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Prevalence and molecular characterization of *Cryptosporidium* spp. in yaks (*Bos grunniens*) in Naqu, China

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ABSTRACT

The intestinal protozoan specie, *Cryptosporidium* causes serious diarrheal syndrome in humans and animals worldwide. However, limited knowledge is known about the infection caused by this specie in yaks in Naqu. About 950 serum and 150 fecal samples were collected and assayed by using commercial ELISA kits and nPCR detection methods to find the prevalence and molecular characterization of *Cryptosporidium* spp. in yaks. Results found that 103 out of 950 (10.8%) serums were uncovered against *C. parvum* antibodies. In different regions, the prevalence of *C. parvum* in yaks were in a range from 9.1% to 16.7%, with obvious difference among the three areas ($P < 0.001$). In male and female yaks, the prevalence of *C. parvum* was found to be 7.2% and 13.3% respectively ($P < 0.001$); and a significant difference ($P < 0.001$) with a range of 9.8%–18.2% was observed among different age groups. Out of 150 fecal samples, only 2 (1.3%) positive samples were detected via nPCR. The positive samples were sequenced and identified to be *C. bovis*. The two isolates were clustered to cattle and yak clade separately. Our results highlight the prevalence and epidemiological status of *Cryptosporidium* spp. in yaks which may contribute towards the prevention and control of this zoonotic disease in Naqu, China.

1. Introduction

Cryptosporidium spp. is one of the widely known intestinal protozoa that causes serious diarrheal syndrome in humans and animals worldwide [1,2]. Cryptosporidiosis caused by this parasite is self-limiting in normal immunity patients; however, in immunocompromised and young individuals, the infection can be fatal and life-threatening [3,4]. Cryptosporidiosis can not only causes chronic, severe and intractable diarrhea to acquired immune deficiency syndrome people, but also significantly shortens their life expectancy [5]. In large ruminant animals like cattle, cryptosporidiosis can cause serious diarrhea, weight loss, delayed growth, even mortality leading to an economic problem [3].

Yak is the symbolic animal species on the Qinghai Tibetan plateau [6,7]. There are approximately 15 million yaks in the world and 90% of its population is living on the high cold plateaus of China [6,8]. Apart from serving as a transportation mean, this bovine ruminate also contribute greatly towards the betterment of local economy [8,9] like dung, fur, wool, milk and meat helping the herdsmen to improve their life quality. Nowadays, Chinese people are under the pressure of the epidemic of African swine fever; so, more and more people are switching to the use of beef meat as an alternative to the dietary protein sources. With the price-hike of meat in China, it is of great importance to keep a stable and healthy development of animal husbandry. Previously, over 38 *Cryptosporidium* species and more than 70 genotypes had been identified [10–12]. The prevalence of *Cryptosporidium* spp. in yaks has been reported to be in a range from 1.98% to 30% through nPCR detection [2,3,10,11,13]. However, until now scarce information is available regarding *Cryptosporidium* spp. infection in yaks in Naqu plateau.

Naqu is located in the north of Tibet, with northern latitudes of 29°55′–36°30′ and eastern longitudes of 83°55′–95°30′. The annual average temperature of this cold region is −2.1 °C and the lowest temperature usually reach to −40 °C. There are about 5.2 million

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livestock animals living in this plateau. Numerous wild-lives and birds such as wild goat, stone sheep, deer, lynx, wild donkey, bear, fox, wolf, finch, brown back crow, pheasant, vulture, wild ducks, swans, black-necked crane and red-crowned crane are found in this high-altitude region (over 4500 m above sea level).

It is necessary and meaningful to investigate the status of *Cryptosporidium* spp. infection in yaks to provide evidence for prevention and control of disease. This can not only contribute to the healthy development of yak industry especially in current problem of African swine fever, but also potently benefit the health of local farmers. Therefore, to improve our information regarding the epidemiology status of cryptosporidiosis in plateau, this study was performed to reveal the prevalence and molecular characterization of *Cryptosporidium* spp. in yaks in Naqu, China.

2. Materials and methods

2.1. Ethics statement

Blood and fecal samples were collected after the permission of the relevant institutions. All procedures were performed under the instructions and approval of Laboratory Animals Research Centre of Hubei province and Tibet in P. R. China, and the Ethics Committee of Huazhong Agricultural University, Wuhan.

2.2. Serum samples

Blood samples (n = 950) of yaks were collected in 2019 from three regions (Jiani 12, Seni 538, Nierong 400) of Naqu Tibet, China (Table 1). The relevant detail information of farm, gender and age was recorded on prescribed form. The blood samples were centrifuged at 4000 × *g* for 15 min, and serum was separated and stored at −80 °C for further analysis.

Table 1
Prevalence of Cryptosporidiosis in yaks in different regions in Tibet, China.

Region	Farm	Male	Female	Total	C.I (95%)
		Prevalence (%) (Positive No./ Samples No.)	Prevalence (%) (Positive No./ Samples No.)	Prevalence (%) (Positive No./ Samples No.)	
Jiani		16.7% (2/12)	0	16.7% (2/12)[a]	2.1–48.4
Seni	S1	2.1% (1/48)	15.4% (8/52)	9.0% (9/100)[b]	4.2–16.4
	S2	0	7.3% (4/55)	7.3% (4/55)	2.0–17.6
	S3	0	11.5% (7/61)	11.5% (7/61)	4.7–22.2
	S4	1.6% (1/62)	7.3% (3/41)	3.9% (4/103)	1.1–9.6
	S5	6.1% (2/33)	8.0% (2/25)	6.9% (4/58)	1.9–16.7
	S6	20.1% (13/54)	7.3% (4/55)	15.6% (17/ 109)	9.4–23.8
	S7	9.7% (3/31)	4.8% (1/21)	7.7% (4/52)	2.1–18.5
Total		8.8% (20/228)	9.4% (29/310)	9.1% (49/538)	6.8–11.9
Nierong	N1	9.5% (4/42)	33.3% (16/48)	22.2% (20/ 90)[c]	14.1–32.2
	N2	0 (0/35)	5.7% (2/35)	2.9% (2/70)	0.3–9.9
	N3	7.4% (2/27)	1.7% (1/60)	3.4% (3/87)	0.7–9.7
	N4	(0/20)	35.3% (12/34)	22.2% (12/54)	12.0–35.6
	N5	0 (0/17)	23.9% (11/46)	17.5% (11/63)	9.1–29.1
	N6	28.6% (2/7)	6.9% (2/29)	11.1% (4/36)	3.1–26.1
Total		5.4% (8/148)	17.5% (44/ 252)	13.0% (52/ 400)	9.9–16.7
Total		7.2% (28/ 388)[d]	13.3% (75/ 562)	10.8% (103/ 950)	8.9–13.0

[a] Significant difference was found in yaks in different regions ($P < 0.001$, $\chi2 = 395.506$).
[b] Difference among farms was uncovered statistically significant in Seni ($P = 0.014 < 0.05$, $\chi2 = 15.937$).
[c] Difference among farms was revealed statistically significant in Nierong ($P < 0.001$, $\chi2 = 32.149$).
[d] Significant difference was found in yaks in different genders ($P < 0.001$, $\chi2 = 121.514$).

2.3. Fecal samples

A total of 150 fecal samples were obtained from yaks in Naqu plateau. Then all of the samples were stored in dry ice and shipped to the clinical lab of College of Veterinary Medicine, Huazhong Agricultural University Wuhan.

2.4. Determination of antibodies against C. Parvum in yaks

Serum antibodies against *Cryptosporidium* spp. were procured by employing commercial enzyme linked immunosorbent assay (ELISA) kit (Bovine *C. parvum* ELISA Kit, Jingmei Biotechnology Co., Ltd, Jiangsu, China) according to the manufacturer's instructions and as described in previous studies [14,15]. The test value was based on the optical density (OD) values of 450 nm. To ensure validity, positive and negative controls were performed twice and the average values of positive and negative controls were set at ≥ 1.00 and ≤0.15 respectively. The critical (cut off) value = the average value of negative controls + 0.15. The results were interpreted as positive when OD 450 ≥ cut off value; and negative when OD 450 < cut off value.

2.5. DNA extraction

Total genomic DNA extraction from fecal samples were performed using fecal DNA extraction reagent kit (TIANgen fecal genomic DNA Kit, DP328, Tiangen Biotech CO., LTD, Beijing, China) according to manufacturer's recommendations. The eluted DNA was stored at −20 °C prior to PCR analysis.

2.6. Gene amplification and DNA electrophoresis

The nPCR amplification approach was used to amplify the 18S SSU rRNA gene of *Cryptosporidium* as previously reported [16]. In primary PCR, the primer pairs (forward: 18SiCF2 5′-GACATATCATTCAAGTTT CTGACC-3′ and reverse: 18SiCR2 5′-CTGAAGGAGTAAGGAACAACC-3′) were used. The PCR mixture contained 12 μL autoclaved distilled water, 2.5 μL PCR Buffer (10 ×), 5 μL dNTPs (2.5 mM), 2.5 μL DNA, 1 μL Taq, 1 μL of each forward and reverse primer (working concentration: 10 μmol/L) in a 25 μL reaction volume. Each of the 35 PCR cycles consisted of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min after an initial hot start at 98 °C for 5 min and ending with 72 °C for 5 min. During second PCR, the primer pairs (forward: 18SiCF1 5′-CCTATCA GCTTTAGACGGTAGG-3′and reverse: 18SiCR1 5′-TCTAAGAATTTCAC CTCTGACTG-3′) were utilized. The PCR mixture contained 24 μL autoclaved distilled water, 5 μL PCR Buffer (10 ×), 10 μL dNTPs (2.5 mM), 5 μL of primary product, 2 μL Taq, 2 μL of each forward and reverse primer (working concentration: 10 μmol/L) in a 50 μL reaction volume. Each of the 35 PCR cycles consisted of 98 °C for 35 s, 58 °C for 35 s, and 72 °C for 1 min after an initial hot start at 98 °C for 10 min and ending with 72 °C for 7 min. Ultimately, PCR products were analyzed on a 2% agarose gel stained with ethidium bromide following electrophoresis. All the positive PCR electrophoresis products were purified by piloting a Hi-TIAN gel Midi Purification Kit (Tiangen Biotech CO., LTD, Beijing, China) according to manufacturer's recommendations.

2.7. Sequencing and analysis

All the purified products were sent for sequencing via illumine at Gene Crest Biological Technology Co., Ltd. (Wuhan, China). Multiple alignments were conducted to the 18s rRNA of current isolates and available references gene of 18s rRNA of *Cryptosporidium* spp. at NCBI database by Lasergene (Version 7.0). These strains were *C. bovis* (MK501766.1, MN540746.1, MH754168.1, MK880572.1, MG972763.1, KY808999.1, MK573327.1, MF142033.1, KY809003.1,

KY809002.1), *C. baileyi* (L19068.1), *C. parvum* (L16996.1), *C. muris* (L19069.1), *C. hominis* (KP280061.1), *C. aviaum* (JQ246415.1), *C. felis* (AF093013.1), *C. meleagridis* (AF381169.1).

2.8. Phylogenetic analysis

The phylogenetic analysis was performed to determine *Cryptosporidium* spp. through MEGA (Version 6.0) by means of the neighbor-joining algorithm, and the distances were computed through the neighbor-joining method. The stability of branches was assessed after bootstrapping with 1000 replicates.

2.9. Statistical analysis

Differences in prevalence were calculated by chi-square test utilizing IBM SPSS Statistics (SPSS 20.0) and P values < 0.05 were considered statistically significant.

3. Results

A total 103 out of 950 (10.8%) serum samples were uncovered against *C. parvum* antibodies. In different regions, the prevalence of *C. parvum* in yaks ranged from 9.1% to 16.7%, with obvious difference among three areas ($P < 0.001$, $\chi2 = 395.506$). The prevalence of *C. parvum* in yaks in Jiani ($P < 0.001$) and Nierong ($P < 0.001$) was found to be prominently higher as compared to Seni region. In Seni, the prevalence of *C. parvum* in yaks ranged from 3.9% to 15.6%, with notable differences among 7 farms ($P < 0.05$, $\chi2 = 15.937$) (Table 1). The prevalence of *C. parvum* in yaks in farm S4 was strikingly lower than that in the farms of S1 ($P < 0.01$), S2 ($P < 0.05$), S3 ($P < 0.05$), S5 ($P < 0.06$) and S6 ($P < 0.001$). In Nierong, the prevalence of *C. parvum* in yaks ranged from 2.9% to 22.2%, with remarkable differences among the six farms ($P < 0.001$, $\chi2 = 32.149$) (Table 1) with higher significance in farms N1 ($P < 0.001$), N3 ($P < 0.05$), N4 ($P < 0.01$), and N5 ($P < 0.01$) as compared to the farm N2.

In male and female yaks, the prevalence of *C. parvum* was 7.2% and 13.3% respectively (Table 1.) with higher occurrence in females than male ones ($P < 0.001$, $\chi2 = 121.514$). In Nierong, the prevalence of female yaks in three farms; N1 ($P < 0.001$), N4 ($P < 0.01$) and N5 ($P < 0.001$) were revealed to be obviously higher than male yaks, while female yaks in farms N3 ($P < 0.05$) and N6 ($P < 0.001$) were both found to be much lower than male ones. In Seni, the prevalence of female yaks in farm S1 ($P < 0.01$) was significant higher that in male yaks, while female yaks in farms S6 ($P < 0.001$) was clearly lower than male yaks.

In different ages, the prevalence of *C. parvum* was ranged from 9.8% to 18.2%, with significant difference among different ages ($P < 0.001$, $\chi2 = 232.644$) (Table 2). In comparison to the yaks of more than four years of age, the prevalence of *C. parvum* were obviously higher both at age < 2 ($P < 0.001$), $2 <$ year ≤ 4 ($P < 0.001$). The prevalence of *C. parvum* in female yaks over 2 years of age was significantly higher than that in male ones ($P < 0.001$).

Out of the 150 fecal samples, 2 samples (1.3%) were found positive through nPCR. These positive samples were sequenced and deposited in GenBank under the accession numbers MN696243 and MN696246. Phylogenetic tree analysis performed via MEAG (6.0) showed the presence of *C. bovis* in both isolates. However, both of them were clustered to cattle and yak clade separately (Fig. 1). By sequencing the multiple alignment of 18S rRNA gene of *C. bovis* by employing Clustal V method via Lasergene (7.0), it was found that the homology of MN696243 with previous isolates was 98.2%–99.6%, while the isogeny of MN696246 with previous isolates was slightly lower (95.7%–96%).

Table 2
Prevalence of Cryptosporidiosis in yaks in different ages in Tibet, China.

Ages (Year)	Male	Female	Total	C.I (95%)
	Prevalence (%) (Positive No./ Samples No.)	Prevalence (%) (Positive No./ Samples No.)	Prevalence (%) (Positive No./ Samples No.)	
< 2	0 (0/7)	50.0% (2/4)	18.2% (2/11)[e]	2.3–51.8
2 < Y ≤ 4	9.2% (11/120)	18.1% (21/116)	13.6% (32/236)	9.5–18.6
4 >	6.5% (17/261)	11.8% (52/442)	9.8% (69/703)	7.7–12.3

[e] Significant difference was found in yaks in different ages ($P < 0.001$, $\chi2 = 232.644$).

4. Discussion

With the sustained economic growth during the past 10 years, there was a growing need of meat products in China. With population of 89.15 and 428.17 million cattle and pigs respectively in 2018 in China (National Bureau of Statistics of China, http://data.stats.gov.cn/easyquery.htm?cn=C01), the prices of meat products are seriously affected especially in case of outbreak of infectious diseases. Therefore, regular monitoring of infectious diseases is of great importance to maintain the development of animal husbandry and raise people's standard of living.

Cryptosporidium infection is a public health problem in many countries and particularly in remote areas [17], so the present study was carried out to investigate the occurrence of this parasite in these areas. Previously, the prevalence of *Cryptosporidium* spp. in dairy calves in Argentina and Italy was reported to be 25.5% and 38.8% respectively [18,19]. In China, its prevalence in cattle, dairy calves and yaks was determined as 14.4%, 16% and 11.3%, respectively in Qinghai, Xinjiang and Qinghai regions which was in parallel with our results in yaks in Naqu [4,11,20]. However, the prevalence of *Cryptosporidium* spp. in our studies was found to be much lower than those in yaks (30.0%) in Qinghai in 2014, and dairy cattle (32.3%), beef cattle (26.5%), water buffaloes (23.8%) in Henan and Shandong regions [3,21]. In the present study, obvious differences of the prevalence of *Cryptosporidium* spp. in yaks were also found in different regions and farms (Table 1). The potential reason may be because of the differences in environmental and climatic factors, water resources, and animal density in this region [7,22].

In current results, the overall prevalence of *C. parvum* in female yaks was clearly higher than those in male yaks. The prevalence of female yaks in farms N1, N4, N5, and S1 were all significantly higher than the male yaks. From the present data, we may infer that female yaks were under higher risk of infection of this parasite and the potential reason may be because of the different immunity and animal density. In different ages, the prevalence of *C. parvum* were obviously higher both in age < 2 ($P < 0.001$), $2 <$ year ≤ 4 ($P < 0.001$), which was in accordance with the results examined in yaks in Qinghai [3], but not in consistent with previous reports as there was an age-associated occurrence of *Cryptosporidium* spp. in cattle [21].

Provious studies have reported the presence of four species *C. parvum, C. bovis, C. andersoni* and *C. ryanae* in cattle [23] and *C. parvum, C. bovis, C. ryanae, C. andersoni, C. ubiquitum, C. xiaoi, C. struthionis, C. hominis and C. canis* infection in yaks [3,10,11,13]. *C. bovis* has been found the dominating species in yaks in Qinghai (33/56, 3/76) and Tibet (3/4) [3,11,13], while *C. parvum* and *C. andersoni* was the main species reported in Dangxiong yaks (4/4) and Qinghai yaks (72/158) respectively [10,24]. From fecal samples of yaks, two *C. bovis* isolates were revealed, which was in accordance with results found in most yaks and cattle in China [25,26]. However, the two isolates were clustered to cattle and yak clade separately (Fig. 1), and the cattle isolate was not reported before. Though the yak isolate was clustered to previous yak isolates, there were more than 20 (4.3%) differential bases in the short 18S rRNA.

The infected yaks may potentially transmit the *Cryptosporidium* spp.

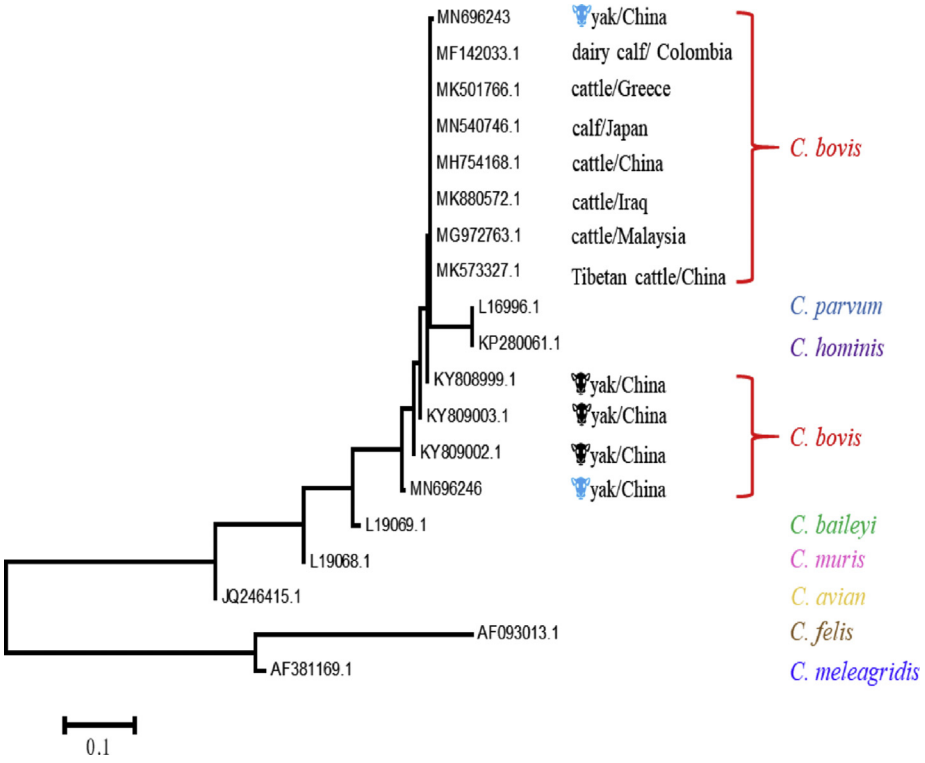

Fig. 1. Phylogenetic tree for *Cryptosporidium* spp. based on partial 18S rRNA gene sequences was constructed using the neighbor-joining method with Kimura two-parameter analysis and bootstrap analysis of 1000 replicates. The numbers on the branches indicate the percentage of replicates that reproduced the topology for each clad. The blue cattle indicate the sequences acquired from the current study.

to other domestic or wild animals, as the social and free-ranged yaks share grasslands and water with large number of lives on high plateau [6,7]. Cattle have been considered a commonly recognized reservoir of *Cryptosporidium* specie causing human infections [21]. Previously *C. parvum* subtypes infecting humans had been isolated from cattle [27]. The local herdsmen are also under high risk of *Cryptosporidium* spp. infection as it is relatively difficult to boil water on the plateau region and people here prefer to eat air dried meat products. Also the shed parasitic oocysts in the environment are difficult to be eliminated due to their high resistance to chemical disinfectants or water treatments such as chlorination [1]. The spread of *Cryptosporidium* spp. usually occurs through the fecal-oral route after the ingestion of contaminated food or water containing infective oocysts or cysts [25]. Therefore, *Cryptosporidium* spp. from yaks may pose a potential health threat to human and other animals on the plateau.

In conclusion, the current research herein reveals the prevalence of *Cryptosporidium* spp. which is one of the causes of diarrhea in yaks. Our results highlight the epidemiological status of *Cryptosporidium* infectious agent in yaks on the high plateau. Therefore, prevention of cryptosporidiosis is necessary in highland area and more attention should be paid to assess the public health risk.

Authors' contribution

KL and JKL designed the study. KL, ZXL, ZBZ, and AYL performed the experiments, and KL, MM and KG analyzed the data. KL and KM wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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