



# The First Case of Diarrhoea in Tibetan Sheep, *Ovis aries*, Caused by *Balantidium coli* in the Qinghai Tibetan Plateau Area, China

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**Abstract:** This study was carried out to determine the pathogen-causing diarrhoea in sheep *Ovis aries* in the Qinghai Tibetan Plateau Area, China. A trophozoite was identified as species of ciliate alveolates infecting the sheep based on morphological characteristics examined by microscope. It was mostly spherical, colourless and transparent, with many vesicles. Macronucleus and contractile vacuoles could not be distinguished. Size of the trophozoite was 80-180 × 70-150 μm and its surface was covered with cilia. Molecular analysis based on sequences of 18S rRNA and ITS genes confirmed the ciliate species as *Balantidium coli*. According to the literature, there have been many epidemiological investigations of *B. coli* infection in pigs, monkeys and humans. To our knowledge, this was the first report of *B. coli* infections in sheep in the Qinghai Tibetan Plateau Area of China, or elsewhere around the world. Importantly, the sheep case was rare but raised our concern that *B. coli* may spread across species and expand its host range.

**Key words:** *Balantidium coli*, sheep, morphological characteristics, molecular method, Qinghai Tibetan Plateau, China

## INTRODUCTION

*Balantidium coli* (*B. coli*, former name, new/novel generic name *Neobalantidium coli*, *N. coli*) is an important zoonotic protozoan parasite, which can infect a wide variety of hosts, including pigs (domestic and wild), humans and non-human primates, rodents, cattle, camels and horses, causing the disease balantidiasis [1]. Balantidiasis is also known as *B. coli* infection and is treated as a neglected tropical zoonotic disease. *B. coli* is parasitic and infects the small intestine, rectum, and caecum of the host. The manifestations of serious infection include severe diarrhoea and other digestive diseases [2]. To our knowledge, pigs are an important reservoir host and humans and other animals can be infected through the faecal-oral route by food and/or water contaminated with *B. coli* [3]. There are some epidemiological reports about the prevalence of *B. coli* infections in pigs [4-6] in China; however, there are no positive data about *B. coli* infections in sheep (goats) except one case in

which *B. coli* trophozoites were found within the lymphatic ducts of the gastric lymph node and the abdominal submucosa of the zoo barbary sheep. Unexpectedly, there were no *B. coli* trophozoites or cysts detected in the faeces [7]. According to the epidemiological report of gastrointestinal protozoan and helminthic infections in cattle, buffaloes, goats and sheep in southern Rajasthan of India, *B. coli* was found only in cattle (10.76%), and not detected in goats and sheep [8].

Therefore, the present study was performed by morphological and molecular methods to identify the species of ciliate alveolates that was rarely found in Tibetan sheep with diarrhoea in the Qinghai Tibetan Plateau Area (QTPA), China. It also raised our concern about this parasite spreading across species and expanding its host range and becoming a zoonotic threat to public health.

## CASE RECORD

One faecal sample from a 2.5-year-old male Tibetan sheep (*Ovis aries*) with diarrhoea was collected and tested in the laboratory. The faecal sample was further processed and purified by the sucrose density gradient centrifugation method. Then, the purified sample was checked microscopically with an optical microscope and captured with a DP 25 digital camera

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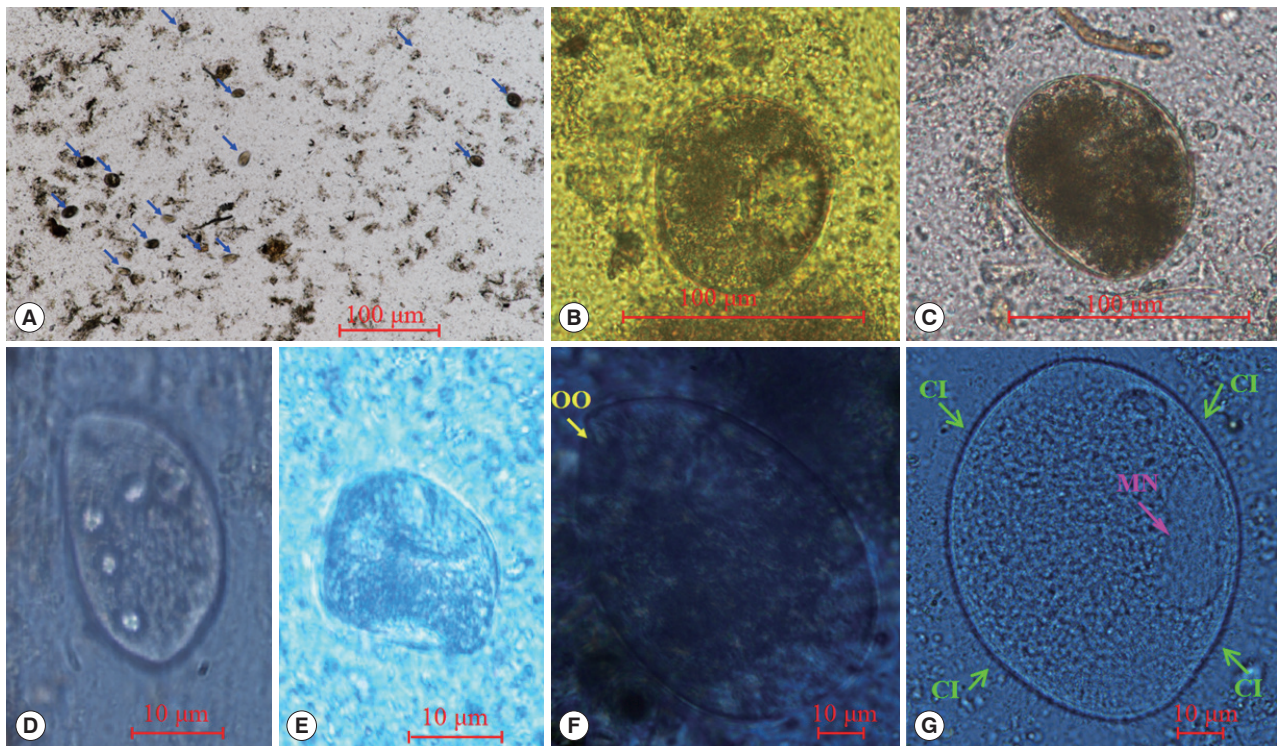
(NIKON, Tokyo, Japan).

The slides were observed with an optical microscope, and it was found that the trophozoites could be identified as the species of ciliate alveolates based on morphological characteristics, which were mostly spherical, colourless and transparent, with many round vesicles. The macronuclear and contractile vacuoles cannot be distinguished. The size of the trophozoite was 80-180 × 70-150 μm. Surface was covered with cilia. The trophozoites spread quickly and the sheep had serious diarrhoea and high-density infections (Fig. 1A). It was also observed the trophozoite was ovoid or spherical in shape, but it became more rounded (Fig. 1B, C). We observed the trophozoites moving very quickly (Fig. 1D, E). They featured an oral opening at the anterior end and were covered with cilia on the whole external surface, and one macro nucleus were present (Fig. 1F, G).

The total genomic DNA was extracted from the stool sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed to amplify the 18S rRNA gene and ITS gene with following primers: *B. coli* 18SF: 5'-TTGTCGCGATAAATCATC-

CA-3' and *B. coli* 18SR: 5'-GCAGGTTAAGGTCTCGTTCG-3' (product=980 bp, Tm=55°C), *B. coli* ITSF: 5'-CCTGCGGAA-GGATCATTAAAC-3' and *B. coli* ITSR: 5'-GCTTTAATTCAGC-GGGTCAT-3' (product=420 bp, Tm=55°C). The amplification products were analyzed by electrophoresis by loading 10 μl of PCR products on a 1.5% agarose gel (Biowest Regular Agarose G-10, manufacturerd to specifications distributed by Gene Company Ltd., Hong Kong, China, origin: Spain) stained with ExRed nucleic acid electrophoresis dye (Beijing Zoman Biotechnology, Beijing, China), Gels were observed and images captured using a WD-9413B gel imaging analysis system (Beijing Liuyi Biotechnology, Beijing, China).

Direct sequencing was performed on the positive PCR products by the Beijing GENEWIZ Company (Beijing, China). The obtained sequences were then aligned with reference sequences available in the GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic relationships of *Balantidium* spp. were constructed by the MEGA 5.05 software with the neighbour-joining method, which was performed using the Kimura 2-parameter model and 2,000 bootstrap replicates.



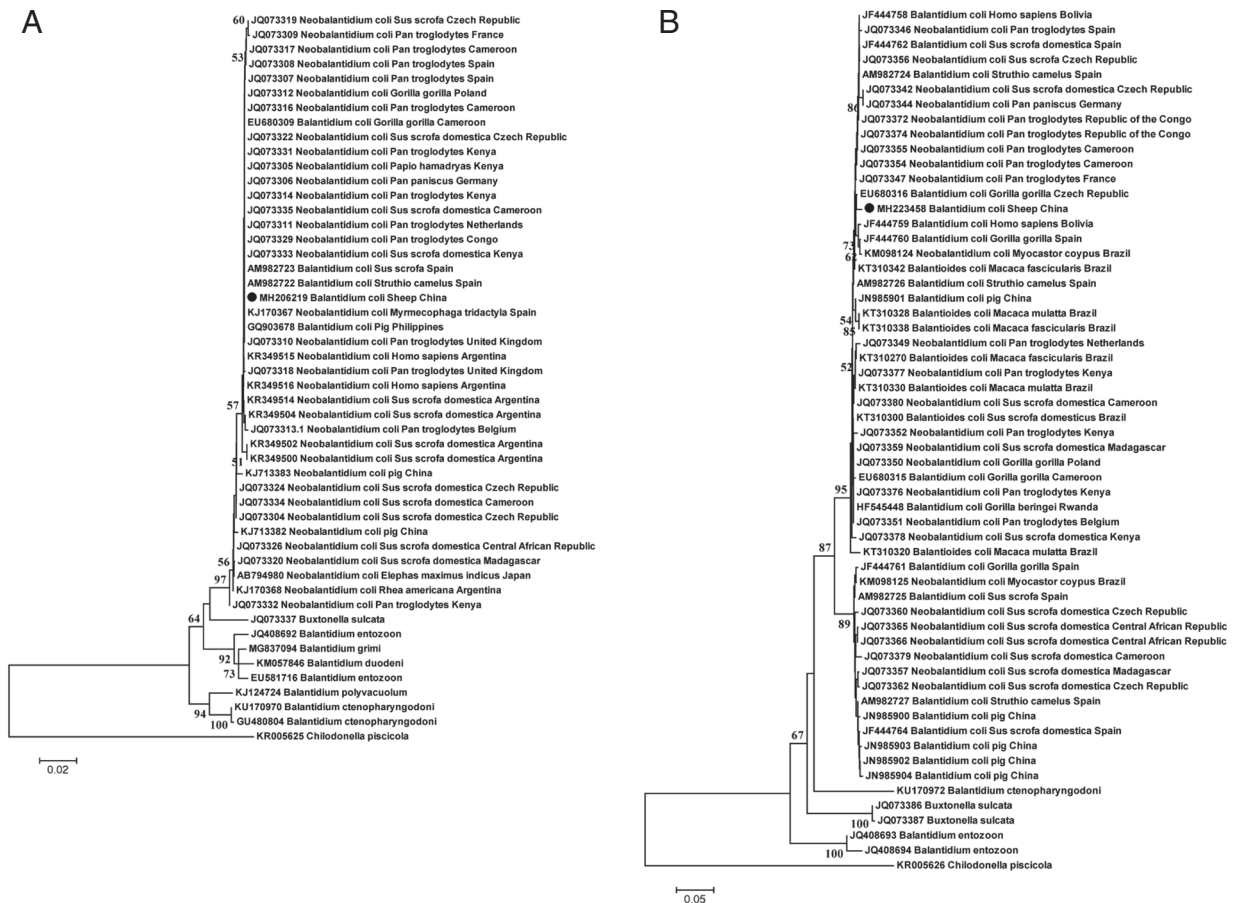
**Fig. 1.** The trophozoite and cyst forms of *Balantidium coli* observed in sheep faecal samples. (A) Trophozoites of *B. coli* (×4) (blue arrows); (B, C) Cysts of *B. coli* (×20); (D, E) The movement state of *B. coli* (×40); (F, G) The oral opening (OO, yellow arrow), cilia (CI, green arrows) and macro nucleus (MN, pink arrow) of the trophozoites of *B. coli* (×100) detected in direct examination of sheep faecal samples.

The species was identified as *B. coli* following the amplification of the fragments of the 18S rRNA gene (GenBank accession No. MH206219) and ITS gene (MH223458), which showed 100% homology to the 18S rRNA gene of *N. coli* (KJ170367/JQ073305/11/12/15/16/22/31/33/35) and *B. coli* (GQ903678/AF029763/EU680309), and 99% homology to the ITS gene of *N. coli* (JQ073346/47/48/50/51/53/54/55/56/59/72/73/74/76/80) and *B. coli* (EU680315/6, HF545448, JF444758, AM982724), when blasted against the NCBI database. Phylogenetic reconstructions with the published *Balantidium* 18S rRNA gene and the ITS gene sequences confirmed that this isolate was closely related to *B. coli* (Fig. 2). Although the phylogenetic trees based on the 18S rRNA gene and ITS gene did not show obvious geographical distribution specificity and host specificity of the *Balantidium* isolates, the isolate from sheep in this study was branched separately from other *Balantidium* isolates located far distances from China (Fig. 2). Conversely, the isolate from sheep

was placed into a sub-clade with primates and domestic pigs from other countries (Fig. 2).

### DISCUSSION

Balantidiasis is an infectious disease commonly occurring in pigs worldwide, but also a serious zoonosis. Pigs are known to be *B. coli* reservoir hosts, which not only causes economic losses in the pig industry, but also exposes humans directly or indirectly resulting in co-infections [1]. *B. coli* has a wide range of hosts, such as humans, primates, wild and domestic pigs, camels and guinea pigs [9]. *B. coli* infections were most prevalent in pigs, with 29.6% prevalence in Swiss pigs [10]; 36.9% prevalence in pigs in Hunan province of subtropical China [3]; a 15.8% prevalence in Cambodian pigs [11]; a 64.7% prevalence in pigs in rural areas of Chungcheongnam-do, Korea [12]; a 10.76% prevalence in cattle in southern Rajasthan, India [8];



**Fig. 2.** Phylogenetic analysis of *Balantidium* based on the sequence of partial 18S rRNA gene (A) or ITS gene (B). *Chilonella piscicola* was used as an outgroup. The black circle represents the *B. coli* in this study. The phylogenetic tree was constructed by using the neighbour-joining method, and the Kimura 2-parameter model with 2,000 bootstrap replicates.

a 2.52% prevalence in cattle from Ludhiana district of Punjab, India [13]; an 18.3% prevalence in donkeys from Lahore, Pakistan [14]; a 52.6% prevalence in captive African great apes [15]; a 62% prevalence in Mole National Park, Ghana [16]; a 64.2% prevalence in bred rhesus monkeys in Guangxi Zhuang Nationality Autonomous Region, southern China [5]; and a 0.7% prevalence in humans in Panama [17]. However, data were lacking on *B. coli* infections in sheep and goats, so the present study is rare but thought-provoking.

In this study, trophozoites were found in the dilute faecal sample from the Tibetan sheep, which had severe diarrhoea and weight loss. For these symptoms in sheep, the bacterial and viral pathogens are usually considered to be the first and chief “culprits”. Parasites are the last to be considered, which results in missing or delaying the best treatment time. In the most cases, the diarrhoea-caused parasites are always parasitic in the intestines of the hosts, and the trophozoites of the parasites are produced in the intestines and excreted in the faeces. Therefore, it is difficult to accurately identify parasitic trophozoites. In this study, we were able to identify the trophozoites as ciliate alveolates morphologically. The size of trophozoite (80-180×70-150 μm) was larger than it was in Schuster and Ramirez-Avila’s study (30×25 μm to 150×120 μm), but it was ovoid or spherical in shape, featured an oral opening at the anterior end and a cytophyge at the posterior end. The trophozoite was covered with cilia on the whole external surface and had one micro and one macro nucleus [1,18,19]. Meanwhile, the cyst form was spherical in shape and the diameter of the cyst (50 to 70 μm) was also larger (40 to 60 μm) [1,18,19]. There were minor differences in the morphological description and comparison analysis, which is rapid and more convenient advantages; however, it is not a very accurate method to identify the exact parasite species. To further determine the exact parasite species, the molecular identification was essential.

The 18S rRNA gene and ITS gene loci sequences were obtained, and the extensive evaluation and polyphyletic analysis of *B. coli* were performed. In the study of Pomajbikova et al. [20], the 2 gene markers SSrDNA and ITS1-5.8SDNA-ITS2 trees all showed the same phylogenetic diversification at the genus level of *B. coli*, which originated from 11 species of captive and wild primates, domestic pigs and wild boars [20]. In the present study, we also analysed our sheep isolate together with the reference sequences of *B. coli* and *N. coli* from humans, nonhuman primates and domestic and wild pigs using the phylogenetic markers: 18S rRNA gene and ITS gene. Our

phylogenetic analyses showed the same phylogenetic character of *B. coli* and *N. coli* gathered into a large clade at the genus level of the tree constructed based on the ITS gene sequences, which appeared to be phylogenetically distinct from *B. entozoon*. In the *B. coli* and *N. coli* clade, the sheep isolate was not clustered with closely related Chinese isolates, but clustered with isolates from other countries, which implied that it may be geographical isolate formed from an adaptive mutation based on the evolutionary analysis of the 18S rRNA gene and ITS gene. Moreover, the sheep isolate was loosely related with humans and non-human primates, which suggested that there was a zoonotic risk of infections. Clearly, balantidiasis is a tropical disease, which is prevalent in tropical and subtropical regions based on epidemiological data analysis [21]. Therefore, the occurrence of balantidiasis is less frequent in cold plateau areas, which was the reason that that *B. coli* was not considered at first. Recently, the development of transportation in the cattle and sheep may increase transmission of the disease. On the other hand, there were many migratory birds in the QTPA (especially Qinghai Lake), and the birds were infected and carried the *B. coli* pathogens [22,23]. Therefore, humans and other animals may be infected by migrating birds excreting faeces.

In conclusion, this is the first report of *B. coli* infection in sheep in the QTPA, China, which indicates that presence of *B. coli*, another new parasitic pathogens causing sheep diarrhoea and it may spread in this area. Therefore, further studies are needed to determine the prevalence of *B. coli* infections in sheep, goats, yaks and birds in the same environment and areas. Although this sheep case was rare, it raised our public health concerns that *B. coli* may spread across species and expand its host range.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to this work.

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