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EXPLORING THE POTENTIAL PARASITIC PATHOGENS CAUSING DIARRHEAL DEATH TO YAK CALVES WITH BLOODY EXCREMENT THROUGH HIGH-THROUGHPUT SEQUENCING

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ABSTRACT

Yaks are of great importance on the high plateau. However, a serious diarrhea disease has emerged in the past few years causing a catastrophic threat to the yak industry. The current study was carried out to explore the parasitic pathogens in involved in diarrheal death of yaks with bloody excrement from Sichuan, China. A total of 12 fresh fecal samples were obtained from 6 healthy yak calves (H group), and 6 yak calves that died from acute bloody diarrheal (B group). High-throughput sequencing was utilized to compare the parasite burden between the H and B yak groups. At phylum level, *Amoebozoa* (P<0.0001), *Apicomplexa* (P<0.0001) and *Blastocysta* (P<0.0001) was discovered to be obviously higher in B yaks, respectively. On the other hand, *Loukozoa* (P<0.0001) and *Parabasalia* (P<0.0001) were both found to be significantly higher in H yaks. At genus level, *Entamoeba* (P<0.0001), *Theileria* (P<0.001), *Tetratrichomonas* (P<0.01), *Blastocystis* (P<0.0001), and *Cryptosporidium* (P<0.0001) were found to be predominantly higher in B yaks, while *Simplicimonas* (P<0.0001), *Hypotrichomonas* (P<0.0001), and *Gregarina* (P<0.01) were higher in H yaks. In conclusion, the current research herein revealed the genus of *Entamoeba*, *Theileria*, *Tetratrichomonas*, *Blastocystis*, and *Cryptosporidium* as potential parasitic pathogens causing the serious emerging bloody diarrheal deaths in yaks on plateau. Our present results provide insightful knowledge that can be important in controlling and preventing diarrhea in bovine animals.

***Keywords*:** Parasitic pathogens, 18S, Diarrheal, Yak, Sequencing

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INTRODUCTION

The long-haired symbolic domestic or wild yak (*Bos grunniens*) is a special high cold plateau bovine species mainly found throughout the Qinghai Tibet plateau (QTP) at altitudes above 3000 m (Li et al. 2014, Han et al. 2017, Li et al. 2018a). According to the statistics, there was approximately 15.5 million yaks in the world, and over 90% of those were in China (Li et al. 2018b, Li et al. 2018c). Those large bovine animals are of very high economic importance for the local herdsmen, because they provide milk, meat, dung and wool, as well as serve as a convenient transportation mode on the high plateau (Li et al. 2015, Li et al. 2018c).

Yak diarrhea has been recorded since 1987 in China (Tang et al. 1987). However, in the past few years, serious diarrhea disease outbreaks in yaks have shown a re-surgence, which is now threating the yak industry. Unlike diarrhea in cattle, the pathogens causing yak diarrhea are difficult to be detect because of the challenging environment, and lack to knowledge, among others. Until now, the perniciousness pathogens were still unknown. Thus far, only work to explore yak bacterial and fungal pathogens via high-throughput sequencing has been performed (Han et al. 2017, Li et al., 2018c). Interestingly reports have indicated that over 40% of diarrhea in cattle in China are caused by parasitic infections (Cai and Zhang 2004). However, there is scarce information regarding the parasitic pathogens associated with diarrhea in yaks. Therefore, the current research was carried out to explore the potential parasitic pathogens that may be causing bloody diarrhea deaths in yak calves in Sichuan, China.

MATERIALS AND METHODS

***Ethics statement*:** Samples were collected under the permission of the relevant institutions in P. R. China. Procedures used in this study were employed under the instructions and approval of Laboratory Animals Research Centre of

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Sichuan and Hubei province.

***Fecal collection*:** A total of twelve fresh fecal samples were obtained from 6 healthy black male yak calves (H group), and 6 black male yak calves that had died due to acute bloody diarrhea (B group) in Hongyuan of Sichuan (altitude >3500 m), China. All the animals in each group were about~3 months old, and similar in weight (~35kg). All the fecal samples were shipped to our lab at Huazhong Agricultural University using drikold.

***Total DNA isolation*:** All the fecal samples were preprocessed by utilizing a similar method as previously described (Han et al. 2017, Li et al. 2018a) with minor modifications. Briefly, equal amounts (20 g) of feces were washed five times with ice cold phosphate buffered saline (PBS) and centrifuged at 5000 g/min for 10 minutes, the supernatant was obtained and placed into a new 50 mL sterile tube. The samples were centrifuged again at 5000 g/min for 20 minutes, the supernatant discarded, and the sediments were re-suspended in 30 mL ice cold PBS and centrifuged twice at 4500 g/min for 10 minutes. The supernatant was discarded, and the sediments were used for total genomic DNA (gDNA) isolation. The extraction of gDNA from the preprocessed samples was performed by employing QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The integrities of all the DNA samples were examined by 1.2% agarose gel electrophoresis.

***18S SSU rRNA gene amplification*:** The target V4 region (~420bp) of the 18S SSU rRNA gene was amplified by using the primers of 454F (5′-CCAGCASCYGCGGTAATTCC-3′) and V4r (5′-ACTTTCGTTCTTGATYRA-3′), as previously reported (Li et al., 2018a). The PCR mixture was composed of 18.0 uL autoclaved distilled water, 10 uL PCR Buffer (5×), 2 uL dNTPs (2.5 mM), 10 uL GC Buffer (5×), 5 uL DNA Template, 1 uL Taq E, 2 uL of each forward and reverse primer (working concentration: 10 uM) in a total reaction volume of 50 uL. Each of the 40 PCR cycles consisted of 98°C for 25 sec, 55°C for 45 sec and 72 °C for 45 sec after an initial hot start at 98°C for 5 min and ending with 72°C for 10 min. All the PCR products were fractionated and analyzed using 1.2% agarose gel electrophoresis.

***Library preparation and sequencing*:** Library preparation and sequencing were performed as previously reported (Han et al. 2017, Li et al. 2018c). In brief, TruSeq Nano DNA LT Library Prep Kit (Illumina) was employed for libraries construction for all the samples. The library quality examination and quantitative assay were performed by utilizing Agilent High Sensitivity DNA Kit via Agilent Bioanalyzer and Quant-iT PicoGrenn dsDNA Assay Kit via Promega QuantiFluor according to the manufacturer's specifications, respectively. High-throughput sequencing (HTS) for the 12 samples was carried out using the Illumina MiSeq platform (MiSeq Reagent Kit V3).

***Parasite diversity analysis*:** After sequencing, FLASH (v1.2.7, http://ccb.jhu.edu/software/FLASH/) and QIIME (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/) were piloted to eliminate the questioned sequences (Caporaso et al. 2010, Magoc and Salzberg 2011). Then all the effective sequences were demarcated as different operational taxonomic units (OTUs) by employing UCLUST (Edgar 2010). Ultimately, BLASTn tool against a curated UNITE database was utilized to perform OTUs classification in Phylum and Genus (Koljalg et al. 2013), and none of the parasite OTUs were eliminated based on taxonomy. Comparison of the parasite difference between the H and B yaks groups at Phylum and Genus level was carried out using one-way analysis of variance followed by Tukey's honest test for continuous variables, and the differences were considered statistically significant if $P<0.05$ through the IBM SPSS Statistics 21.0 (SPSS Somers, NY).

RESULTS AND DISCUSSION

In the present study, the classified OTUs in phyla, classes, orders, families and genera levels in H and B yaks were shown in Fig. 1. At phylum level, Amoebozoa ($P<0.0001$), Apicomplexa ($P<0.0001$) and Blastocysta ($P<0.0001$) were discovered to be obviously higher in B yaks, while Loukozoa ($P<0.0001$) and Parabasalia ($P<0.0001$) were found to be significantly higher in H yaks (Fig. 2).

At genus level, Entamoeba ($P<0.0001$), Theileria ($P<0.001$), Tetratrichomonas ($P<0.01$), Blastocystis ($P<0.0001$), and Cryptosporidium ($P<0.0001$) were found to be prominently higher in B yaks group, while Simplicimonas ($P<0.0001$), Hypotrichomonas ($P<0.0001$), and Gregarina ($P<0.01$) was were higher in H yaks group (Fig. 3).

The globally distributed bovine diarrhea disease seriously harms the health of animals, causing tremendous economic losses to the cattle industry (Brar et al. 2017, Han et al. 2017, Ribeiro et al. 2017, Li et al. 2018a). A lot of measures including vaccinations, medications and herd management have been taken with the go of decreasing the economic losses caused by diarrhea, however, the overall effect has been limited (Cho and Yoon 2014, Li et al. 2018a).

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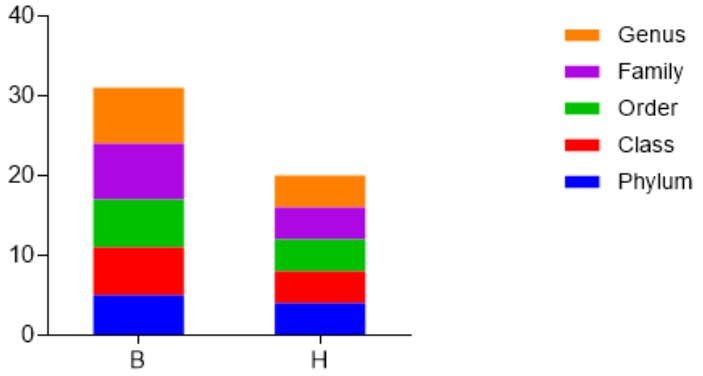

Fig. 1: Statistics of OTUs (Operational Taxonomic Units) classification at Phylum, Class, Order, Family and Genus levels in different yak groups.

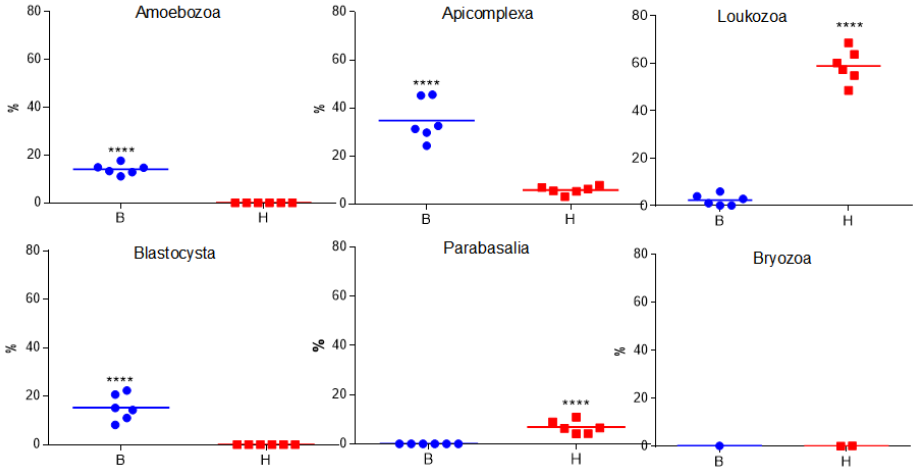

Fig. 2: Comparison of the parasitic diversity at phylum level in different yak groups. **** P<0.0001.

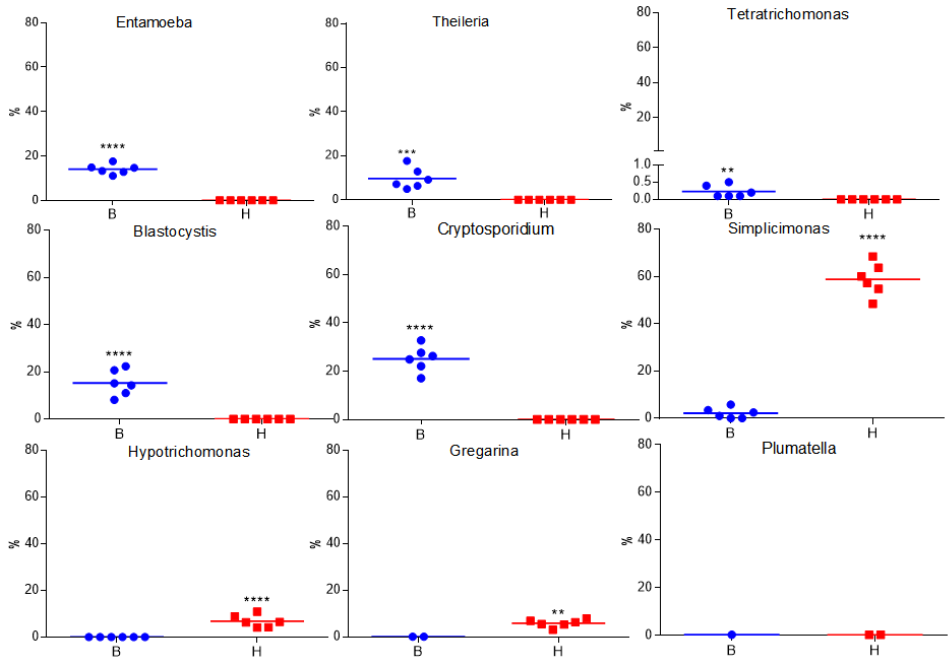

Fig. 3: Comparison of the parasitic diversity at genus level in different yak groups. **** P<0.0001, *** P<0.001, ** P<0.01.

The yak contributes significantly to the economic development in the western China. So, any yak disease may lead to serious economic and cultural difficulties in those remote high plateau regions (Li et al. 2018b). However, the knowledge regarding the emerging diarrhea disease in yaks is rather limited. Previously, HTS was utilized to examine the unicellular eukaryotes and helminths from environmental samples (Cannon et al. 2018).

The present research was preformed HTS to explore the parasitic pathogens causing bloody diarrhea deaths among yaks in Sichuan located on the high plateau. Compared with the H group yaks, Entamoeba was found to be common in B yak group ($P<0.0001$) (Fig 3). Worldwide, *Entamoeba* has been associated with amoebic dysentery (Fonseca et al. 2019), which may indicate that *Entamoeba* is related to the diarrhea in yaks. *Theileria* was uncovered to be conspicuously higher in B yaks ($P<0.001$). *Theileria* has been reported to lead to serious hemolysis in infected animals (Kim et al. 2017), which may reveal the reason for bloody excrement in yak. In comparison to H yaks, the genus *Tetratrichomonas* was obviously higher in B yaks ($P<0.01$), which may indicate that the this parasite genus is related to the diarrhea in yaks, as *Tetratrichomonas* flagellates have been previously shown to cause diarrhea in giant anteate (Ibañez-Escribano et al. 2013).

Compared with the H yaks, *Blastocystis* was uncovered to be remarkably high in B yaks ($P<0.0001$). *Blastocystis* is zoonotic and was found to significantly increase the fever rate in dengue human patients (Greige et al. 2019, Thergarajan et al. 2019). *Blastocystis* has also been reported to be linked to common gastrointestinal illnesses (Greige et al. 2019), which may suggest that *Blastocystis* contribute to the diarrhea in yaks. The genus *Cryptosporidium* is known to commonly cause serious diarrhea in animals and children (Witola et al. 2017, Li et al. 2018b, Li et al. 2019). However, there are limited effective drugs against *Cryptosporidium* (Zhang et al. 2018, Li et al. 2019). This may explain the difficulties associated with controlling the serious emerging diarrhea in yaks. Though genera *Simplicimonas* ($P<0.0001$), *Hypotrichomonas* ($P<0.0001$), and *Gregarina* ($P<0.001$) were found to be higher in the H than in the B group yaks. However, those genera are not been shown to be associated with diarrheal syndromes in aniamls. Even though the unicellular organism *Gregarina* is common in the environment, it infects mainly invertebrates (Rueckert et al. 2017). Further, the genus *Hypotrichomonas* have only been reported to be found in lizards and birds (Céza et al. 2015). To the best of our knowledge, there is no information that associates the genus *Simplicimonas* with diarrhea.

Conclusion: The current research herein reveals the genera of *Entamoeba*, *Theileria*, *Tetratrichomonas*, *Blastocystis*, and *Cryptosporidium* as potential parasitic pathogens causing the serious emerging bloody diarrhea syndrome causing death in yaks on the high cold plateau. The present results provide insightful knowledge that can be important in controlling and preventing diarrhea in yaks and other bovines.

Authors' contributions: KL and JKL conceived and designed experiments. KL performed the experiments. KL, HQL and KM performed statistical analyses of experimental data. KL prepared the draft of the manuscript. KL and MS edited the manuscript. All authors critically revised the manuscript and approved the final version.

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