First report on the epidemiology of Buxtonella sulcata in bovines in Pakistan

Muhammad Adnan Sabir Mughal
University of Agriculture Faisalabad
Muhammad Kasib Khan (mkkhan@uaf.edu.pk)
University of Agriculture Faisalabad
Zaheer Abbas
University of Agriculture Faisalabad
Abdullah Khalid Chatha
University of Agriculture Faisalabad
Rao Zahid Abbas
University of Agriculture Faisalabad
Anas Sarwar Qureshi
University of Agriculture Faisalabad
Muhammad Shahid Mahmood
University of Agriculture Faisalabad
Sultan Ali
University of Agriculture Faisalabad
Zid-ud-Din Sindhu
University of Agriculture Faisalabad
Arsalan Zafar
University of Agriculture Faisalabad
Muhammad Nadeem
University of Agriculture Faisalabad
Hamza Tariq
University of Agriculture Faisalabad

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Abstract

Gastro-intestinal parasites are prevalent across the world especially in developing countries. The prevalence is high in rural and semi-urban areas. *Buxtonella (B.) sulcata* is an opportunistic protozoan parasite causing gastrointestinal (GI) problems in cattle and buffaloes which serve as the reservoir host for this zoonotic pathogen. The oocysts of this parasite are resistant to chemical treatment and survive well in harsh environmental conditions like temperature, humidity etc. outside the host. In Pakistan, limited published data is available on molecular studies on prevalence of this parasite in bovines and associated risk factors. Briefly, 384 faecal samples (as determined through epidemiological sample size calculation formula) were collected from cattle and buffaloes from four towns of District Faisalabad through simple random sampling method. On a pre-designed questionnaire with closed ended questions, data on certain associated risk factors of GI protozoal infections was gathered. Parasitic oocysts were separated from faeces by using centrifugal floatation technique. DNA extraction was done from isolated oocysts of parasites followed by PCR analysis using genus specific primers (18S rRNA gene). Out of total samples collected, 78 samples were found positive for *Buxtonella spp.* with prevalence rate of 20.31%. The highest prevalence was observed in Lyallpur town 26.17% followed by Iqbal town 19.59%, Jinnah town 14.13% and Madina town 10.23%, respectively. The infection was found higher in buffaloes as compared to cattle in the study zone. Upon phylogenetic analysis, *B. sulcata* showed 90% similarity with *Balantidium (B.) coli* isolated from pigs (Accession No. MT191774.1) through Minimum Evolution Method by using Bootstrap test of phylogeny. In conclusion, we need appropriate preventive measures in order to prevent its zoonotic transmission.

Introduction

Pakistan is endowed with a large proportion of livestock population, including best dairy and meat producing breeds. More than 70% of rural population is raising their income through livestock which share nearly 11% in the total GDP and 56.3% in agricultural GDP (Rehman et al., 2017). In Pakistan, livestock is countering with different infectious and non-infectious risk factors such as parasitic exposure, poor environmental conditions, and lack of awareness. In developing countries, major portion of the livestock is not showing yield to the acceptable level due to various parasitic diseases which play a major role in poor performance. These parasitic diseases are responsible for great financial losses to livestock either directly by disturbing the production parameters or indirectly by the limiting international movement, treatment and control measures of diseases (Ahmed et al., 2020).

Buxtonella (B.) sulcata is a ciliated intestinal protozoan parasite belongs to the phylum *Ciliophora* and has worldwide distribution. It is found in the caecum and colon of water buffaloes and cows (Grim et al., 2015). Morphologically, it is similar to a ciliate protozoan *Balantidium (B.) coli* that is found in the caecum and colon of human, pigs and non-human primates (Al-Bakri et al., 2010). There is a list of gastrointestinal protozoa, responsible for causing diarrhea and death in young animals specially in bovines. *B. sulcata* is an opportunistic parasite responsible of causing infection in immunocompromised animals, production losses and even death if animal left untreated (Goz et al., 2006; Al-Zubaidi and Al-

Mayah, 2011). After ingestion, cyst enter in the small intestine where intestinal pH, proteases and bicarbonates stimulate cysts and results in trophozoites release which then colonize and invade in the colonic wall ultimately leads to diarrhea (El-Ashram et al., 2019).

Previously, *B. sulcata* has been reported from various countries including Poland; 87.9% (Diarrheal animals) (Tomczuk et al., 2005), Turkey; 9.5% (Göz et al., 2006), Iraq; 24.16% (Al-Saffar et al., 2013), Costa Rica; 21.5% (Jimenez et al., 2010), Baghdad; 43.2% (Al-Zubaidi and Al-Mayah, 2011), Nepal; 27% (Adhikari et al., 2013), Egypt; 41.6% (Sultan et al., 2013), Taiwan; 61.7% (Huang et al., 2014) and India; 35% (B. Kumar et al., 2017).

Apart from microscopy, the parasite can also be detected by molecular methods i.e., PCR showing high sensitivity and specificity and confirmed by the DNA sequencing. Sequencing provide confirmation regarding identification of parasite which remain unclear after microscopy (Dianso et al., 2018).

Although it has been reported in various regions of the world but in Pakistan, it is still considered as neglected parasite in bovine as no published data is available on its molecular detection indicating a dire need for conducting this study and confirming parasite by sequence analysis.

Methodology

Study Area

The study was conducted in major towns of District Faisalabad, Punjab including Iqbal town, Jinnah town, Lyallpur town, and Madina town. The study area was selected due to hot and humid climate which favor the growth of parasite. Moreover, a large-scale cattle market is present in the region which increases the chances of animals to get infected.

Collection and Processing of samples

A total of 384 faecal samples were collected from cattle and buffaloes through simple random sampling method. Sample size was calculated through epidemiological formula by taking expected prevalence as 50%, confidence interval as 95% and precision as 5%. These faecal samples were taken in sterile bottles having 10% formalin as a preservative. The samples were further processed by centrifugal floatation technique for collection of oocysts. Briefly, the samples were stirred in the tab water and sieve through triple fold muslin cloth. Centrifugation was done by taking 3ml faecal sample and 9ml concentrated salt solution in collection tube. Oocysts get floated on the surface, as these have less specific gravity then salt solution. From these tubes, 200µl of each sample was taken in Eppendrof tubes for DNA extraction.

Molecular Investigation

DNA extraction was done by using Stool DNA Isolation Mini Kit (FAVORGEN). 200µl of stool sample were taken in 2ml bead tube containing 200mg glass beads. The remaining extraction procedure was done using manufacturer instructions and the extracted DNA was stored at − 20°C until PCR was performed.

For identification of selected parasite, the 18S rRNA gene of *Buxtonella* was amplified through PCR using newly designed genus specific primers (Forward Primer: GTTGATCCTGCCAGTAGTC, Reverse Primer: CCTACGGAAACCTTGTTACG). PCR reaction mixture was comprising of 10µl of 2X master mixture, 1µl forward primer, 1µl reverse primer, 5µl DNA sample and 3µl distil water, making up a total of 20µl mixture. By using 1.5% agar rose gel, these amplified products were separated through gel electrophoresis. For visualization of bands, gel documentation system was used. The targeted bands were isolated from gel using commercially available kit and purified products were subjected to sequence.

Phylogenetic Analysis

The obtained sequences were subjected to BLASTn search at NCBI in order to find similarity indices and related sequences were downloaded (Altschul et al., 1990). After adding query sequence and subject sequences in FASTA file, Multiple Sequence Alignment (MLA) was performed using Clustal X software. The aligned sequences were edited in Bioedit software, including removal of gaps and deletion of extra sequences (Hall, 1999). The jModel Test was used for selection of best-fit model of nucleotide substitution for data (Posada, 2008). The phylogenetic tree was constructed by using Minimum Evolution method in MEGA X software (S. Kumar et al., 2018).

Statistical Analysis

The risk factors linked with occurrence of *Buxtonella* were statistically analyzed by using multiple logistic regression method (Thrusfield, 2018). Moreover, pair wise comparison of associated risk factors was performed through odds ratio using SAS statistical package (SAS, 2010) at 95% level of confidence.

Results

The sample were identified as *Buxtonella (B.) sulcata* upon microscopic examination based on the presence of cytostome and macronucleus (Fig. 1). The collected DNA samples from positive samples were amplified by using genus specific primers (18S rRNA) of genus *Buxtonella*. The PCR product was detected at 684 bp molecular weight (Fig. 2). The overall prevalence detected for *B. sulcata* in district Faisalabad was 20.31% (Table 1). A total of 384 faecal samples were examined and 78 samples were found positive for *B. sulcata*. Host specie, age, sex, drinking and feeding pattern are the few factors which were taken in to consideration as associated risk factors. According to sex wise prevalence ($P < 0.05$), female animals were found highly infected (60/238; 25.21%) as compared to male animals (18/146; 12.33%) (Table 1). Samples were taken from both cattle and buffaloes of different age groups. A total of 171 samples were taken from animals less than 1 year of age, 140 samples from 1 to 5 years age group and 73 samples were taken from the animals greater than 5 years of age. The highest prevalence ($P < 0.05$) was noted in less than 1 year age group (51/171; 29.82%) followed by the animals greater than 5 year of age (16/73; 21.92%) and 1 to 5 years age group (11/140; 7.86%) (Table 1). According to drinking water habits three categories were made, one which were drinking pond water and other from tap water and canal water. Based on these drinking patterns the prevalence ($P < 0.05$) was found higher in pond water (50/147; 34.01%), followed by animals that were drinking canal water (21/140; 15.00%) and tap

water (7/97; 7.22%) (Table 1). According to specie-wise prevalence (P < 0.05), the infection was found higher in the buffaloes (49/192; 25.52%) as compared to cattle (29/192; 15.10%). Based on feeding pattern (P < 0.05), grazer animals were found more infected (56/236; 23.73%) than the stall feeders (22/148; 14.86%). (Table 1).

Table-1: Multiple parameters-based prevalence of *Buxtonella* in selected towns of Faisalabad

Parameters	Factors	Total Samples Collected (N)	Positive Samples (n)	Prevalence %	Odds Ratio (OR)	P Value
Gender	Male	146	18	12.33	_	_
	Female	238	60	25.21	0.4172	0.0028
Age	Less than 1 year	171	51	29.82	0.2006	<0.0001
	1-5 years	140	11	7.86	_	_
	More than 5 years	73	16	21.92	0.3038	0.0048
Drinking Water	Canal Water	140	21	15.00	0.4407	0.0738
	Tap Water	97	7	7.22	_	_
	Pond Water	147	50	34.01	0.0585	0.0546
Species	Buffaloes	192	49	25.52	0.5192	0.0120
	Cattle	192	29	15.10	_	_
Feeding Pattern	Grazer	236	56	23.73	0.5612	0.0371
	Stall Feeder	148	22	14.86	_	_

Phylogenetic and Sequence Analysis

The positive samples were sequenced by using forward and reverse primers subjected to PCR. After BLAST analysis, the sequence was recognized as *B. sulcata* similar in morphology to *B. coli* (Fig. 3). The subject sequence showed 95.8% similarity with *Balantioides (B.) coli/Balantidium (B.) coli* isolated from pigs (Accession No. MT191774.1; MT127237.1). The above-mentioned sequences were isolated from Guinea pig in Vietnam and from Guinea pig in China after amplification of genomic DNA. Beside this, to further explore the diversity phylogenetic tree was constructed through Minimum Evolution Method by using Bootstrap method as a test of phylogeny. Upon phylogenetic analysis, the presence of *B. sulcata* in same clade indicated high similarity with *B. coli* (Accession No. MT191774.1). The clade was descendent to sequence of *Buxtonella monkey* isolated from Monkeys in China (Accession No. MG989271.1).

Isotricha prostoma (Accession No. AM158454.1) was used as an outgroup for constructing phylogenetic tree.

Discussion

Livestock sector in Pakistan has been facing the issue of parasitic infections from decades with huge economic losses either directly by reducing production or indirectly by limiting international trade of dairy and meat products (Masood et al., 2013). *Buxtonella* is one of the major protozoan parasites inhabiting in the gastrointestinal tract of dairy and meat animals with scanty information and contradictious reports about its pathogenicity (Omeragic and Crnkic, 2015). It has not been reported in Pakistan previously. The basic reason is that *Buxtonella* is morphologically similar to *Balantidium coli* and there are greater chances of misdiagnosing buxtonellosis in microscopic examination. So, the present study was designed to estimate the load of infection in cattle and buffaloes which serve as reservoir of these infections. After DNA extraction from feacal sample, the infection load was estimated through PCR.

This is first longitudinal study in Faisalabad to describe the data related to prevalence of *Buxtonella* and its molecular detection in bovines. The occurrence of infection is affected by a range of risk factors like feeding and drinking pattern, host specie, age and sex. In present study, the overall prevalence detected for *Buxtonella* spp. was 20.31%. Similarly, (El-Ashram et al., 2019) also detected prevalence of *Buxtonella* as 30.15% through floatation technique and modified Ziehl-Neelsen technique in different localities of El-Minia Province, Egypt. It often causes diarrhea, anorexia, abdominal pain and depression in animals leading to decrease in production and heavy economic losses to farmer. Another similar epidemiological study by (Kalkal and Sangwan, 2019) indicated the prevalence of *Buxtonella* in buffaloes as 54.5% in Hisar district, Haryana through sedimentation technique.

In current study, infection was found significantly ($P < 0.05$) higher in female animals (25.21%) as compared to male animals (12.33%). This significant difference may be related to immunocompromised issues in female animals. Similarly, (Hasheminasab et al., 2015) detected higher infection rate of buxtonellosis in female animals (47.32%) as compared to male animals (38.46%) in Snandaj province, Iran. Alternatively, (Al-Zubaidi and Al-Mayah, 2011) found slightly higher rate of *Buxtonella* infection in male animals (43.6%) as compared to female (42.8%) in Baghdad.

In present study, significantly ($P < 0.05$) higher infection was detected in younger animals less than 1 year of age (29.82%) and in buffaloes (25.52%). Similarly, (Ganai et al., 2015) also found higher rate of Buxtonellosis in bovines in RS Pura, Jammu having the high infection rate was detected in cattle (23.6%) as compared to buffaloes (18.5%) and younger animals showed more infection rate (33.1%) as compare to adult (13.9%). The infection rate decreases with advancement in age because of increased resistance, immunity and relocation of the animals to a less contaminated environment after weaning (Al-Saffar et al., 2013).

BLAST analysis of obtained sequence showed 96.64% similarity with *Buxtonella* from monkey (Accession No. MG989271.1), 95.8% similarity with *B. coli* isolated from pigs (Accession No.

MT991774.1) and 95.80% similarity with *B. coli* from bear (Accession No. KJ170368.1). The presence of *B. sulcata* in same clade indicated high similarity with *B. coli*. Similarly, (Dianso et al., 2018) conducted molecular phylogenetic analysis of *B. sulcata* in water buffalo in Philippines and found three isolates that were present in same clade and showing bootstrap value of 100%. These isolates showed no resemblance with other protozoan parasite registered on GenBank.

Conclusion

In conclusion, *Buxtonella* infection was found more in female animals as compared to male animals due to immune-compromised issues during pregnancy. The more infection rate in younger animals less than 1 year of age is due to presence of low immunity and less resistance to parasite. The animals drinking water from pond water showed high infection as compared to tap water due to contamination of pond water with faeces, manure, and other pollutants. Buffaloes and grazer animals showed higher infection due to its particular feeding pattern. The data generated from the present study could help in formulating effective control strategies against gastrointestinal parasites particularly *B. sulcata*. A large-scale study is needed in future to clear the infection situation in different agro-climatic zones of Punjab, Pakistan.

References

1. Adhikari, B. B., Rana, H. B., Sultan, K. M. I., Devkotal, B., Nakao, T., Kobayashi, K., Sato, H., & Dhakal, I. P. (2013). Prevalence of Buxtonella sulcata in water buffaloes and cows in Chitwan Valley, southern Nepal. Japanese Journal of Veterinary Parasitology, *11*(2).
2. Ahmed, A., Ijaz, M., Ayyub, R. M., Ghaffar, A., Ghauri, H. N., Aziz, M. U., Ali, S., Altaf, M., Awais, M., Naveed, M., & Nawab, Y. (2020). Balantidium coli in domestic animals: An emerging protozoan pathogen of zoonotic significance. Acta Trópica, *203*, 105298.
3. Al-Saffar, T. M., Al-Taee, A. F., Hadi, E. D., & Suleiman, E. G. (2013). Diagnostic study of Buxtonella sulcata in buffaloes in Mosul, Iraq. Iraqi Journal of Veterinary Science, *24*(1), 27–30.
4. Al-Zubaidi, M. T., & Al-Mayah, K. S. (2011). Prevalence of Buxtonella sulcata in neonatal and young calves in Al-Nasir station and some regions in Baghdad (Al-Shuala snd Gazaliya). Iraqi Journal of Science, *52*(4), 420–424.
5. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of molecular biology, *215*(3), 403–410.
6. Dianso, J. A., Garcia, G. G., Belotindos, L. P., & Mingala, C. N. (2018). Molecular identification of Buxtonella sulcata from associated-diarrhea in water buffaloes (Bubalus bubalis) in the Philippines. Annals of parasitology, *64*(2).
7. El-Ashram, S., Aboelhadid, S. M., Kamel, A. A., Mahrous, L. N., & Abdelwahab, K. H. (2019). Diversity of parasitic diarrhea associated with Buxtonella sulcata in cattle and buffalo calves with control of buxtonellosis. Animals, *9*(5), 259.

8. Ganai, A., Parveen, S., Kaur, D., Katoch, R., Yadav, A., Godara, R., & Ahamed, I. (2015). Incidence of Buxtonella sulcata in bovines in RS Pura, Jammu. Journal of parasitic diseases, *39*(3), 446–447.
9. Goz, Y., Altug, N., Yuksek, N., & Ozkan, C. (2006). Parasites detected in neonatal and young calves with diarrhoea. Bulletin-Veterinary Institute in Pulawy, *50*(3), 345.
10. Grim, J. N., Jirku-Pomajbikova, K., & Ponce-Gordo, F. (2015). Light microscopic morphometrics, ultrastructure, and molecular phylogeny of the putative pycnotrichid ciliate, Buxtonella sulcata. European journal of protistology, *51*(5), 425–436.
11. Hall, T. A. (1999). BioEdit software, version 5.0. 9. *North Carolina State University, Raleigh, NC*.
12. Hasheminasab, S. S., Moradi, P., Talvar, H. M., Wright, I., & Darbandi, M. S. (2015). Buxtonella spp. like infection in cattle in Sanandaj province, Iran. Annals of parasitology, *61*(4).
13. Huang, C.-C., Wang, L.-C., Pan, C.-H., Yang, C.-H., & Lai, C.-H. (2014). Investigation of gastrointestinal parasites of dairy cattle around Taiwan. Journal of Microbiology, Immunology and Infection, *47*(1), 70–74.
14. Jiménez, A. E., Fernández, A., Alfaro, R., Dolz, G., Vargas, B., Epe, C., & Schnieder, T. (2010). A cross-sectional survey of gastrointestinal parasites with dispersal stages in feces from Costa Rican dairy calves. Veterinary Parasitology, *173*(3–4), 236–246.
15. Kalkal, H., & Sangwan, A. K. (2019). Epidemiological investigations of Buxtonella sulcata in buffaloes of Hisar district, Haryana.
16. Kumar, B., Maharana, B. R., Prashad, A., Joseph, J. P., & Patel, B. R. (2017). Incidence of Buxtonella sulcata in Jaffrabadi buffaloes of south-western Gujarat, India. Buffalo Bulletin, *36*(4), 623–628.
17. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution, *35*(6), 1547.
18. Omeragić, J., & Crnkić, Ć. (2015). Diarrhoea in cattle caused by Buxtonella sulcata in Sarajevo area. Veterinaria, *64*(2), 50–54.
19. Posada, D. (2008). jModelTest: phylogenetic model averaging. Molecular biology and evolution, *25*(7), 1253–1256.
20. Rehman, A., Jingdong, L., Chandio, A. A., & Hussain, I. (2017). Livestock production and population census in Pakistan: Determining their relationship with agricultural GDP using econometric analysis. Information Processing in Agriculture, *4*(2), 168–177.
21. S Al-Bakri, H., G Suliman, E., & M Al-Saffar, T. (2010). Prevalence of intestinal ciliate Buxtonella sulcata in cattle in Mosul. Iraqi Journal of Veterinary Sciences, *24*(1), 27–30.
22. Sultan, K., Khalafalla, R. E., & Elseify, M. A. (2013). Preliminary investigation on Buxtonella sulcata (Jameson, 1926)(Ciliphora: Trichostomatidae) in Egyptian Ruminants. Journal of Veterinary Medical Research, *22*(1), 91–94.
23. Thrusfield, M. (2018). *Veterinary epidemiology*. John Wiley & Sons.
24. Tomczuk, K., Kurek, L., Stec, A., Studzinska, M., & Mochol, J. (2005). Incidence and clinical aspects of colon ciliate Buxtonella sulcata infection in cattle. Bull Veterinary Institute Pulawy, *49*(1), 29–33.

Figures

Figure 1

Microscopic image showing oocyst of *Buxtonella sulcata* in faecal samples of cattle and buffaloes (A= Cytostome, B= Macronucleus)

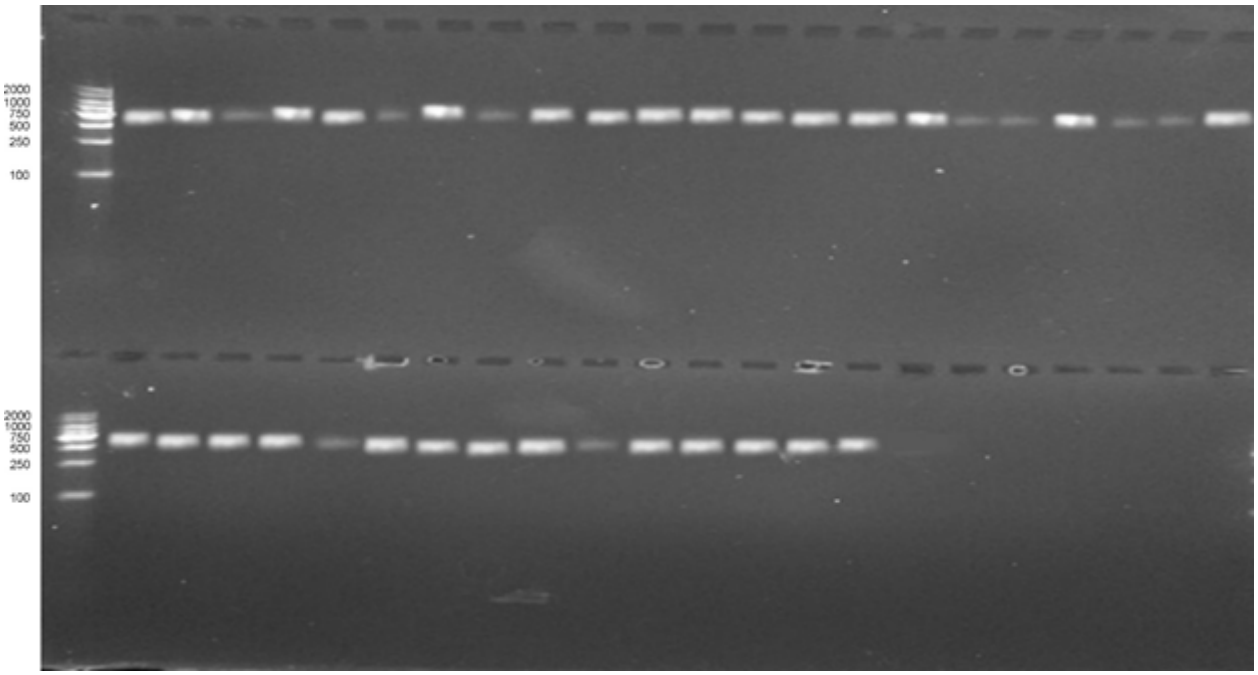

Figure 2

Gel electrophoresis showing positive bands of *Buxtonella sulcata* DNA in faecal samples of cattle and buffaloes (Product size = 684bp)

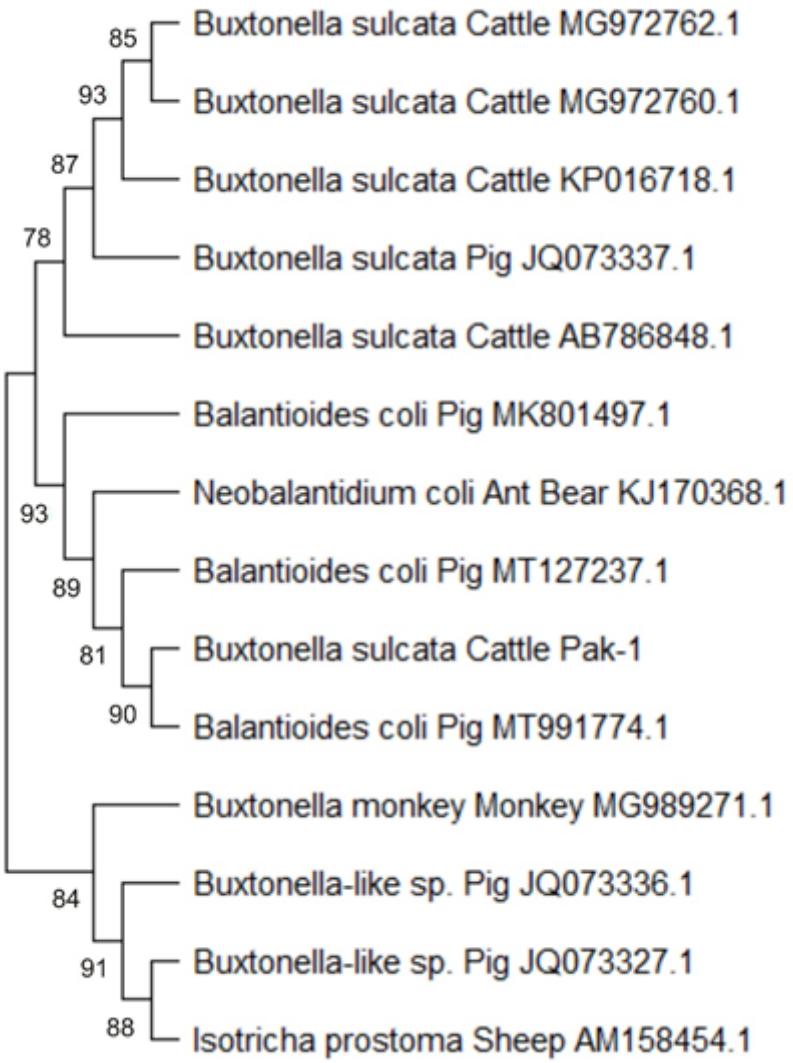

Figure 3

Phylogenetic relationship of 18S rRNA gene of *Buxtonella sulcata* identified in present study with closely related species already reported worldwide

Supplementary Files

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