Original Paper

Prevalence of *Buxtonella sulcata* in water buffaloes and cows in Chitwan Valley, southern Nepal

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ABSTRACT

In November 2009, 104 crossed Murrah breed of water buffaloes (*Bubalus bubalis*) and 144 cows suspected of having reproductive problems were brought by farmers to an 'infertility camp' held at four villages in Chitwan Valley, southern Nepal. Fecal samples were collected arbitrarily from 45 water buffaloes and 66 cows. *Buxtonella sulcata* cysts were detected in 12 of the 45 samples (27%) from water buffaloes and 14 of the 66 samples (21%) from cows. Simultaneously, eimerian oocysts, nematode eggs of strongylids, *Trichuris* and Capillariinae, and trematode eggs of *Fasciola* and amphistomes were found; however, their prevalences were relatively low (0-11%) except for eimerian oocysts (47% and 33% for buffaloes and cows, respectively). The small subunit ribosomal RNA gene of *B. sulcata* was successfully sequenced (DDBJ/EMBL/GenBank databases accession no. AB786848), revealing it to be closely related to that of *Balantidium coli* among ciliates of the subclass Trichostomatia (Identity 96.7% along 1,594-bp length).

Keywords : *Buxtonella sulcata*, buffalo, cattle, Nepal, SSU rDNA

1. INTRODUCTION

In Nepal, there are 4.5 million water buffaloes (*Bubalus bubalis*) and 7.1 million cattle, with the former livestock producing 71% of total milk and 53% of total meat production in the country [16]. It is estimated that about a half of the households in the rural areas of Nepal maintain buffaloes, not only for milk and meat, but also for draft power, manure, hides, etc. The Institute of Agriculture and Animal Science (IAAS), Tribhuvan University, in association with local municipalities, conducts a regular 'infertility camp' in southern Nepal in order to provide veterinary care for buffaloes and cows suspected of having reproductive problems. The present study reports the prevalence of parasitic infections in water buffaloes and cows brought to these infertility camps in November 2009, with special reference to *Buxtonella sulcata* infection in both types of domestic ruminant livestock.

2. MATERIALS AND METHODS

In November 2009, 104 crossed Murrah breed of water buffaloes and 144 cows were brought by farmers to 'Infertility Camps' held at Bijayanagar, Gaurigunj, Pithuwa, and Birendranagar villages in Chitwan Valley, southern Nepal. These domestic ruminants were kept on small-scale farms holding either a single animal or only a few livestock (geomean 2.1, range 1-8). Fecal samples were collected from 45 water buffaloes and 66 cows prior to clinical examination assessing reproductive parameters. The samples were kept at 4℃

in a cool box and transported to the IAAS laboratory within 6 hours of collection. Each fecal sample was divided into two parts and fixed separately in 10% neutral-buffered formalin and 70% alcohol.

Parasitological examination of approximately 1g of formalin-fixed feces was conducted using a formalin-ether sedimentation technique [6] and the sediments were examined under a light microscope. When necessary, Lugol's iodine solution was added to sediments at a ratio of 1:5.

For the genetic analysis, alcohol-fixed fecal samples positive for *B. sulcata* were washed in sterile water and applied to a formalin-free sedimentation technique. From the sediments, *B. sulcata* cysts were collected under a stereomicroscope: 150 cysts from a cow kept in Bijayanagar; 30 cysts from a buffalo kept in Pithuwa; and 100 cysts each from two buffaloes kept in Birendranagar. Parasite DNA was extracted from these *B. sulcata* cysts using an Illustra™ Tissue and Cell Genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Extracted DNA was used as a template for PCR amplification of almost the whole length of the small subunit ribosomal RNA gene (SSU rDNA) in a 20-μl reaction mixture containing DNA polymerase (Blend Taq -Plus-, TOYOBO, Osaka, Japan) and a combination of universal eukaryotic primers, forward Eurib1 (5′-ACCTGGTTGATC CTGCCAG-3′) and reverse Eurib2 (5′-CTTCCGCTGGTT CACCTACGG-3′) [13]. The PCR cycling protocol was 2 min at 95 ℃, then 35 cycles of 1 min at 95℃, 1 min at 48℃, and 90 s at 72 ℃, followed by a final extension at 72℃ for 7 min. The PCR products were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany), then the amplicon was cloned into a plasmid vector, pTA2 (Target Clone™, TOYOBO), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a High Pure Plasmid Cleanup Micro Kit (Roche Diagnostics GmbH) and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. The nucleotide sequence reported in the present study is available in the DDBJ/ EMBL/GenBank databases under accession no. AB786848.

The newly obtained sequence and deposited sequence of *Balantidium coli* (DDBJ/EMBL/GenBank accession nos. AM982722, AM982723, and AF029763), as well as 26 deposited SSU rDNA sequences greater than 1,570 bp in length of related ciliates of the class Litostomatea from the DDBJ/EMBL/GenBank databases, were aligned using the ClustalW multiple alignment program [20], with subsequent manual adjustment. Regions judged to be poorly aligned and characters with a gap in any sequences were excluded from subsequent analyses; 1,512 characters, of which 279 were variable, remained for the phylogenetic analysis. Maximum likelihood (ML) analysis was performed with the program PhyML [4, 8], provided on the Phylogeny.fr website (http://www.phylogeny.fr/). The probability of the inferred branch was assessed by the approximate likelihood-ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support [3]. Five taxa of free-living ciliates belonging to the order Spathidiida (subclass Haptoria) were used as an outgroup for construction of a phylogenetic tree of endosymbiotic ciliates classified in the subclass Trichostomatia [15].

3. RESULTS

The results of the fecal examination are shown in Table 1. The most prevalent parasites found in the feces of the studied buffaloes and cows were oocysts of *Eimeria* spp. (47% and 33%, respectively), followed by *Buxtonella sulcata* (27% and 21%, respectively). In addition, eggs of strongylids, *Trichuris*, Capillariinae, *Fasciola*, and amphistomes were found at low prevalences (0-11%). Cysts of *B. sulcata* were round with a diameter ranging 70-105 μm (Fig. 1). The vegetative forms found in the fecal samples measured 88-110 μm by 64-93 μm, with a curved groove running from the anterior end to the posterior end. These morphological features are consistent with those previously reported for the species [9, 12, 14, 18]. A portion of the collected specimens is deposited in the National Museum of Nature and Science, Tokyo, Japan, under specimen

Table 1. Prevalence of parasites in buffaloes and cattle in Nepal by fecal examineation

Host	Locality (no. of samples examined)	Results of fecal examination*						
		Buxtonella sulcata cyst	*Eimeria* oocyst	Strongylid egg	*Trichuris* egg	Capillariinae egg	*Fasciola* egg	*Paramphistoma* egg
Buffalo								
	I. Madhavpur (n=5)	0	2 (40%)	0	0	0	0	0
	II. Bijayanagar (n=16)	4 (25%)	8 (50%)	1 (6%)	0	0	1 (6%)	3 (19%)
	III. Birendranagar (n=14)	4 (29%)	6 (43%)	3 (21%)	0	1 (7%)	0	1 (7%)
	IV. Gauriganj (n=10)	4 (40%)	5 (50%)	1 (10%)	0	0	0	0
	Total (n=45)	12 (27%)	21 (47%)	5 (11%)	0	1 (2%)	1 (2%)	4 (9%)
Cattle								
	I. Madhavpur (n=31)	6 (19%)	10 (32%)	6 (19%)	1 (3%)	0	1 (3.2%)	0
	II. Bijayanagar (n=14)	5 (36%)	6 (43%)	0	0	1 (7%)	0	3 (21%)
	III. Birendranagar (n=10)	2 (20%)	4 (40%)	1 (10%)	0	0	0	3 (30%)
	IV. Gauriganj (n=11)	1 (9%)	2 (18%)	0	0	0	0	0
	Total (n=66)	14 (21%)	22 (33%)	6 (9%)	1 (2%)	1 (2%)	1 (2%)	6 (9%)

*Number of positive samples (percentage)

numbers NSMT-Pr 338 - Pr 341.

Independently obtained nucleotide sequences of the SSU rDNA of *B. sulcata* from four hosts were 1,594-bp long and absolutely identical. As shown in Fig. 2, taxa belonging to the families Balantidiidae and Amylovoracidae formed two independent clades from other families of the subclass Trichostomatia. *Buxtonella sulcata* had the most closely related SSU rDNA sequence to *Balantidium coli*. The identity of SSU rDNA sequences of these two species was 96.7%; 50 base substitutions with 2 gaps over comparable sequences 1,596 bp in length.

4. Discussion

During a fecal survey of gastrointestinal parasites in water buffaloes and cows in the Chitwan Valley of southern Nepal, a high prevalence of *B. sulcata* was found for the first time in both types of domestic ruminant livestock in the country. *Buxtonella sulcata* is a common non-pathogenic ciliate of large ruminants (cattle and buffaloes) in temperate regions of the world [1, 2, 5, 7, 9, 10, 12, 17, 18, 21]. The prevalence of *B. sulcata* in domestic ruminants in Nepal reported in the present study is comparable to such previous reports.

Since no SSU rDNA nucleotide sequence of this ciliate

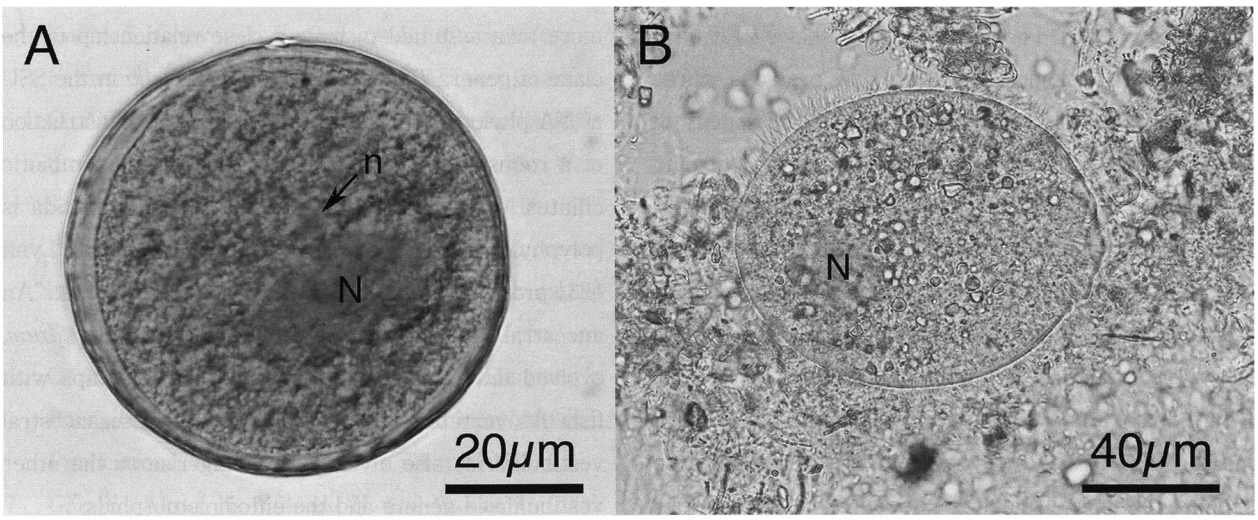

Fig. 1. An iodine-stained cyst (A) and a non-stained vegetative form (B) of *Buxtonella sulcata* from the feces of a buffalo. N, macronucleus; n, micronucleus.

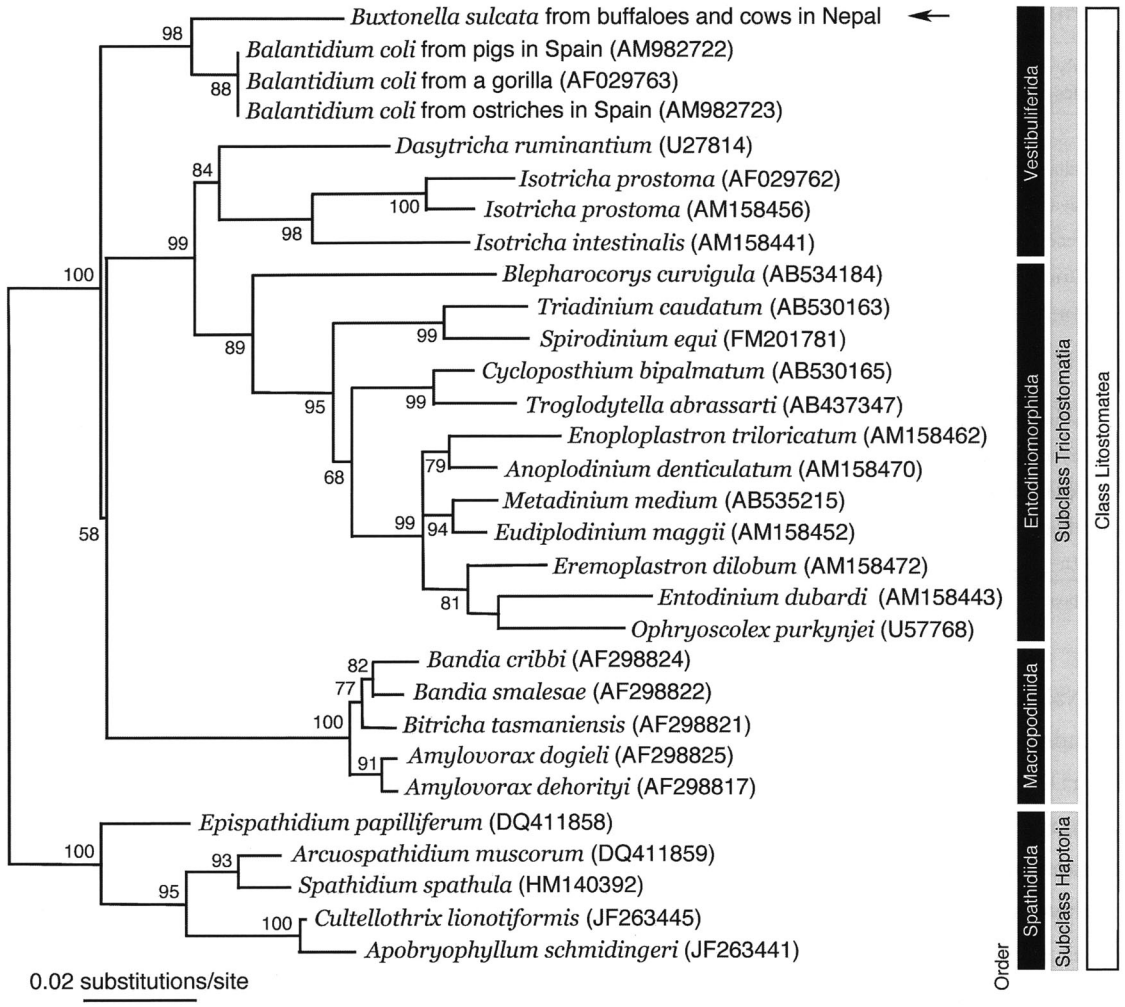

Fig. 2. A PhyML phylogenetic tree based on the SSU rDNA sequence. Branch support scores calculated by aLRT (see text) and expressed after multiplication by 100 are given at nodes. An arrow denotes newly obtained sequences from the present study.

has been reported to date, its molecular phylogenetic position has not been indicated. The present study's molecular tree based on the SSU rDNA shows essentially an identical topological framework to previous works focusing on the phylogenetic SSU rDNA phylogeny of ciliates of the class Litostomatea [11, 19, 22], placing *B. sulcata* closest to *Balantidium coli* (Vestibuliferida, Balantidiidae). Lynn [15] classified *B. sulcata* as 'incertae sedis' in the family Pycnotrichidae (order Vestibuliferida). Pycnotrichidae contains seven genera – *Collinina*, *Infundibulorium*, *Muniziella*, *Nicollella*, *Pycnothrix*, *Taliaferria*, and *Vestibulongum* – at present, and no SSU rDNA sequences of any representative species classified in such genera have been recorded. Regarding the family Balantidiidae, only SSU rDNA sequences of the genus *Balantidium* are available, and no molecular genetic analyses have been conducted on the genera *Dilleria* and *Metacollinia*. The addition of more taxa assumed to have a close relationship to the clade of genera *Balantidium* and *Buxtonella* in the SSU rDNA phylogenetic tree is required for the construction of a robust phylogenetic tree of these endosymbiotic ciliates. Concerning the point why Vestibuliferida is polyphyletic in the phylogenetic tree, Wright and Lynn [23] provided an interesting hypothesis as follows: "An ancestral holotrichous vestibuliferid, like *Balantidium*, evolved first, establishing symbiotic relationships with fish. As vertebrate groups diversified, these ancestral vestibuliferids also diversified, giving rise to the other vestibuliferid genera and the entodiniomorphids.".

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ネパールの水牛と牛での *Buxtonella sulcata* の感染状況

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要　約

2009年11月、ネパール南部のチトワン地域の4村で水牛45頭と牛66頭の直腸便を集め寄生虫検査に供した。水牛12頭（27%）および牛14頭（21%）の便に *Buxtonella sulcata* のシストを検出した。他に、*Eimeria* 属オーシストが高率に検出されたが（水牛で47%、牛で33%が陽性）、円虫卵、鞭虫卵、毛細線虫卵、肝蛭卵や双口吸虫卵の検出率はいずれの動物種でも低かった（0-11%）。*B. sulcata* のSSUリボソームRNA遺伝子について塩基配列を検討したところ（DDBJ/EMBL/GenBank データベース登録番号 AB786848）、比較できた寄生性繊毛虫類の中では *Balantidium coli* と高い近縁性を示した（塩基配列同一性は1,594塩基対で96.7%）。

Keywords : 繊毛虫、*Buxtonella sulcata*、水牛、牛、ネパール、SSU rDNA