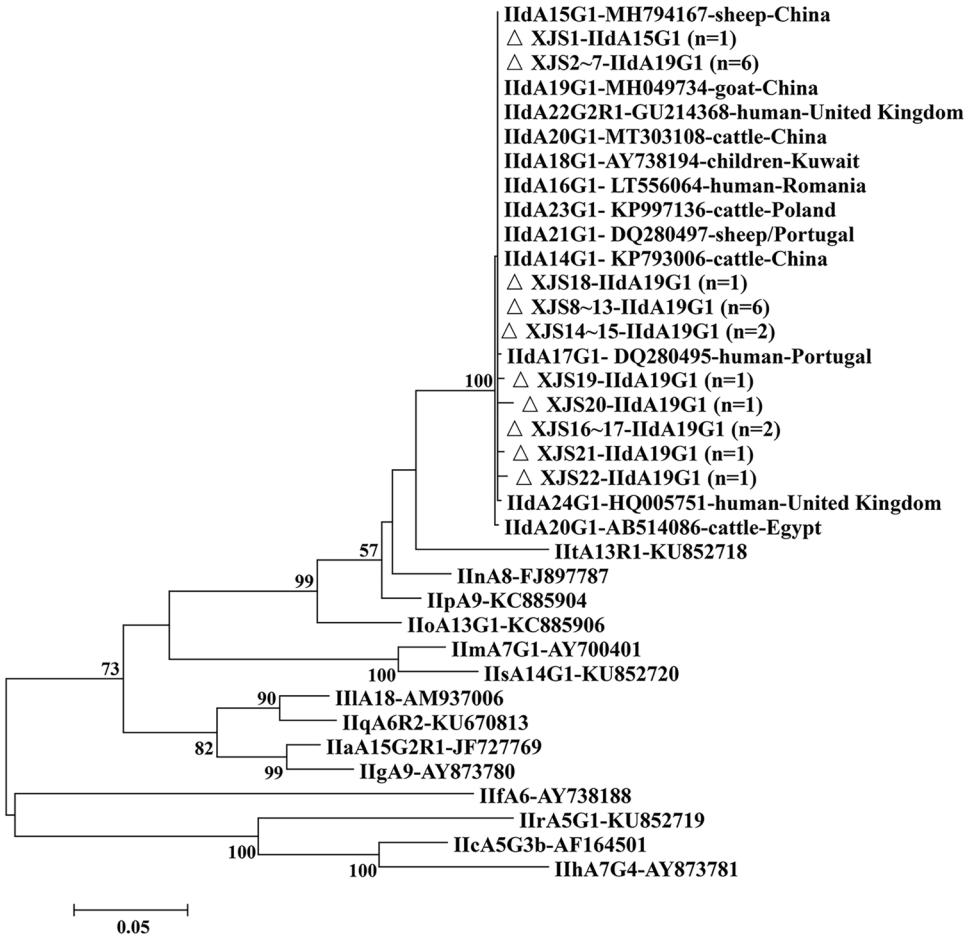

Fig. 3 Phylogenetic relationships of *Cryptosporidium parvum* from sheep based on the partial sequence of the *gp60* gene. The host, region of identification, and GenBank accession number of each isolate are shown. White hollow triangles represent the *C. parvum* isolates in this study

and humans, were found only in four and three of the farms, respectively. This was similar to the results of two previous related surveys in Xinjiang (Qi et al. 2019; Mi et al. 2018). The results from Qi et al. 2019 showed that only one of the 15 sheep grazing areas was *C. parvum* positive. Similarly, in the survey results of Mi et al. in 2018 from two large-scale sheep farms in Xinjiang, only *C. xiaoi* and *C. ubiquitum* were found to have high infection rates, while no cases of *C. parvum* infection were observed. Once colonized on large-scale farms, the species is often not easily eradicated, causing the animals and environment of the positive farms to act as sources for a long time (Cai et al. 2017; Li et al. 2019a; Santin et al. 2008; Sweeny et al. 2011b; Yang et al. 2014). In particular, a 1-year longitudinal survey of newborn calves on two farms in Xinjiang showed that *C. parvum* infection was basically the same and at a high level all year round, accompanied by the occurrence of herd diarrhoea in calves, suggesting its strong survival and pathogenic ability (Zhang et al. 2022).

All *C. ubiquitum* isolates in this study were subtyped as XIIa, which was consistent with previous results in sheep from multiple provinces (Qinghai, Beijing, Inner Mongolia, Jilin, Ningxia, Shanghai, and Xinjiang) in China (Li et al. 2019b; Mi et al. 2018) and has also been reported in sheep from Australia, Ghana, and Spain (Ramo et al. 2016; Squire et al. 2017; Yang et al. 2014), suggesting that subtype XIIa of *C. ubiquitum* is the most common subtype in sheep worldwide. *C. parvum* subtypes IIdA15G1 ($n = 1$) and IIdA19G1 ($n = 21$) were identified in this study which was reported in previous studies to cause large-scale outbreaks of diarrheal disease in newborn calves, resulting in mass mortality in China (Guo et al. 2021; Li et al. 2019a). To date, only one case related to IIdA15G1 has been identified in sheep in China, and several IIdA15G1 isolates from sheep were found in Greece (Papanikolopoulou et al. 2018; Qi et al. 2019). Subtype IIdA19G1 was found only in a small number of sheep in eastern China (Shandong and Shanghai) and has not been reported abroad, although it has previously appeared in humans and other animals such as goats and cattle (Guo et al. 2021; Santin 2020). This study also found that the IIdA19G1 subtype could be divided into several haplotypes based on SNPs, indicating that it had intra-subtype genetic diversity.

In conclusion, this is the first large-scale survey of *Cryptosporidium* in sheep in southern Xinjiang, which confirmed that *Cryptosporidium* infection was common

in sheep on local intensive farms. The infection rate of *Cryptosporidium* decreased with the age of the host, including the zoonotic subtypes IIdA19G1 and IIdA15G1 of *C. parvum* and the subtype XIIa of *C. ubiquitum.*

Author contribution Zhengrong Wang, Xia Peng, Xinwen Bo and Bowen Zhang collected the faecal samples. Zhengrong Wang, Yanyan Zhang, Fuchang Yu and Aiyun Zhao carried out the PCR assays and sequence analyses. Zhenjie Zhang and Meng Qi designed the study and drafted the current manuscript.

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Data availability The nucleotide sequences reported in this study have been deposited in the GenBank database at the National Center for Biotechnology Information under accession numbers OR361825-OR361831.

Declarations

Consent to participate Not applicable.

Consent for publication All the authors consent to publication of this article.

Conflict of interest The authors declare no competing interests.

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