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SHORT COMMUNICATION

Epidemiological Investigation of *Cryptosporidium* Infection in Yaks in Chamdo, China

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A B S T R A C T

Cryptosporidium is a parasitic protozoan that can infect a wide range of animals across different geographical areas. The current incidence of Cryptosporidium infection in yaks in Chamdo remains uncertain. Our objective was to determine the prevalence of Cryptosporidium in the study areas through the use of both serology and molecular inquiry. The ELISA and nested-PCR techniques were utilized to analyze blood (n=691) and fecal samples (n=91) from yaks obtained from various farms over the specified period. The nucleic acid-based analysis focused on the 18S rRNA gene. The ELISA approach indicated that the total prevalence of Cryptosporidium infection was 12.7%. The prevalence of Cryptosporidium in fecal samples, as revealed by nPCR, was 33.0% (30 out of 91). The sequencing of selectively amplified samples revealed the presence of *C. bovis* (n=5) and *C. parvum* (n=2). The study utilized logistic regression analysis to examine the correlation between Cryptosporidium infection and the yak's location (farms), health condition, age, and sex. The findings indicated that age, farm (with varying management practices), and health status were risk factors that influenced the frequency of Cryptosporidium in animals in Chamdo. Overall, the results gained help to develop control methods for Cryptosporidium infections in yaks.

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INTRODUCTION

Cryptosporidium is a zoonotically important intracellular parasite capable of infecting all vertebrates (Zhao *et al*., 2024). It causes diarrhea in calves along with celialgia, puking, naupathia and weight loss (Li *et al*., 2020). In healthy animals this parasite reduces production however, in immunocompromised animals it often leads to death (Zhao *et al*., 2024). *Cryptosporidium* causes serious health concerns to all vertebrates as few treatment methods have been discovered for its cure. Scientists have discovered more than forty known species of *Cryptosporidium* out of which *C. parvum*, *C. bovis*, *C. andersoni* and *C. ryanae* are relatively common in cattle (Li *et al*., 2021).

Chamdo is a farming area in Tibet having 1.54 million cattle out of which are 1.45 million yaks which are considered important ruminants on the Qinghai-Xizang plateau in China as they provide meat, milk and fur to the people living in that area. Moreover, yaks in that area are also used for draft purposes therefore, maintaining their health is of significance importance (Wang *et al*., 2021).

Cattle have been reported to be natural reservoirs of *Cryptosporidium* in Shanxi, Jiangxi and Yunnan (Li *et al*., 2021). However, the prevalence of *Cryptosporidium* infection in yaks at Chamdo, China has not been reported as yet. Keeping in view the importance of *Cryptosporidium* infection in yaks, this study aims to detect the prevalence of *Cryptosporidium* in yaks of Chamdo, China.

MATERIALS AND METHODS

Ethics statement: The current experiment was performed following the procedures laid down by the Ethics Committee of Nanjing Agricultural University (NJAU.No20220520108).

Yak samples: Sample collection was done from yaks of 4 different farms with each farm having free ranged 500 yaks. Farms were located at different geographical locations of Chamdo and the distance between two consecutive farms was not less than 50 kilometers. Moreover, yaks belonged to different age groups (yaks that were 0–1-year-old were consider as un-weaned calves, yaks that were 1–2-year-old were considered as puberty yaks, 2–4-year-old yaks were considered as yaks of latency period and yaks that were more than 4-year-old were considered as adult yaks). Blood (n= 691) and fecal samples (n= 91) were collected from yaks in Chamdo, China. The details of yaks related to difference of geographical location of farm, fecal status (which was considered by checking the consistency of feces either normal or watery), gender and age were recorded. Serum was harvested from blood by centrifuging at 4000×g for 20 min and then stored at −80°C.

Examination of *Cryptosporidium* spp. in yaks: Serums samples were tested for antibodies-against protozoon through bovine *Cryptosporidium spp.* enzyme linked immunosorbent assay (ELISA) kit (Dongge Boye Biotechnology, Beijing, China) as described by Li *et al*. (2020). Results were calculated according to the optical density $(OD)_{450\ nm}$ value and critical value= mean value of negative controls+0.15. Samples that were only having $OD_{450nm} \geq$ critical value were considered as positive. All samples were tested thrice along with positive and negative controls. The value of positive controls ≥ 1.00 and negative controls ≤ 0.15 were set to ensure their validity.

Regarding fecal samples nested PCR (n-PCR) amplification to screen positive samples was performed. The genomic DNA from fecal samples was extracted by stool genomic DNA extraction kit (Beijing Solarbio Science & Technology Co., Ltd, China). The obtained products were utilized for n-PCR amplification targeting 18S rRNA (Qin *et al*., 2024). Agarose gel (1.5%) was used for n-PCR product evaluating and the primers used were 18SiCF2:5′-GACATATCATTCAAGTTTCTGAC C-3′ and 18SiCR2:5′-CTGAAGGAGTAAGGAACAAC C-3′, 18SiCF1:5′-CCTATCAGCTTTAGACGGTAGG-3′ and 18SiCR1:5′-TCTAAGAATTTCACCTCTGACTG-3′.

Sequencing and phylogenetic analysis: *Cryptosporidium*-positive products from yaks were sent to Sangon Biotech (Shanghai, China) for sequencing. Multiple alignments of achieved *Cryptosporidium spp.* sequences with reference 18s rRNA sequences were launched through Mega (Version 6.0). Phylogenetic tree analysis of *Cryptosporidium* isolates of yaks with reference sequences was implemented via Mega using maximum likelihood method whereas, the firmness of branches was evaluated by bootstrapping of 1000 replicates. Moreover, the primers used were 18SiCF1:5′-CCTATCAGCTTTAGACGGTAGG-3′ and 18SiCR1:5′-TCTAAGAATTTCACCTCTGACTG-3′.

Statistical analysis: Multivariable logistic regression model was conducted to screen factors of different farms (having different geographical location), fecal status, gender and age that possibly affected the exposure of *Cryptosporidium spp.* in yaks. Statistically difference was considered at $P<0.05$ and odds-ratio with 95% confidence interval was calculated by Statistical Package for Social Sciences (SPSS for windows version 26.0).

RESULTS AND DISCUSSION

Prevalence of *Cryptosporidium* infection in yaks: In serum samples the prevalence of *Cryptosporidium* infection in yaks was 12.7%. This is higher than that reported in in Iran (5%) (Mahmoudi *et al*., 2021) and Uganda (7.7%) (Witto *et al*., 2021) while, it is lower than Jiangxi, China (24.3%) (Li *et al*., 2021), China (29.9%) (Zhao *et al*., 2024) and Turkey (35.5%) (Yildirima *et al*., 2020). The difference between the results is because of different climatic conditions under which the animals were being reared and difference of diagnostic methods among the said studies (Li *et al*., 2021). In different farms the prevalence ranged from 5.2-22.2%. In heathy and diarrhea animals the prevalence was 8.7% and 39.6% respectively. In male and female animals, the prevalence was found to be 12.2% and 13.1%, respectively. Moreover, the prevalence was 6.2-22.4% in yaks of different age groups (Table 1). Results of logistic regression revealed that difference of age, difference of farm (having different geographical locations), fecal status and year of age were the risk factors affecting prevalence of *Cryptosporidium* in Chamdo yaks. Animals suffering from diarrhea were more positive compared to non-diarrheal animals. Yaks of ages of 1-2 years and 2-4 years had three times higher risk of infection compared to animals that were more than 4 years old (6.2%) whereas, yaks that were 0-1 year of age (22.4%) had four times higher risk of infection compared to older yaks (Fig. 1A-D). The higher prevalence was detected in yaks suffering from diarrhea which is in line with the results reported in cattle of Shanxi (Zhao *et al*., 2024). Prevalence of *Cryptosporidium* in yaks of different ages was 6.2%-22.4% whereas, higher prevalence was found in yaks that were less than four years old especially in 0–1-year-old yaks which is in relation with meta-analysis results that indicate higher prevalence in young yaks (Geng *et al.*, 2021). These results confirm that the fecal status and age are the risk factors of *Cryptosporidium* infection in yaks. No significance was observed among gender regarding the prevalence of *Cryptosporidium* infection which is not in line with the results documented regarding cattle of Western Uganda (Witto *et al*., 2021). However, significance was noticed among male and female yaks at farm D which supports the finding of Witto *et al*. (2021) that gender affect the infection of *Cryptosporidium* (Fig. 2A-C). In fecal samples the prevalence of *Cryptosporidium*-positive samples was 33% (30/91) (95% CI: 23.5-43.6) whereas, in heathy and diarrheal yaks' prevalence was 8.0% (95% CI: 1.0-26.0) and 42.4% (95% CI: 30.3-55.2) respectively (Table 2).

Sequencing and phylogenetic analysis: All *Cryptosporidium*-positive samples were sequenced out of which seven were successfully sequenced. All seven sequences were deposited in NCBI database with accession numbers: PP345956-PP345962. It was found that five of the sequences were 99.28%-99.76% similar to *C. bovis* (OR460649.1) whereas, other two were 99.64%

Table 1: Prevalence of *Cryptosporidium* in serum samples in yaks

Variables	Category	No. tested	No. positive	% (95% CI)	*P*-value	OR (95% CI)
Farm	C	271	14	5.2 (2.9-8.5)		Reference
	B	102	15	14.7 (8.5-23.1)	0.002	3.165 (1.469-6.821)
	D	169	26	15.4 (10.3-21.7)	<0.001	3.338 (1.689-6.596)
	A	149	33	22.2 (15.8-29.7)	<0.001	5.222 (2.693-10.129)
Animal Health Status	Health	600	52	8.7 (6.5-11.2)		Reference
	Diarrhea	91	36	39.6 (29.5-50.4)	<0.001	6.898 (4.153-11.457)
Gender	Male	303	37	12.2 (8.7-16.4)		Reference
	Female	388	51	13.1 (9.9-16.9)	0.715	1.088 (0.692-1.711)
Age	>4 year	356	22	6.2 (3.9-9.2)		Reference
	2-4 year	161	32	19.9 (14.0-26.9)	<0.001	3.766 (2.109-6.724)
	1-2 year	89	15	16.9 (9.8-26.3)	0.001	3.077 (1.524-6.215)
	0-1 year	85	19	22.4 (14.0-32.7)	<0.001	4.371 (2.240-8.526)
Total		691	88	12.7 (10.3-15.5)		

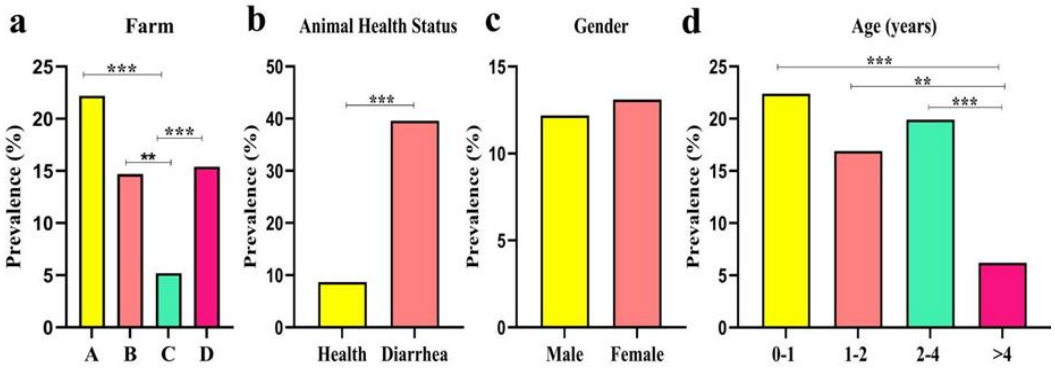

Fig. 1: Prevalence of *Cryptosporidium* in serum samples in yaks in different farms (a), animal health status (b), genders (c) and ages (d).

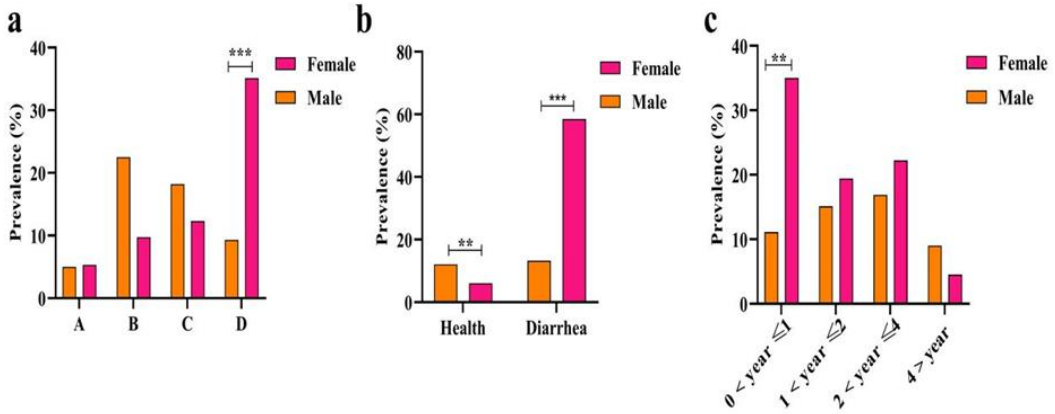

Fig. 2: Prevalence of *Cryptosporidium* in yaks in different genders. (a) Different Farm, (b) Health Status, (c) Age.

Table 2: Prevalence of *Cryptosporidium* in fecal samples in yaks

Yaks	Samples	Positive samples	Prevalence (95% CI)
Healthy	25	2	8.0 (1.0-26.0)
Diarrhea	66	28	42.4 (30.3-55.2)
Total	91	30	33.0 (23.5-43.6)

similar to *C. parvum* (OR460692.1), these results are in agreement with results reported in previous studies (Li *et al*., 2020). Multiple sequences alignment revealed that the distance between current Chamdo isolates, and available *Cryptosporidium* references was 0.004-0.098 whereas, the distance of yak isolates was 0.002-0.016 (Fig. 3). Phylogenetic tree analysis in Fig. 3 describes that PP345956-PP345960 were clustered together with *C. bovis* (OR460649.1, KT922231.1, OQ456122.1, OP861767.1), whereas PP345961 and PP345962 were clustered with *C. parvum* (OR460692.1, OR460699.1, OR350494.1) (Fig. 4). However, we cannot find *C. andersoni* and *C. ryanae*, which is due to the limited fecal samples in current study. *C. parvum* is a highly prevalent zoonotic parasite causing fatal diarrhea in kid animals (Li *et al*., 2021). Positive animals are potential threat to the health of other healthy animals and herdsmen as yaks are inhabiting on the plateau with numerous wild and domestic animals (Mahmoudi *et al*., 2021). These protozoa are mainly transmitted through intake of oocyst in polluted food or water by host (Mahmoudi *et al*., 2021). As animals and native people share water resources on this plateau and grazing yaks provide milk products to local people, therefore increase in the infection of *C. parvum* in this plateau is notable. *C. bovis* was mostly detected in post-weaned calves (Zhao *et al*., 2024) however, it has also been reported in pre-weaned animals (Geng *et al*., 2021). Although no clinical symptoms are observed in the infection of *C. bovis* nevertheless it causes chronic infection that negatively affects feed conversion efficiency and weight gain of animals (Qin *et al*., 2024).

The findings of this study indicate that the occurrence of Cryptosporidium in yaks poses a significant concern in Chamdo, China. The predominant species found in yaks are *Cryptosporidium bovis* and *Cryptosporidium parvum*. The findings of our study provide valuable insights for the development of preventative and control strategies for Cryptosporidiosis infection in yaks of Chamdo, China.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. PP345956		0.002	0.002	0.002	0.003	0.016	0.016	0.002	0.002	0.002	0.002	0.016	0.016	0.016	0.010	0.022	0.019	0.019	0.011	0.009	0.047
2. PP345957	0.005		0.002	0.003	0.003	0.016	0.017	0.002	0.002	0.002	0.002	0.016	0.016	0.016	0.010	0.023	0.019	0.019	0.011	0.010	0.047
3. PP345958	0.005	0.002		0.002	0.004	0.016	0.016	0.002	0.002	0.002	0.002	0.015	0.015	0.015	0.010	0.022	0.018	0.019	0.010	0.009	0.047
4. PP345959	0.004	0.006	0.004		0.003	0.016	0.016	0.002	0.002	0.002	0.002	0.016	0.016	0.016	0.010	0.022	0.018	0.019	0.010	0.010	0.048
5. PP345960	0.009	0.007	0.010	0.007		0.017	0.018	0.003	0.003	0.003	0.003	0.017	0.017	0.017	0.011	0.024	0.020	0.020	0.011	0.011	0.049
6. PP345961	0.067	0.067	0.067	0.068	0.073		0.003	0.016	0.016	0.016	0.016	0.002	0.002	0.002	0.016	0.030	0.026	0.026	0.009	0.013	0.053
7. PP345962	0.071	0.071	0.068	0.070	0.077	0.006		0.016	0.016	0.016	0.016	0.002	0.002	0.002	0.016	0.031	0.026	0.026	0.009	0.013	0.054
8. OR460649.1	0.004	0.005	0.002	0.002	0.007	0.068	0.070		0.000	0.000	0.000	0.016	0.016	0.016	0.009	0.022	0.018	0.018	0.010	0.009	0.047
9. KT922231.1	0.004	0.005	0.002	0.002	0.007	0.068	0.070	0.000		0.000	0.000	0.016	0.016	0.016	0.009	0.022	0.018	0.018	0.010	0.009	0.047
10. OQ456122.1	0.004	0.005	0.002	0.002	0.007	0.068	0.070	0.000	0.000		0.000	0.016	0.016	0.016	0.009	0.022	0.018	0.018	0.010	0.009	0.047
11. OP861767.1	0.004	0.005	0.002	0.002	0.007	0.068	0.070	0.000	0.000	0.000		0.016	0.016	0.016	0.009	0.022	0.018	0.018	0.010	0.009	0.047
12. OR460692.1	0.068	0.068	0.066	0.067	0.074	0.004	0.002	0.067	0.067	0.067	0.067		0.000	0.000	0.015	0.030	0.025	0.026	0.008	0.012	0.053
13. OR460699.1	0.068	0.068	0.066	0.067	0.074	0.004	0.002	0.067	0.067	0.067	0.067	0.000		0.000	0.015	0.030	0.025	0.026	0.008	0.012	0.053
14. OR350494.1	0.068	0.068	0.066	0.067	0.074	0.004	0.002	0.067	0.067	0.067	0.067	0.000	0.000		0.015	0.030	0.025	0.026	0.008	0.012	0.053
15. L19068.1	0.039	0.040	0.038	0.038	0.042	0.068	0.070	0.035	0.035	0.035	0.035	0.067	0.067	0.067		0.019	0.014	0.015	0.010	0.009	0.042
16. EU162754.1	0.098	0.099	0.098	0.098	0.102	0.130	0.133	0.097	0.097	0.097	0.097	0.130	0.130	0.130	0.080		0.013	0.013	0.024	0.023	0.040
17. L19069.1	0.082	0.083	0.080	0.080	0.084	0.112	0.113	0.078	0.078	0.078	0.078	0.110	0.110	0.110	0.060	0.051		0.002	0.018	0.019	0.040
18. KF826312.1	0.084	0.085	0.082	0.083	0.087	0.113	0.115	0.080	0.080	0.080	0.080	0.112	0.112	0.112	0.063	0.053	0.002		0.019	0.020	0.040
19. AF115378.1	0.043	0.044	0.042	0.042	0.046	0.037	0.035	0.039	0.039	0.039	0.039	0.033	0.033	0.033	0.040	0.103	0.079	0.082		0.007	0.044
20. AF112576.1	0.038	0.042	0.039	0.039	0.043	0.054	0.055	0.036	0.036	0.036	0.036	0.052	0.052	0.052	0.038	0.097	0.081	0.083	0.026		0.045
21. L24381.1	0.190	0.190	0.188	0.191	0.195	0.207	0.208	0.188	0.188	0.188	0.188	0.205	0.205	0.205	0.173	0.166	0.164	0.166	0.179	0.180	

Fig. 3: Analysis of *Cryptosporidium spp*. yak isolates with reference sequences.

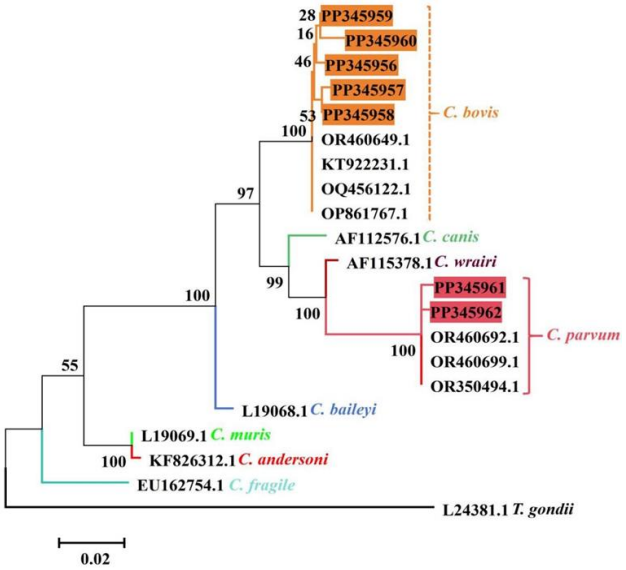

Fig. 4: Phylogenetic tree of *Cryptosporidium spp*. isolates using 18S rRNA gene sequences.

Author contributions: SP, CX and KL carried out the conceptual and experimental work. KL wrote the first draft of the manuscript. PS, CX, MUS, WB, AI and KL contributed to the writing and review of the manuscript. KL supervised the study. All authors have approved the manuscript for publication.

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Conflicts of Interest: None.

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