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Occurrence and molecular characterization of *Cryptosporidium* spp. in yaks (*Bos grunniens*) in China

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ABSTRACT

Compared with dairy and beef cattle, few data are available on the occurrence and distribution of *Cryptosporidium* species in yaks, which live in a very different habitat. In this study, 327 fecal specimens were collected from yaks in 4 counties in Qinghai Province of China and screened for *Cryptosporidium* by nested PCR analysis of the 18S rRNA gene. A total of 98 (30.0%) specimens were positive for *Cryptosporidium*. The occurrence of *Cryptosporidium* varied significantly among age groups; infection rates were 49.3% in weaned calves, 31.7% in yearlings, and 17.4% in adults. PCR products of *all Cryptosporidium*-positive specimens were successfully sequenced, with 56 specimens (57.1%) having *C. bovis*, 33 (33.7%) having *C. ryanae*, 2 (2.0%) having *C. andersoni*, 1 (1.0%) having *C. ubiquitum*, 1 (1.0%) having *C. xiaoi*, 2 (2.0%) having a novel genotype, and 3 (3.1%) having mixed infections of *C. bovis* and *C. ryanae*. There were some age-related differences in the distribution of *Cryptosporidium* species in post-weaned yaks examined. To our knowledge, this is the first report of *C. andersoni*, *C. ubiquitum*, *C. xiaoi* and a novel *Cryptosporidium* genotype in yaks.

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1. Introduction

Cryptosporidium spp. are common parasites of humans, farm animals, and other vertebrates, causing the disease cryptosporidiosis (Fayer, 2010; Plutzer and Karanis, 2009; Xiao, 2010). *Cryptosporidium* oocysts are released in feces by infected hosts and transmitted via direct contact with infected persons or animals or through contaminated food or water (Baldursson and Karanis, 2011; Chalmers and Davies, 2010). Cryptosporidiosis is self-limiting in immunocompetent patients but can be life-threatening in immunocompromised and young individuals (Chalmers and Davies, 2010). Cryptosporidiosis is difficult to control as effective treatment is not widely available and *Cryptosporidium* oocysts are resistant to many environmental conditions and disinfection treatments (Cacciò and Pozio, 2006).

In cattle, cryptosporidiosis is associated with the occurrence of diarrhea, weight loss and delayed growth, and sometimes mortality of calves, leading to economic losses (Anderson, 1998; Singh et al., 2006). *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium andersoni* are the four major *Cryptosporidium* species in dairy cattle. There is an age-related distribution of the four species in cattle in most areas: *C. parvum* predominates in pre-weaned calves, *C. bovis* and

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C. ryanae in post-weaned calves, and *C. andersoni* in adult cattle (Santin, 2013). In addition, contact with pre-weaned calves is a major risk factor for *C. parvum* infections in humans (Chalmers and Giles, 2010).

Yaks (*Bos grunniens*) belong to the genus *Bos*, thus are related to cattle. Yaks living on the Qinghai-Tibetan Plateau of China (about 3000 m above sea level) make up 90% of the global yak population (13 million). Domesticated yaks are usually kept in close contact with local residents, primarily for milk and meat, and as draft animals, raising public health concerns (Liu et al., 2008).

The free-range nature and cold habitat of yak farming suggest that the distribution of *Cryptosporidium* species in these animals could in theory be different from the widely studied dairy cattle. Thus far, few studies have been done to genotype *Cryptosporidium* spp. in yaks. Most cryptosporidiosis studies in yaks have been done by microscopy or enzyme immunoassays in China (Bai et al., 2001; Dou, 2007; Ma et al., 2011; Wang and Liu, 2007; Zhang et al., 2006; Zhou et al., 2009). Only three studies used molecular tools to characterize *Cryptosporidium* spp. in yaks and identified three species: *C. parvum*, *C. bovis*, and *C. ryanae* (Feng et al., 2007; Karanis et al., 2007; Mi et al., 2013). In this study, we used an 18S rRNA-based genotyping tool to characterize *Cryptosporidium* spp. in post-weaned yaks in Qinghai Province of China, and identified the occurrence of *C. andersoni*, a novel *Cryptosporidium* genotype, and two species common in sheep: *Cryptosporidium ubiquitum* and *Cryptosporidium xiaoi*.

2. Materials and methods

2.1. Study area and sample collection

Fresh fecal specimens were collected from May to July 2013 from yaks in Dari, Yushu, Qilian and Chendi Counties in Qinghai Province (31–39°N, 88–103°E), Northwestern China. The average altitude, annual temperature, and annual rainfall of the 4 counties range from 3100 to 4600 m, −1.0 to 8.5 °C, and 360 to 550 mm, respectively. Yaks in the areas were mostly kept outdoor year around, and shared pastures with sheep and wild animals. A total of 327 fecal specimens were collected from yaks of <1 year (weaned calves), 1–2 years (yearlings), and >2 years (adults) of age in this study. No clinical signs were observed in the sampled animals. Specimens were stored in 2.5% potassium dichromate at 4 °C before molecular analysis.

2.2. DNA extraction

DNA was extracted from 500 μl of fecal material after the specimens were washed off potassium dichromate with distilled water by centrifugation at 2000 × *g* for 10 min. The FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) was used for extraction of genomic DNA as previously described (Jiang et al., 2005).

2.3. PCR detection of Cryptosporidium

For the detection of *Cryptosporidium*, a nested PCR protocol was used to amplify an ~587-bp fragment of the 18S rRNA gene as previously described (Ryan et al., 2003). Some PCR-positive specimens were re-analyzed by a nested PCR targeting an ~830 bp fragment of the 18S rRNA (Feng et al., 2007). For both assays, the PCR mixture consisted of 1 μl DNA (primary PCR) or 2 μl primary PCR product (secondary PCR), 3 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Thermo Scientific, Pittsburgh, PA), 1× PCR buffer, 0.2 mM of each dNTP, 200 nM primers and 400 ng/μl of bovine serum albumin (Sigma–Aldrich, St. Louis, MO) in a final volume of 50 μl. Each specimen was analyzed at least twice using DNA of *C. baileyi* as the positive control and reagent water as the negative control. PCR products were examined by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

2.4. Sequence and phylogenetic analysis

All positive secondary PCR products of the 18S rRNA gene were sequenced in both directions with the secondary primers using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences obtained were aligned with each other and published 18S rRNA gene sequences of *Cryptosporidium* spp. using the software ClustalX (http://www.clustal.org/) to determine *Cryptosporidium* species. Phylogenetic relationship of the new *Cryptosporidium* genotype to established species and common genotypes was assessed using the Neighbor-Joining (NJ) analysis implemented in MEGA 5.0 (http://www.megasoftware.net/) based on genetic distances calculated by the Kimura 2-parameter model. The sequence generated from PCR analysis of the ~830 bp fragment of the 18S rRNA gene was used in the phylogenetic analysis. Representative nucleotide sequences (from the ~830 bp PCR product for *C. bovis*, *C. ryanae*, and the novel genotype, and ~587 bp PCR products for other species) generated in the study were deposited in the GenBank database under accession numbers KF971356 and KJ531688-KJ531692.

2.5. Statistical analysis

Data were analyzed using SPSS 19.0 for Windows (SPSS Inc., Chicago, USA). The χ^2 test was used to analyze differences in infection rates among different counties and age groups. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Occurrence of Cryptosporidium spp.

Of the 327 fecal specimens examined, 98 (30.0%) were positive for *Cryptosporidium*. *Cryptosporidium* infection rates in the 4 study locations ranged from 8.0% to 44.7% (Table 1). Higher infection rates were recorded in Dari (44.7%) and Qilian counties (28.0%) than Chengdi (17.3%) and Yushu counties (8.0%). Results of the χ^2 test showed that the difference in infection rates was statistically significant ($P < 0.05$). *Cryptosporidium* infection rates also varied significantly among age groups (Table 2). Weaned