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SHORT COMMUNICATION

First Detection and Molecular Identification of *Entamoeba* in Yaks from China

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Abstract

Background Yak, a predominant livestock of plateau areas, is known as a host to many parasites. And the genus *Entamoeba*, the third-common cause of the mortality worldwide from parasitic diseases, was discovered in yaks once.

Methods We investigated the distribution and species of *Entamoeba* spp. from yaks in Qinghai province, northwestern China, by collecting 1027 yak fecal samples. All samples were divided according to seven geographical sites, four seasons, and two age groups of yaks. After extracting DNA, polymerase chain reaction (PCR) was performed to amplify the 18S rRNA gene, and sequences were analyzed with phylogenetic method.

Results We observed an overall *Entamoeba* positive rate of 36.32% (373/1027) in yaks from Qinghai province. The common species included *Entamoeba bovis* (284/373), *Entamoeba* sp. MG107/BEL (79/373), *Entamoeba* sp. ribosomal lineage (RL) two (8/373), and *Entamoeba* sp. RL9 (2/373). According to the result of statistical analysis, *Entamoeba* infection rate was the highest in summer and significantly differed from that observed during other seasons ($P < 0.05$). The yaks from Golog had the highest prevalence of *Entamoeba* among all geographical origins in Qinghai province ($P < 0.05$). However, no significant difference was observed ($P > 0.05$) among different age groups, as evident from a positive rate of 39.58% in ≤ 6-month and 36.16% in > 6-month yaks.

Conclusion These results indicate the prevalence and predominant species of *Entamoeba* in yaks. To our knowledge, this is the first study to report *E. bovis*, *Entamoeba* sp. RL2, and *Entamoeba* sp. RL9 in Chinese yaks.

Keywords Qinghai · Yak · *Entamoeba* · 18S rRNA · Phylogeny

Abbreviations

PCR	Polymerase chain reaction
RL	Ribosomal lineages
χ^2 test	Chi-square test/Chi-square goodness-of-fit test
BLAST	Basic local alignment search tool

Introduction

Yak, known as "the boat of plateau" has lived in high-altitude areas in a long history [1]. Given their tolerance to bad weather, yaks are the most important animals for local residents and a source of milk, wool, fur, and other life necessities. The Qinghai province in northwest China has

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approximately 5.9 million yaks, much more than any other area in the world [2]. As a predominant livestock in plateau areas, yak is known to be a host to many kinds of protozoa such as *Cryptosporidium*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Toxoplasma gondii* [3–6]. In 2018, *Entamoeba* was detected in yaks in Gannan Tibetan Autonomous Prefecture, northwestern China, by high-throughput sequencing [7], but no specific species of this genus was mentioned.

In recent years, *Entamoeba* has been discovered in amphibians (toads), fish (eel) and insects (cockroaches), along with many other hosts, including humans, non-human primates, birds, mammals, and reptiles, and has been listed as the third most common cause of parasite disease-associated mortality [8–18]. *Entamoeba* species can be distinguished based on morphological characteristics into uninucleate (*E. bovis*-like group), quadrinucleate (*E. histolytica*-like group), octonucleate (*E. coli*-like group) and non-cyst-producing (*E. gingivalis*-like group) groups [19]. The vast majority of *Entamoeba* species are not pathogenic except for *E. histolytica*, very possibly *E. bangladeshi*, *E. invadens* and the one causing deaths in toads (accession number MH890608). Normally most *Entamoeba* species exist asymptomatically in the gut of animals and are considered part of the microbiome. According to Stensvold et al., ribosomal lineage (RL) has been used to identify organisms with branches within phylogenetic trees that do not show a strong affinity with previously described *Entamoeba* species [20]. So far, 11 RLs have been established.

The dominant species found in ruminants are *E. bovis*, *E. ovis*, *E. histolytica*, *E. dilimani*, and *E. bubalus*, of which *E. bovis* was mostly discovered in cattle and *E. ovis* in sheep. A report indicated that *E. ovis* was just a junior synonym of *E. bovis* and that *E. bovis* can infect both cattle and sheep [11, 17]. Several other *E. bovis* hosts have been found, including white-tailed deer [12], reindeer [20], gnu [21], and Bay duiker [22]. A uninucleate *E. bovis*-like *Entamoeba* sp. was recently identified from rangeland goat [23]. So far, *E. bovis* has been reported in eight countries, namely, Australia, Costa Rica, Icelan, Japan, Libya, Swedend, Uganda and UK [17, 19, 20, 23–26].

In the present study, we explored and detected the prevalence of *Entamoeba* species in Chinese yak in Qinghai province by the amplification of the 18S rRNA gene locus. Further, the most prevalent geographical origins, seasons, and host age were discussed by statistical analysis.

Materials and Methods

Sample Sources

In total, 1027 fecal samples of free-range yaks from seven geographical locations in Qinghai province, northwestern China, were collected in plastic bags after receiving owners' permission orally from 2016 to 2017 (Table 1). Each stool sample was collected from the top layer of the feces immediately after excretion to prevent any sample material that had contacted the ground. Each animal was sampled only once. The collected samples were sorted as per collecting site, season, and yak age. All samples were transferred to laboratory in polyfoam boxes containing ice packs. A part of fecal specimens was partly transferred into 15-mL centrifuge tubes with 2.5% potassium dichromate and immediately stored in a refrigerator (4 °C) until DNA extraction was performed. The rest of each specimen was reserved at – 20 °C.

DNA Extraction and Amplification of 18S rRNA Locus

About 0.5 g of each refrigerated fecal specimen was washed five times with distilled water by centrifugation at 13,000 × *g* for 1 min to remove 2.5% potassium dichromate. DNA was extracted from the washed fecal samples by the E.Z.N.A.™ Stool DNA Kit (D4015-02; Omega Bio-Tek Inc., Norcross, GA, USA). All operations were implemented as per the manufacturer's instructions. The extracted DNA was stored at – 20 °C until further analysis.

The 18S rRNA gene sequence was amplified through polymerase chain reaction (PCR) using specific primers (forward, JVF 5′-GTT GAT CCT GCC AGT ATT ATA TG-3′ and reverse, DSPR2 5′-CAC TAT TGG AGC TGG AAT TAC-3′) [27], synthesized by Sangon Biotech Co., Ltd, Shanghai, China. PCR was performed as follows: 5 min at 95 °C, 40 cycles for 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C, followed by final steps for 2 min at 72 °C and 1 min at 16 °C. PCR products (550 bp approximately), stored at 4 °C before the next step, were tested by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Sequencing and Phylogenetic Analysis

After gel extraction, the nucleotide sequences of all positive amplicons were detected by Sangon Biotech Co., Ltd. Then, after assembling the obtained sequence of the 18S rRNA locus for each sample by DNAStar 5.0 [28], all sequences were submitted to Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the reference sequences from the GenBank database. ClustalX 1.83 [29] and on-line Castresana Lab (https://molevol.cmima.csic.es/castresana/Gblocks_server.html) (Gblocks server) under default settings were used to align all sequences and trim the alignment. MEGA 7.0.26 [30] was applied to construct a Maximum Likelihood (ML) tree containing representative sequences from our study and reference sequences from the database. Nucleotide sequence data reported in this paper are available in the GenBank database

under the accession numbers MH127441 to MH127457, MT734113 to MT734386, MT734571 to MT734647, and MT734656 to MT734660.

Statistical Analysis

The χ^2 test (Chi-square test/Chi-square goodness-of-fit test) was performed using SPSS 21.0 for Windows (SPSS Inc., Chicago, USA) to analyze data on the positive rate of *Entamoeba* and determine any differences between sampling sites, seasons, and ages. Differences were considered significant at $P < 0.05$.

Results

PCR amplification and sequencing revealed *Entamoeba*-positive specimens. Of 1027 samples, 373 samples were successfully sequenced, and an overall prevalence of 36.32% was observed among yaks from Qinghai province. Of these sequences, four known *Entamoeba* species were identified, including *E. bovis* (76.14%, 284/373), *Entamoeba* sp. MG107/BEl (21.18%, 79/373), *Entamoeba* sp. RL2 (2.14%, 8/373), and *Entamoeba* sp. RL9 (0.54%, 2/373) from the result of phylogenetic tree analysis (Table 1). These sequences were submitted to GenBank under the accession numbers mentioned above.

The prevalence of species in different locations, seasons, and host ages is shown in Table 1. A statistically significant difference was observed among different geographical origins ($P < 0.05$), with the highest and lowest prevalence reported in the yaks from Golog (56.58%) and Haibei (16.84%), respectively. We also noted significant differences among samples from different seasons ($P < 0.05$) but not between yaks of two age groups.

Through the phylogenetic analysis (ML method), the sequence Lc73 and the reference sequence *Entamoeba* sp. RL2 (FR686363) in the GenBank database are on the same branch; the sequences Lc1 and the reference sequence *Entamoeba* sp. MG107/BEL (GU437825) are on the same branch; the sequence Hc24 and Mc12 are on the same branch with the reference sequence *E. bovis* (FN666250 and FN666252), while Ac4 is on the same branch with *Entamoeba* sp. RL9 (KR025407) (Fig. 1).

Discussion

In China, *Entamoeba* has been mostly detected and studied in macaques and humans. In the past few years, *E. nuttalli*, *E. coli*, and *E. polecki* subtype two have been discovered from Tibetan macaques and rhesus macaques in Mount Long-hu and Gui-yang using PCR [31]. *E. histolytica* was also isolated from the residents of seven provinces in China (Beijing, Shanghai, Guangxi, Sichuan, Guizhou, Qinghai, and Sinkiang) [32], and from diarrheal patients in Shanghai using an enzyme-linked immunosorbent assay [33]. However, we failed to detect *E. histolytica* in yaks in the present study, consistent with the results of a previous study that reported *E. histolytica* primarily in humans [34, 35]. In our study, the predominant *Entamoeba* species discovered in yaks from Qinghai province was *E. bovis*, which is recognized as generally infectious to ruminants, especially cattle

Table 1 Occurrence of *Entamoeba* in yaks from Qinghai province

Factor	Categories	Sample size	Prevalence % (No. of positive)	Species (*n*)			
				E. bovis	*Entamoeba* sp. MG107/BEL	*Entamoeba* sp. RL2	*Entamoeba* sp. RL9
Host age	≤6 months	48	39.58 (19)	15	4		
	>6 months	979	36.16 (354)	269	75	8	2
Seasons	Spring	215	39.07 (84)	61	21		2
	Summer	355	45.63 (162)	136	24	2	
	Autumn	254	32.68 (83)	64	16	3	
	Winter	203	21.67 (44)	23	18	3	
Locations	Xining	192	48.96 (94)	71	16	5	2
	Haibei	190	16.84 (32)	23	9		
	Hainan	162	25.93 (42)	29	12	1	
	Haixi	47	55.32 (26)	25	1		
	Huangnan	270	39.26 (106)	65	40	1	
	Yushu	90	33.33 (30)	29	1		
	Golog	76	56.58 (43)	42		1	
Total		1027	36.32 (373)	284	79	8	2

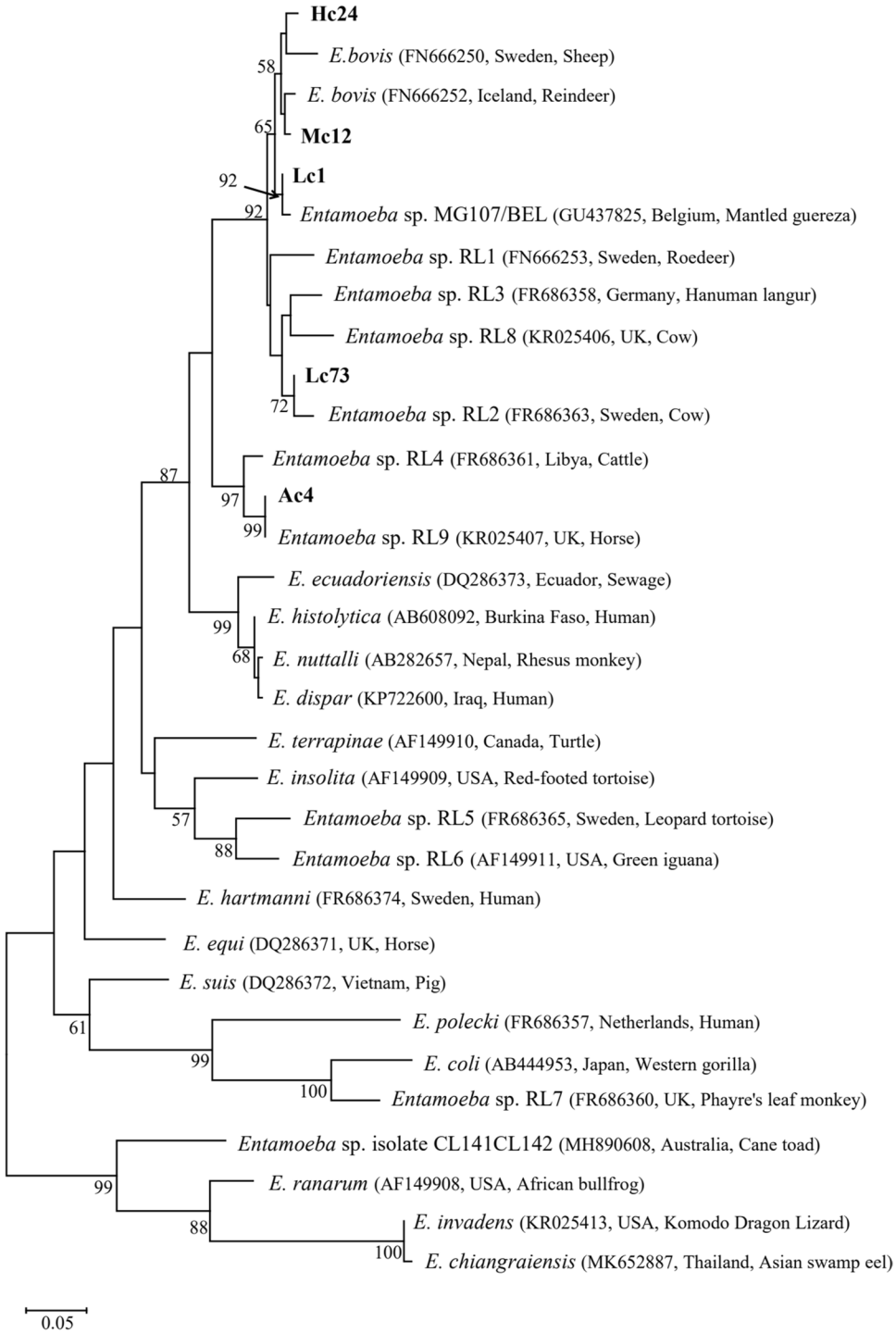

Fig. 1 A phylogenetic tree based on the partial 18S rRNA sequences of representative *Entamoeba* members constructed using the Maximum Likelihood (ML) method. The GenBank accession numbers of all the sequences are indicated after their taxon names. New sequences are indicated in bold text. The scale bar represents 0.05 substitutions per nucleotide

[19]. This species, *E. bovis*, is considered as not pathogenic [26].

This is the first study to investigate the prevalence of *Entamoeba* in Chinese yaks. The *Entamoeba*-positive rate in our study was 36.32% (373/1027), close to a rate of 44.40% reported in cattle [17] and higher than that reported in dairy cattle and beef cattle (2.50% and 1.10%, respectively) in Costa Rica [24]. One possible reason underlying this variation may be the differences in the detection methods adopted. These authors employed the flotation technique using a hyper-saturated sugar solution to detect *Entamoeba* cysts, while we chose a molecular PCR-based strategy. The comparison of results suggests that molecular method is more precise than morphological analysis to detect intestinal protozoa. The prevalence noted in our study was much lower than that observed in cattle from Japan and Uganda (72.00%

and 80.00%, respectively). The samples used in these two studies were collected from farms where animals thrived in a small area [25, 26] and those collected in our study were from yaks raised in free range, explaining the differences in results.

According to the results of statistical analysis, summer was the most prevalent season for *Entamoeba* in yaks from Qinghai province, consistent with a previous report by Zhang et al. stating summer as the high-risk season for *E. histolytica* infections [33]. Thus, the surveillance of *Entamoeba* should be particularly strengthened in summer. In addition, the yaks from Golog had the highest positive rate of *Entamoeba* infections as compared with those from other locations, probably owing to many factors such as the weather and altitude.

While processing the 18S rRNA gene sequence of *E. bovis*, we observed high variations in a band of about 30 bp. These ambiguous bases were previously discovered and reported by Stensvold et al. [19, 20] and considered as differences between individual gene copies among isolates. However, 18S rRNA gene has always been a highly conserved locus and widely used for the molecular characterization of many parasites such as *Cryptosporidium*, *Sarcocystis*, *Giardia*, and *Toxoplasma* [4, 36, 37]. No variation was observed in these parasites; thus, further study is warranted to explore the mechanism underlying nucleotide difference in this genus.

We performed phylogenetic analysis analysis of the 18S rRNA gene sequence and found 284 *Entamoeba* sequences as *E. bovis*. Besides, 79 sequences showed high similarity with *Entamoeba* sp. MG107/BEL (GU437825). This species found by Levecke et al. *Entamoeba* sp. MG107/BEL, belong to *Entamoeba* spp. but fail to match with all the other known *Entamoeba* spp.[38]. In addition, eight and two sequences were considered to be *Entamoeba* sp. RL2 (FR686363) and *Entamoeba* sp. RL9 (KR025407), respectively; however, we cannot confirm yet if these RLs have been described before or serve as new species. In previous studies, *Entamoeba* sp. RL9 was only detected in horses. To our best knowledge, this is the first study to report *Entamoeba* sp. MG107/BEL and *Entamoeba* sp. RL9 in ruminants.

In conclusion, the present study explored the prevalence of *Entamoeba* spp. in yaks depending on geographical origins, seasons, and host age. This is the first time that *E. bovis*, *Entamoeba* sp. MG107/BEL, *Entamoeba* sp. RL2 and *Entamoeba* sp. RL9 have been detected in yaks.

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Author Contributions MR helped with the collection of the fecal samples, performed the experiments of DNA extracting and PCR amplification, the data analyses and the writing of this manuscript. FY and JMG helped with the sample collection and the experiments. QL designed the experiment and helped with the final manuscript. PXW, MZ and XHZ helped with the experiments and the data analyses. All authors read and approved the final manuscript.

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Availability of Data and Materials The data of the current study would be available from the corresponding author on a reasonable request.

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interests.

Ethical Approval This study was conducted strictly as per the legal requirements of guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and approved by the Research Ethics Committee of Northwest A&F University. Sampling was permitted by yak owners, and no specific authority was needed for sample collection.

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