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RESEARCH ARTICLE

Molecular Epidemiological Investigation of *Cryptosporidium sp*., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis sp*. Infection in Free-ranged Yaks and Tibetan Pigs on the Plateau

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A B S T R A C T

Intestinal parasites are of great economic importance in livestock. However, scarcity of data has been found about the prevalence of four important intestinal parasites including *Cryptosporidium sp*., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis sp*. infection in free-ranged yaks and Tibetan pigs during the winter season on the plateau. Fecal samples of yaks (n=40) and Tibetan pigs (n=60) were collected and molecular identification of these parasites was performed through nested PCR amplification. Positive PCR samples were sequenced and further phylogenetic analysis was performed. Results found that the prevalence of *Cryptosporidium sp*., *G. duodenalis*, *E. bieneusi* and *Blastocystis sp*. was 10.0, 7.5, 12.5, 7.5% in yaks, and 18.3, 0, 56.7, 50.0% in Tibetan pigs, respectively. Co-infections was found between 2.5-7.5% in yaks and 0-26.7% in Tibetan pigs, respectively. The sequenced samples were identified to be *Cryptosporidium sp.* and was identified as pig genotype II. Findings of this study will provide an insight to the prevention and control for these important parasites on the high plateau.

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INTRODUCTION

Intestinal parasites like *Cryptosporidium sp*., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis sp*. are ubiquitous important parasitic pathogens infection in various vertebrate species (Wu *et al*., 2020; Masuda *et al*., 2022). C*ryptosporidium* causes severe diarrhea in infants and neonatal animals with high morbidity and mortality (Němejc *et al.,* 2012; Li *et al*., 2019). The animals infected with intestinal parasites were commonly found of weight loss and growth retardation, which results in huge economic losses in the livestock industry (Chikweto *et al.,* 2018). *E. bieneusi* is unicellular enteric pathogen leading to gastrointestinal infection in people and animal (Liu *et al*., 2021). Besides causing diarrhea and other diseases in cattle and pigs, growing evidences suggest that *E. bieneusi* is a potential threat to HIV/AIDS and other immunodeficient people with high morbidity and mortality (Liu *et al*., 2021; Li *et al*., 2022). The infection of *G. duodenalis* in children is generally recognized as an important health issue as it usually leads to serious nutritional deficiency, retarded growth, and even

cognitive impairments (Lee *et al*., 2020). In animals, *G. duodenalis* usually present clinical symptom of diarrhea, dehydration, vomiting and weight loss, which ultimately result in large economic impacts in the industry (Wang *et al*., 2017). The infection of protozoa *Blastocystis sp.* commonly led to intestinal diseases like irritable bowel syndrome, diarrhea, nausea, vomiting, abdominal pain and inflammatory bowel disease (Wang *et al*., 2021; Li *et al*., 2022; Masuda *et al.*, 2022). This parasite can also be regarded as an indicator of intestinal health (Rossen *et al.*, 2015).

The plateau symbolic long-haired bovine ruminant yak (*Bos grunniens*) with gigantic body is typically found in over 3000 m regions in the Himalayan plateau areas of the South-Central Asia (Li *et al*., 2020a). Among the world's total 15 million yaks' population, approximately 90% of them are living on the Chinese plateau regions (Li *et al*., 2020a). Yaks are used for local transportation and they give valuable products including dung, fur, wool, milk and meat thus making this an important animal to the local aborigines (Li *et al*., 2020a). Tibetan pig is a local cold plateau swine species mostly found in South-East of Tibetan Plateau, China (Li *et al*., 2016). This animal long-time inhibited in the rugged environment with lower temperature, insufficient oxygen and food scarcity etc., which contribute to the formation of special characteristics like disease resistance, being adaptive to hypoxia, lean carcass with high-quality nutritious food products, etc. (Li *et al*., 2016; Wang *et al*., 2020). Therefore, any infectious disease in the economically important yaks and Tibetan pigs may not only affect people's lives quality, but also potentially threaten public health.

Though, there were previous studies reported intestinal parasites in yaks and Tibetan pigs (Zheng *et al*., 2019; Li *et al*., 2020b; Wu *et al*., 2020), most of them were performed with samples collected during summer. During winter especially in the snow season, plateau animals experience starvation and bitter cold, which may increase the opportunity for animals to be infected by pathogens. Therefore, we carried out this study to investigate four commonly known parasites infection in two economically important food animals on the plateau during winter season.

MATERIALS AND METHODS

Fecal samples: Fresh fecal samples (n=100) were obtained in the November to March from yaks (n=40) in Qinghai and Tibetan pigs (n=60) in Tibetan plateau, respectively in December 2021 (Table 1). Samples were kept in dry cold ice and transported to the laboratory of Traditional Chinese Veterinary Medicine, Nanjing Agricultural University for further analysis.

Ethics statement: Fresh fecal samples from animals were collected under the guidelines and approval of LARC (laboratory animals research centre) of Jiangsu, Qinghai and Tibet, and the ethics committee of Nanjing Agricultural University (NJAU.No20220305025).

DNA extraction, Gene amplification and DNA electrophoresis: Total genomic DNA (gDNA) from all samples was collected by employing commercial DNA extraction kit (Universal Genomic DNA Extraction Kit, Item number: D2100, Solarbio Science & Technology Co., Ltd). The eluted yak and Tibetan pig DNA samples were stored at -20°C prior to PCR analysis.

Nested PCR (nPCR) was employed for the gene amplification of 18S SSU rRNA of *Cryptosporidium sp.* and *G. duodenalis*, ITS gene of *E. bieneusi*, respectively. PCR for *Blastocystis sp*. was performed to amplify *Blastocystis sp.* 18S gene sequence from yaks and Tibetan pigs. The primer pairs used in the current study were designed by following the previous studies, shown in Table 2 (Sulaiman *et al*., 2004; Xue *et al*., 2020; Wang *et al*., 2020; Liu *et al*., 2021). For each amplification reaction, the PCR reaction mixture was composed of 25µL PCR Buffer (2×), 10µL dNTPs (2.5 mM), 2µL DNA, 1µL Taq, 3.0µL of primer pairs, and autoclaved distilled water was added up to a 50µL reaction volume. The PCR amplification contained 35 PCR cycles with 95°C for 35s, Tm (showed in Table 2) for 50 s, and 72°C for 60 s in each cycle after an initial hot start at 95°C for 3 min and ending with 72°C for 5 min. After that, all the achieved amplified products were examined through 2.0% agarose gel electrophoresis. Then all of the positive PCR amplified bands with expected size were purified by using PureLink® Quick Gel Extraction Kit (Catalog numbers K2100-12, Thermo Fisher Scientific Inc.) as suggested by the manufacturer's explanatory memorandum.

Sequencing analysis and Phylogenetic analysis: Purified PCR samples were further subjected for bidirectional gene sequencing via ABI 3730 DNA analyzer at Tsingke Biotechnology Co., Ltd. (Nanjing, China). Multiple sequence alignments of 18S SSU rRNA genes were preformed between Tibetan pig isolate and references genes of SSU rRNA of *Cryptosporidium sp*. available at NCBI database by piloting Lasergene (V7.1). The current used reference strains were *Cryptosporidium sp*. pig genotype II isolate SHAO48 (JQ936498.1), *Cryptosporidium sp.* pig genotype II isolate QL02 (KU668898.1), *Cryptosporidium sp*. isolate Chikkaballapur (OL691173.1), *Cryptosporidium sp*. pig genotype II isolate JD-1 (JF710246.1), *Cryptosporidium parvum* isolate KSU-1 (AF308600.1), *Cryptosporidium bovis* isolate DH150 (OL912801.1), *Cryptosporidium ryanae* isolate DH68 (OL912799.1), *Cryptosporidium baileyi* (L19068.1), *Cryptosporidium hominis* strain CHZF1 (EF570921.1), *Toxoplasma gondii* strain MAS 5S (AF158095.1) (out group). All of the alignment gaps and missing position were eliminated as previous study suggested (Lam *et al*., 2021), then multiple sequence alignment results were further processed for phylogenetic relationship analysis.

The phylogenetic relationship analysis between 18S SSU rRNA gene of *Cryptosporidium sp.* Tibetan pig isolate and nine reference genes (JQ936498.1, KU668898.1, JF710246.1, AF308600.1, OL912801.1, OL912799.1, L19068.1, EF570921.1 and AF158095.1) were carried out by using MEGA (Version 6.0, http://www.megasoftware.net/) through neighbor-joining (NJ) methods (Li *et al*., 2022) and bootstrap analysis was performed using Kimura 2-parameter model. All the stability of branches used in the present phylogenetic tree were assessed after bootstrapping replicates (n= 1000).

Table 1: Prevalence of *Cryptosporidium sp*., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis sp*. infection in free-ranged yaks and tibetan pigs on the plateau

	Yaks		Tibetan pigs	
Parasites	No. tested/No. positive	Prevalence (%)	No. tested/No. positive	Prevalence (%)
Cryptosporidium sp.	4/40	10.0%	11/60	18.3%
Giardia duodenalis	3/40[a]	7.5%	0/60	0
Enterocytozoon bieneusi	5/40	12.5%	34/60[b]	56.7%
Blastocystis sp.	3/40	7.5%	33/60[c]	50.0%
Cryptosporidium sp. + Giardia duodenalis	3/40[d]	7.5%	0/60	0
Cryptosporidium sp. + Enterocytozoon bieneusi	2/40	5.0%	10/60	16.7%
Cryptosporidium sp. + Blastocystis sp.	1/40	2.5%	5/60	8.3%
Giardia duodenalis + Enterocytozoon bieneusi	2/40	5.0%	0/60	0
Giardia duodenalis + Blastocystis sp.	1/40	2.5%	0/60	0
Enterocytozoon bieneusi + Blastocystis sp.	3/40	7.5%	16/60[e]	26.7%
Cryptosporidium sp. + Giardia duodenalis + Enterocytozoon bieneusi	2/40	5.0%	0/60	0
Cryptosporidium sp. + Giardia duodenalis + Blastocystis sp.	1/40	2.5%	0/60	0
Giardia duodenalis + Enterocytozoon bieneusi + Blastocystis sp.	1/40	2.5%	0/60	0
Cryptosporidium sp. + Enterocytozoon bieneusi + Blastocystis sp.	1/40	2.5%	5/60	8.3%
Cryptosporidium sp. + Giardia duodenalis + Enterocytozoon bieneusi + Blastocystis sp.	1/40	2.5%	0/60	0

[a] significant difference was found in the prevalence of *Giardia duodenalis* between yaks and Tibetan pigs (p=0.031<0.05, χ^2=4.639): [b]Significant difference was found in the prevalence of *Enterocytozoon bieneusi* between yaks and Tibetan pigs (P<0.001, χ^2=19.679): [c]Significant difference was found in the prevalence of *Blastocystis sp*. between yaks and Tibetan pigs (P<0.001, χ^2=23.503): [d]Significant difference was found in the prevalence of *Cryptosporidium sp. + Giardia duodenalis* between yaks and Tibetan pigs (p=0.031<0.05, χ^2=4.639): [e]Significant difference was found in the prevalence of *Enterocytozoon bieneusi + Blastocystis sp*. between yaks and Tibetan pigs (p=0.017<0.05, χ^2=5.729).

Table 2: Primary pairs used in the current study

Gene	Primer	Sequence (5'-3')	Annealing temperature (◦C)	Fragment length (bp)	Reference
SSU rRNA	CP-SSU-F2	TTCTAGAGCTAATACATGCG	52	830	(Xue *et al.*, 2020)
	CP-SSU-R2	CCCATTTCCTTCGAAACAGGA			
	CP-SSU-F3	GGAAGGGTTGTATTTATTAGATAAAG	51	800	
	CP-SSU-R4	CTCATAAGGTGCTGAAGGAGTA			
SSU rRNA	GD-Gia2029	AAGTGTGGTGCAGACGGACTC	60	497	(Wang *et al.*, 2020)
	GD-Gia2150c	CTGCTGCCGTCCTTGGATGT			
	GD-RH11	CATCCGGTCGATCCTGCC	63	292	
	GD-RH4	AGTCGAACCCTGATTCTCCGCCCAGG			
ITS	EB-AL4037-F1	GATGGTCATAGGGATGAAGAGCTT	53	410	(Sulaiman *et al.*, 2004)
	EB-AL4039-R1	TATGCTTAAGTCCAGGGAG			
	EB-AL4038-F2	AGGGATGAAGAGCTTCGGCTCTG	55	392	
	EB-AL4040-R2	AGTGATCCTGTATTAGGGATATT			
SSU rRNA	B-RD5--F	ATCTGGTTGATCCTGCCAGT	54	600	(Liu *et al.*, 2021)
	B-BhRDr-R	GAGCTTTTTAACTGCAACAACG			

Statistical analysis: Statistical difference in prevalence of different parasites among animals were examined by the chi-square test utilizing IBM SPSS Statistics (SPSS 24.0). P values less than 0.05 were accepted as statistically significant.

RESULTS

Prevalence of four parasite species in yaks and Tibetan pigs: From all the fecal samples from yaks, a total of 4 (10.0%) were found to be positive of *Cryptosporidium sp.* 3 (7.5%) of *G. duodenalis*, 5 (12.5%) *E. bieneusi* and 3 (7.5%) *Blastocystis sp.* The prevalence of two species mixed infection was ranging 2.5% to 7.5%. The prevalence of three species mixed infection was ranging2.5% to 5.0%. The prevalence of co-infection of *Cryptosporidium sp. + G. duodenalis + E. bieneusi + Blastocystis sp.* was 2.5% in yaks on the plateau (Fig 1, Table 2).

In Tibetan pigs, the prevalence of *Cryptosporidium sp.* was 18.3%. None of the samples was detected to be positive to *G. duodenalis*, which was obviously lower than prevalence in yaks (P<0.05). The prevalence of *E. bieneusi* and *Blastocystis sp.* was 56.7% and 50% in yaks, which were both significantly higher than that in yaks (P<0.001). The prevalence of two species mixed infection ranged from 0 to 26.7%. Also, statistically significant difference was found for *E. bieneusi + Blastocystis sp.* prevalence in Tibetan pigs than yaks (P<0.05). The prevalence of co-infection of *Cryptosporidium sp. + E. bieneusi + Blastocystis sp.* in Tibetan pigs was 8.3% (Fig 1, Table 1). The prevalence of both *E. bieneusi* and *Blastocystis sp.* in Tibetan pigs was note worthily higher than *Cryptosporidium sp*. (P<0.001) and *G. duodenalis* (P<0.001). The prevalence of *G. duodenalis* was significantly lower than *Cryptosporidium sp.* (P<0.05), *E. bieneusi* (P<0.001) and *Blastocystis sp.* (P<0.001), respectively (Fig 2a). The prevalence of mixed infection of *Cryptosporidium sp. + Giardia duodenalis, Giardia duodenalis + Enterocytozoon bieneusi* and *Giardia duodenalis + Blastocystis sp.* were all obviously lower than *Cryptosporidium sp. + E. bieneusi* (P<0.01), *Cryptosporidium sp. + Blastocystis sp.* (P<0.05) and *E. bieneusi + Blastocystis sp.* (P<0.001), respectively (Fig 2b). The prevalence of co-infection of *Cryptosporidium sp. + E. bieneusi + Blastocystis sp.* Was significantly higher than *Cryptosporidium sp. + G. duodenalis + E. bieneusi* (P<0.05), *Cryptosporidium sp. + G. duodenalis + Blastocystis sp.* (P<0.05) and *G. duodenalis + E. bieneusi + Blastocystis sp.* (P<0.05), respectively (Fig 2c).

Multiple alignments and phylogenetic analysis of different *Cryptosporidium sp*. Isolates: Only one positive PCR product was successfully sequenced and deposited in NCBI database with GeneBank accession

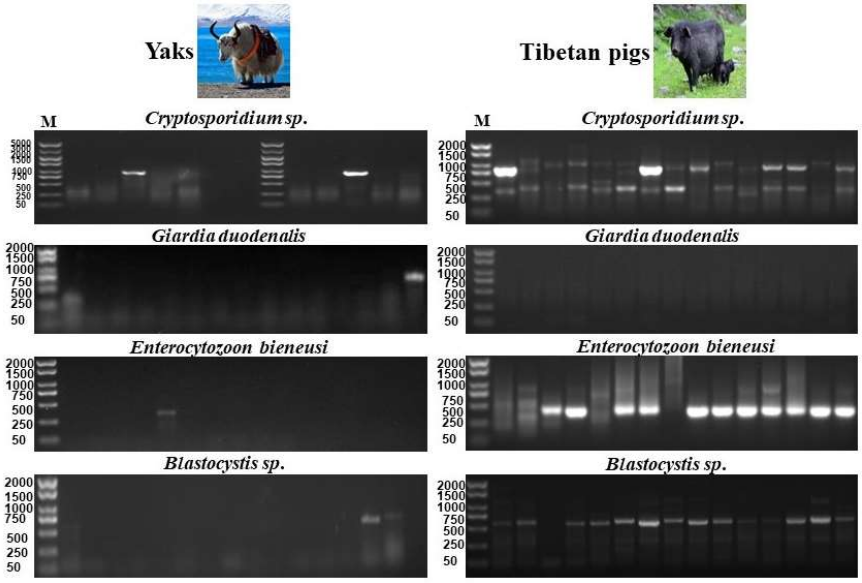

Fig. 1: PCR amplification results of SSU RRNA and its genes from different parasites. marker ladders from upside to downside were 2000, 1500, 1000, 750, 500, 250 and 50 bp or 5000, 3000, 2000, 1500, 1000, 750, 500, 250, 50 bp.

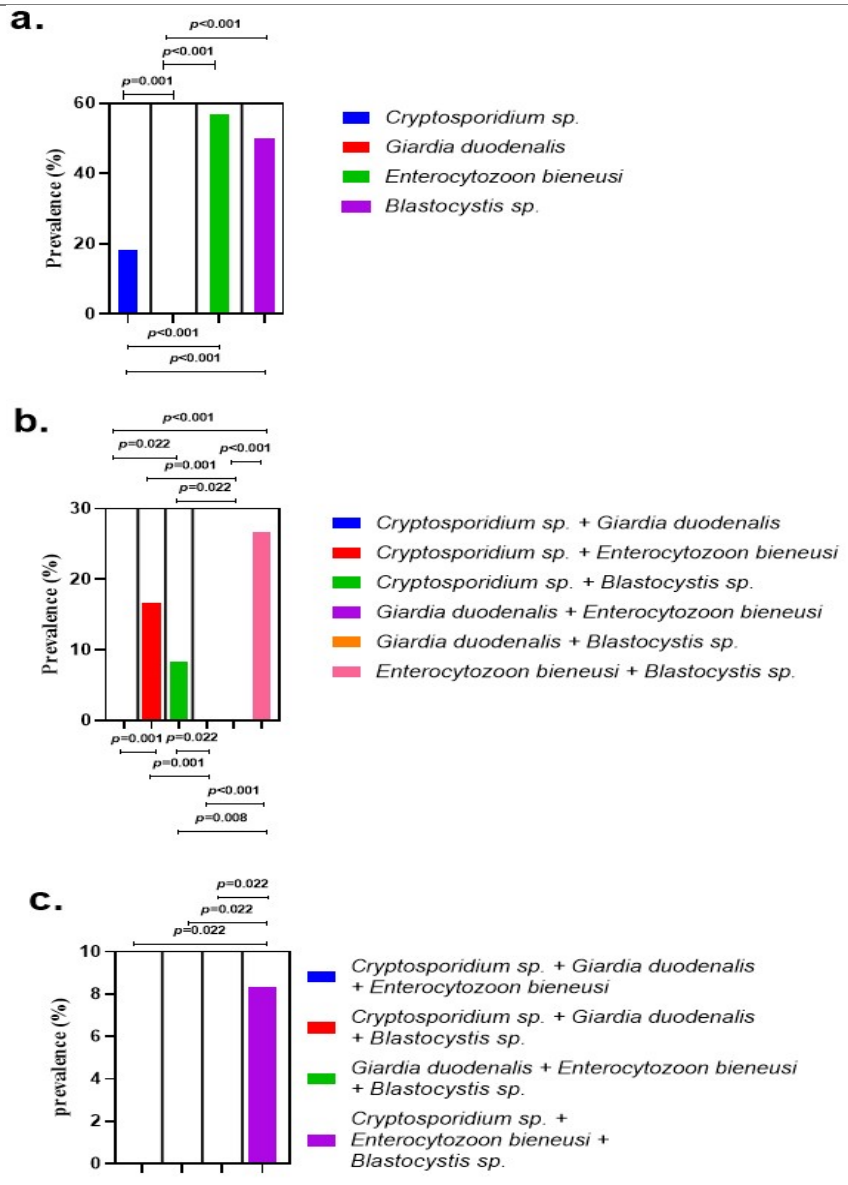

Fig. 2: Comparing the prevalence of parasites infection in Tibetan pigs. a: single species infection, b: two species mixed infections, c: three species mixed infections.

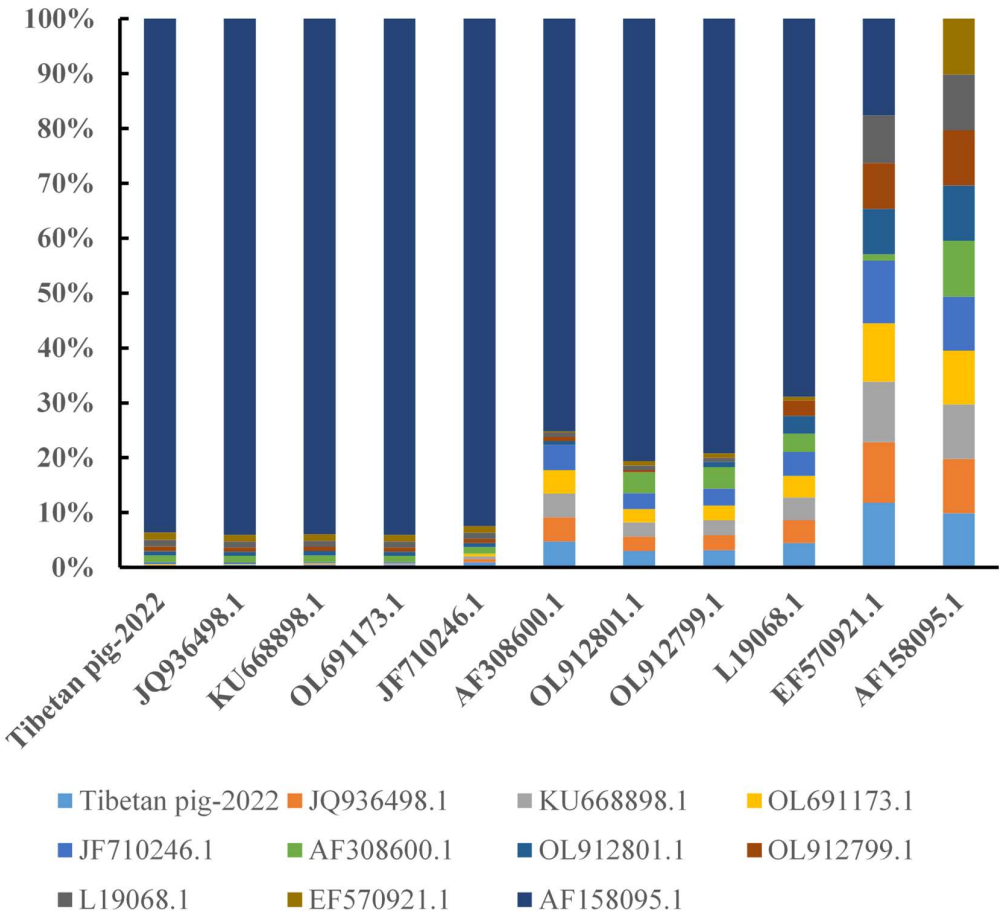

Fig. 3: Multiple sequence alignments analysis of *Cryptosporidium sp*. Tibetan isolate with reference strains.

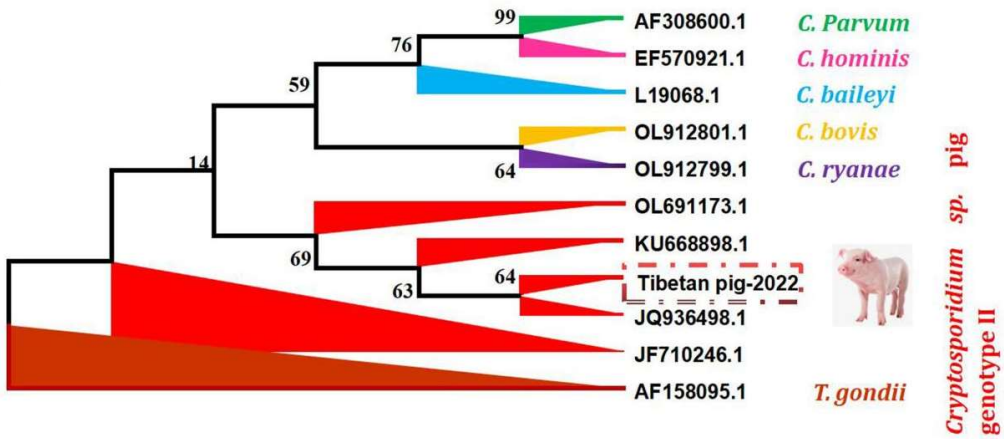

Fig. 4: The phylogenetic relationships of SSU rRNA gene between *Cryptosporidium sp*. sequence derived from Tibetan pigs and reference sequences by employing a Neighbor-Joining (NJ) methods via Kimura two-parameter analysis. The number of nodes indicates the bootstrap values. Bootstrap values > 50% from 1000 replicates, shown on the nodes.

number of OM149377. Multiple alignments analysis found that the current *Cryptosporidium sp.* Tibetan pig isolate was highly homologous to *Cryptosporidium sp.* Pig genotype II isolate from Zhejiang, China (99.8%, JQ936498.1), wild boar in Sichuan, China (99.8%, KU668898.1), Karnataka, India (99.8%, OL691173.1) and Shanghai, China (99.8%, JF710246.1) (Fig 3). Phylogenetic relationships among *Cryptosporidium sp.* Tibetan pig isolates and reference isolates analysis showed that the present pig strain was most similar to *Cryptosporidium sp.* Pig genotype II isolate from Zhejiang, China (99.8%, JQ936498.1) (Fig 4).

DISCUSSION

Cattle and pigs are important food animals, which provide people nutritious products. However, livestock are usually acting as reservoirs for infectious

microorganisms, which may potentially share pathogens to human beings (Lee *et al*., 2020; Wang *et al*., 2020Li *et al*., 2022).

The S*Cryptosporidium sp., G. duodenalis, E. bieneusi* and *Blastocystis sp.* have been detected and reported world-wide (Fiuza *et al*., 2015; Lee *et al*., 2020; Wang *et al*., 2020; Li *et al*., 2022). In the current study, the prevalence of *G. duodenalis* in Tibetan pigs was 0, which is lower than pigs detected in Korea (14.8%) (Lee *et al*., 2020), Shaanxi, China (8%) (Wang *et al*., 2017), Taiwan, China (4.26%) (Lam *et al*., 2021). The prevalence of *G. duodenalis* in yaks was 7.5%, which is higher than yellow cattle in Tibet, China (3.8%) (Wu *et al*., 2020), lower than cattle in Yunnan, China (10.49) (Liu *et al*., 2021), Taiwan, China (19.87%) (Lam *et al*., 2021). The prevalence of *E. bieneusi* in Tibetan pigs was 56.7%, which is higher than pigs in Ningbo, China (25.0%) (Liu *et al*., 2021), Hainan, China (48.8%) (Zhou *et al*., 2020), lower than pigs in Brazil (59.3%) (Fiuza *et al.*, 2015). The prevalence of *E. bieneusi* in yaks was 12.5%, which is higher than the prevalence in cattle in Heilongjiang, China (7.09%) (Xue *et al*., 2020), Jiangxi, China (5.4%) (Li *et al*., 2022), the United States (9.5%) (Sulaiman *et al.,* 2004), Bangladesh (7.9%) (Karim *et al*., 2021), yellow cattle in Tibet, China (2.5%) (Wu *et al.*, 2020). The prevalence of *Cryptosporidium sp.* in pigs was 18.30% in plateau, which is in-line with pigs in Zhejiang, China (17.0%) (Yin *et al*., 2011), higher than pigs reported in Ningbo, China (0.9%) (Liu *et al*., 2021), Shaanxi, China (3.3%) (Lin *et al*., 2015), Henan, China (1.2%) (Zheng *et al*., 2019), Tibetan pigs in Tibet, China (0.49) (Zheng *et al*., 2019). The prevalence of *Cryptosporidium sp.* in yaks was 10.0%, which is higher than cattle in Heilongjiang, China (6.38%) (Xue *et al*., 2020), yaks in Tibet, China (1.3%) (Li *et al*., 2020b), yellow cattle in Tibet, China (0.7%) (Wu *et al*., 2020), and cattle in Yunnan, China (0.77%) (Liang *et al*., 2021). The prevalence of in *Blastocystis sp.* yaks was 7.5%, which is lower than cattle in Jiangxi, China (54.9%) (Li *et al*., 2022), higher than cattle in China (2.11%) (Wang *et al*., 2021). The prevalence of in *Blastocystis sp.* Tibetan pigs was 50.0%, which is higher than pigs in China (31.4%) (Wang *et al*., 2021) and Slovakia (12%) (Danišová and Valenčáková, 2021). The prevalence difference between the current results and previous studies may be because of different climate, geographical location, sample numbers, and animal density, etc. (Li *et al*., 2020a). Previous studies reported multiple infections in livestock, however seldom of them documented the mixed infections (Xue *et al*., 2020; Wu *et al*., 2020; Wang *et al*., 2020). Here, the co-infection of two to four parasitic pathogens was found in present study with significant difference.

Previously, *C. parvum*, *C. suis*, *C. scrofarum* and pig genotype II were found in pigs or wild boars (Liu *et al*., 2021; Lin *et al*., 2015). However, here we only achieved one positive sample sequence and revealed it to be *Cryptosporidium sp.* pig genotype II with highest similarity to strain derived from Zhejiang, China (99.8%, JQ936498.1). The infected yaks and pigs could potentially transmit these infectious parasites to other plateau animals and herdsmen, as these pathogens potentially transmit by fecal-oral route via polluted water and food (Li *et al*., 2022; Karim *et al*., 2021; Liang *et al*., 2021), as previously *Cryptosporidium sp.* and *G. duodenalis* in vegetables, slaughterhouse, sewage and river waters were found in plateau (Ma *et al*., 2019; Li *et al*., 2020). Also, consumption of raw vegetables and intaking contaminate water can result in *Cryptosporidium* and/or *Giardia* infection (Li *et al*., 2020). Nowadays, increasing attention has been paid to animal husbandry due to zoonotic diseases (Lam *et al*., 2021). Therefore, it is of great importance to monitor the status of infectious pathogens in farm animals.

Though, previous studies had reported *Cryptosporidium sp., G. duodenalis and E. bieneusi* in yaks and Tibetan pigs (Zheng *et al*., 2019; Li *et al*., 2020b; Wu *et al*., 2020), samples from their study were collected during summer when there is sufficient food for animals. Plateau animals like yaks and Tibetan pigs have to bear insufficient feedings and snowstorm weather during November to next March, which reduces their ability to counter parasitic pathogens (Zhong, 2021). Previously a study found higher prevalence of *G. duodenalis* and *Blastocystis sp.* in cattle in winter in a plateau region in Yunnan, China (Yue, 2021). Therefor our study may provide new insights regarding parasitic pathogens infection in plateau animals.

Conclusions: It was revealed that important parasitic infection in yaks and Tibet pigs are prevalent during winter season. *Cryptosporidium sp.* pig genotype II in Tibetan pigs was identified. This study signifies the presence of potentially zoonotic parasitic infection in two economically important food animals during winter season. Findings of the current study will give insight to the prevention and control for these important parasites on the high plateau.

Authors contributions: XSC, JXD and HLD carried out the conceptual and experimental work. XSC, MFAK, ZAB and JGL wrote the first draft of the manuscript. XSC, KM, MMA, NMS, II, JYZ and KL contributed to the writing and review of the manuscript. KL and QXW supervised the study. All authors have approved the manuscript for publication.

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Conflict of interest: The authors state that there are no competing interests.

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