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RESEARCH ARTICLE

Prevalence and Characterization of *Buxtonella sulcata* in Bovines in Pakistan Using Morphological and PCR-Based Approaches

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A B S T R A C T

Gastrointestinal (GI) 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 GI problems in cattle and buffaloes which serve as the reservoir host for this zoonotic pathogen. In Pakistan, limited published data is available on molecular studies on prevalence of this parasite in bovines and associated risk factors. Briefly, 384 fecal 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 buxtonellosis was gathered. Parasitic oocysts were separated from feces by using centrifugal sedimentation technique. DNA extraction was done from isolated oocysts of parasites followed by PCR analysis using genus specific primers (18S rRNA gene). The overall prevalence of *Buxtonella* spp. was 12.24 and 20.31% using microscopic and molecular techniques, respectively. Regarding the associated risk factors, the infection was found to be significantly more prevalent in females ($P<0.05$) compared to male animals, in younger animals compared to older animals, and in cattle compared to buffaloes. Among other factors, the prevalence of *Buxtonella* spp. was significantly higher ($P<0.05$) in grazing animals consuming pond water as compared to stall feeders consuming canal or tap water. Upon phylogenetic analysis, the obtained sequences showed 97.48% similarity with *B. sulcata* isolated from cattle and buffalo through Neighbor Joining Method. In conclusion, molecular techniques were found to be more suitable to detect *Buxtonella* spp. compared to microscopic techniques. Moreover, we need appropriate preventive measures to prevent its zoonotic transmission.

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INTRODUCTION

Pakistan is endowed with a significant livestock population, including some of the best dairy and meat-producing breeds. More than 70% of rural population generates their income through livestock, which contributes nearly 11% to the total GDP and 56.3% to agricultural GDP (Rehman *et al.*, 2017). However, livestock in Pakistan faces various infectious and non-infectious risk factors, such as parasitic exposure, poor environmental conditions, and a lack of awareness. In developing countries, a significant portion of livestock does not yield at acceptable levels due to various parasitic diseases, which play a major role in poor performance (Rafiullah *et al.*, 2011; Bachaya *et al*., 2015; Javed and Alkheraije, 2023; Qamar and Alkheraije, 2023). These parasitic diseases are responsible for causing great financial losses to livestock, either directly by disrupting

production parameters or indirectly by limiting international movement, treatment, and control measures for diseases (Masood *et al.*, 2013: Ahmed *et al.*, 2020; Basit *et al*., 2022; Khan *et al*., 2023).

Buxtonella (B.) sulcata is a ciliated intestinal protozoan parasite belonging 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; Kuraa and Malek, 2024). Morphologically, it is similar to the ciliate protozoan *Balantidium coli*, which is found in the caecum and colon of humans, pigs and non-human primates (Al-Bakri *et al.*, 2010). A variety of GI protozoa are responsible for causing diarrhea and death in young animals, especially in bovines (Bhanot *et al.*, 2022; Shibitov and Abdelhamid, 2022). *B. sulcata* is an opportunistic parasite that can cause infection in immunocompromised animals, leading to production losses and even death if left untreated (Goz *et al.*, 2006; Al-Zubaidi and Al-Mayah, 2011). After ingestion, cysts enter the small intestine, where intestinal pH, proteases, and bicarbonates stimulate cysts, resulting in the release of trophozoites that then colonize and invade the colonic wall, ultimately leading to diarrhea (Abd EL-Tawab *et al.*, 2018).

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

Based on microscopy, it is difficult to differentiate between the oocysts of *Balantidium* spp. and *Buxtonella* spp. due to their similar morphological characteristics. In addition to microscopy, the parasite can also be detected by molecular methods, such as PCR, which demonstrates high sensitivity and specificity and is confirmed by DNA sequencing. Sequencing provides confirmation regarding the identification of the parasite, which may remain unclear after microscopy (Dianso *et al.*, 2018).

Although *B. sulcata* has been reported in various regions of the world, it is still considered a neglected parasite in bovines in Pakistan, as no published data is available on its molecular detection. This highlights the urgent need for conducting this study and confirming the parasite through sequence analysis.

MATERIALS AND METHODS

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 its hot and humid climate which favors the growth of parasites. Additionally, a large-scale cattle market in the region increases the chances of animal infections.

Sample and data collection: A total of 384 fecal samples were collected from cattle and buffaloes using a simple random sampling method. The sample size was calculated using an epidemiological formula, assuming an expected prevalence of 50%, a confidence interval of 95% and a precision of 5%. These fecal samples were collected in sterile bottles containing 10% formalin as a preservative. The association of risk factors with the occurrence of *Buxtonella* spp. in the study area was assessed by collecting information through a pre-designed questionnaire, which included factors such as gender, age, source of drinking water, and feeding patterns.

Microscopic investigation: The samples were processed using the centrifugal sedimentation technique for the identification of oocysts (Iqbal *et al.*, 2006). Briefly, 1g feces was mixed with small amount of tape water (10-15mL) and strained through cheesecloth. Then, a 5mL filtrate was taken and transferred to a 15mL conical centrifuge tube. Water was then added to the tube to achieve a final volume of 15mL. The tube containing the suspension was centrifuged at 1500rpm for 5 min. After centrifugation, the supernatant was discarded, and 10mL of water was added to the tube. This step was repeated several times until the supernatant became clear. Finally, the supernatant was discarded, and a drop of suspension from the upper layer of the sediment was placed on a clean glass slide. A coverslip was then added, and the sample was examined under a microscope for identification based on morphological characteristics, including thin wall, granular cytoplasm, a kidney-shaped macronucleus, and a micronucleus (Iqbal *et al.*, 2006).

Molecular investigation: DNA was extracted using the Stool DNA Isolation Mini Kit (FAVORGEN). Briefly, 200µL of fecal suspension from upper layer of sediment, obtained after processing through centrifugal sedimentation method, was taken in 2mL bead tube containing 200mg of glass beads. The remaining extraction procedure was carried out according to the manufacturer's instructions, and the extracted DNA was stored at −20°C for further analysis.

For the identification of the selected parasite, the 18S rRNA gene of *Buxtonella* spp. was amplified using PCR with newly designed genus-specific primers (Forward Primer: GTTGATCCTGCCAGTAGTC, Reverse Primer: CCTACGGAAACCTTGTTACG). The PCR reaction mixture comprised 10µL of 2X master mix (WizPure™, WizBio Solutions), 1µL of forward primer, 1µL of reverse primer, 5µL of DNA sample, and 3µL of distilled water, resulting in a total volume of 20µL. The amplified products from PCR were separated on a 1.5% agar rose gel using gel electrophoresis. A gel documentation system was employed for visualization of the bands. The specific product bands (684bp) were extracted from gel using gel extraction kit (QIAquick®, QIAGEN), and the purified products were subjected to sequencing.

Phylogenetic analysis: The obtained sequences were subjected to BLASTn search at NCBI to find similarity indices, and related sequences were downloaded (Altschul *et al.*, 1990). After adding the query sequence and subject sequences to a FASTA file, Multiple Sequence Alignment (MLA) was performed using Clustal-X software. The aligned sequences were edited in BioEdit software, which included the removal of gaps and deletion of extra sequences (Hall, 1999). The jModelTest was used to select the best-fit model of nucleotide substitution for the data (Posada, 2008). The phylogenetic tree was constructed using the Minimum Evolution method in MEGA X software (Kumar *et al.*, 2018).

Statistical analysis: The risk factors linked to the occurrence of *Buxtonella* spp. were statistically analyzed using the multiple logistic regression method (Thrusfield, 2018). Additionally, pairwise comparisons of associated risk factors were conducted through odds ratios using SAS statistical package (SAS, 2010) at a 95% level of confidence.

RESULTS

Microscopic determination of *Buxtonella* spp.: The *Buxtonella* oocysts' size ranged from 70-80µm in length and 55-80µm in width (Fig. 1). The overall microscopic prevalence of *Buxtonella* spp. detected in district Faisalabad was 12.24% (47/384). The highest prevalence of *Buxtonella* spp. was observed in Lyallpur town (17.76%), followed in decreasing order by Iqbal town (14.43%), Madina town (9.09%), and Jinnah town (6.52%). Further details regarding the number of samples, odds ratio, confidence intervals and p-values are given in Table 1.

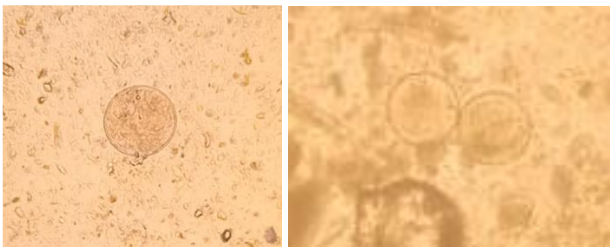

Fig. 1: *Buxtonella* oocysts identified in fecal samples of cattle and buffaloes in District Faisalabad, Pakistan

Molecular detection of *Buxtonella* spp.: In the molecular analysis, the 18S rRNA gene of *Buxtonella* spp. was amplified using newly designed genus-specific primers that target a partial gene sequence of 684bp. The overall prevalence of *Buxtonella* spp. in district Faisalabad was 20.31% (78/384). A similar trend in town-wise prevalence was observed as in microscopic analysis. Further details regarding prevalence percentages, the number of samples, odds ratio, confidence intervals, and p-values are provided in Table 2.

Associated risk factors: Host species, age, gender, drinking, and feeding patterns were considered as associated risk factors. The microscopic observation revealed that the female animals (37/238; 15.55%) were highly infected (P<0.05) compared to male animals (10/146; 6.85%). Samples were collected from both cattle and buffaloes across different age groups: 171 samples from animals less than 1 year of age, 140 samples from 1 to 5-year age group, and 73 samples from animals older than 5 years. The highest prevalence (P<0.05) was noted in the group of animals less than 1-year-old (30/171; 17.54%), followed by those older than 5 years (9/73; 12.33%) and 1 to 5-year age group (8/140; 5.71%). Based on drinking water habits, three categories were established: animals drinking pond water, those drinking tap water and those drinking canal water. The prevalence was higher (P<0.05) in animals drinking pond water (28/147; 19.05%), followed by those drinking canal water (14/140; 10%) and tap water (5/97; 5.15%). According to host species-wise prevalence, the infection rate was higher (P<0.05) in the cattle (31/192; 16.15%) compared to buffaloes (16/192; 8.33%). Based on feeding patterns, grazing animals (33/236; 13.99%) were found to be more infected (P<0.05) than the stall-fed animals (14/148; 9.46%) (Table 3).

Table 1: Prevalence of *Buxtonella* spp. in district Faisalabad, Pakistan based on microscopic detection of oocysts.

Town	Total samples	Positive samples (% prevalence)	CI	Odds ratio	P-value
Jinnah Town	92	6 (6.52)	_	_	_
Lyallpur Town	107	19 (17.76)	0.1231-0.8480	0.3231	0.0217
Madina Town	88	8 (9.09)	0.2319-2.0989	0.6977	0.5218
Iqbal Town	97	14 (14.43)	0.1517-1.1275	0.4136	0.0844
Total	384	47 (12.24)	_	_	_

Table 2: Prevalence of *Buxtonella* spp. in district Faisalabad, Pakistan based on PCR amplification of 18S rRNA gene.

Town	Total samples	Positive samples (% prevalence)	CI	Odds ratio	P-value
Jinnah Town	92	12 (13.04)	_	_	_
Lyallpur Town	107	30 (28.04)	0.1839-0.8061	0.3850	0.0114
Madina Town	88	13 (14.77)	0.3715-2.0156	0.8654	0.7375
Iqbal Town	97	23 (23.71)	0.2243-1.0384	0.4826	0.0624
Total	384	78 (20.31)	_	_	_

The molecular prevalence of *Buxtonella* spp., as determined by amplifying the 18S rRNA gene from Faisalabad district was 20.31%, showing a similar trend in relation to associated risk factors (Table 4).

Phylogenetic and sequence analysis: A total of four samples were randomly selected out of 78 samples for sequencing by the Sanger sequencing method by using forward and reverse primers. After BLAST analysis, the sequence was recognized as *B. sulcata.* Due to higher similarity with each other, all the selected sequences were included in the same clade. The obtained sequences showed 97.48% similarity with *B. sulcata* isolated from cattle from Malaysia (MG972760.1 & MG972761.1) and 97.28% similarity with *B. sulcata* isolated from buffalo from Belgium (JQ073337.1) and from buffalo from Uttar Pradesh, India (PP590352.1). To explore genetic diversity, a phylogenetic tree was constructed using the neighbor joining method. All the subject sequences appeared in the same clade. The clade was ascendent to sequence of *B. sulcata* isolated from Buffalo from India (PP590549.1) and descendent to sequence of *B. sulcata* isolated from Cattle in Malaysia (MG972761.1). *Isotricha prostoma* (AM158454.1) was used as an outgroup for constructing phylogenetic tree (Fig. 2).

DISCUSSION

The present study was conducted to estimate the occurrence of buxtonellosis in buffaloes and cows in district Faisalabad through oocyst quantification and PCR by amplifying partial sequence of 18S rRNA gene using newly designed primers. This is the first longitudinal study

Table 3: Assessment of risk factors influencing *Buxtonella sulcata* prevalence in the study area using microscopic oocyst identification.

Parameters	Factors	Total Samples Collected (N)	Positive Samples (n)	Prevalence %	Odds Ratio (OR)	P-value
Gender	Male	146	10	6.85	_	_
	Female	238	37	15.55	0.3994	0.0140
Age	Less than 1 year	171	30	17.54	0.2848	0.0025
	1-5 years	140	8	5.71	_	_
	More than 5 years	73	9	12.33	0.4310	0.0984
Drinking Water	Canal Water	140	14	10	0.4891	0.1844
	Tap Water	97	5	5.15	_	_
	Pond Water	147	28	19.05	0.2310	0.0037
Species	Cattle	192	31	16.15	0.4721	0.0216
	Buffaloes	192	16	8.33	_	_
Feeding Pattern	Grazer	236	33	13.99	0.6427	0.1907
	Stall Feeder	148	14	9.46	_	_

Table 4: Assessment of risk factors influencing *Buxtonella sulcata* prevalence in the study area using PCR

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	Cattle	192	49	25.52	0.5192	0.0120
	Buffaloes	192	29	15.10	_	_
Feeding Pattern	Grazer	236	56	23.73	0.5612	0.0371
	Stall Feeder	148	22	14.86	_	_

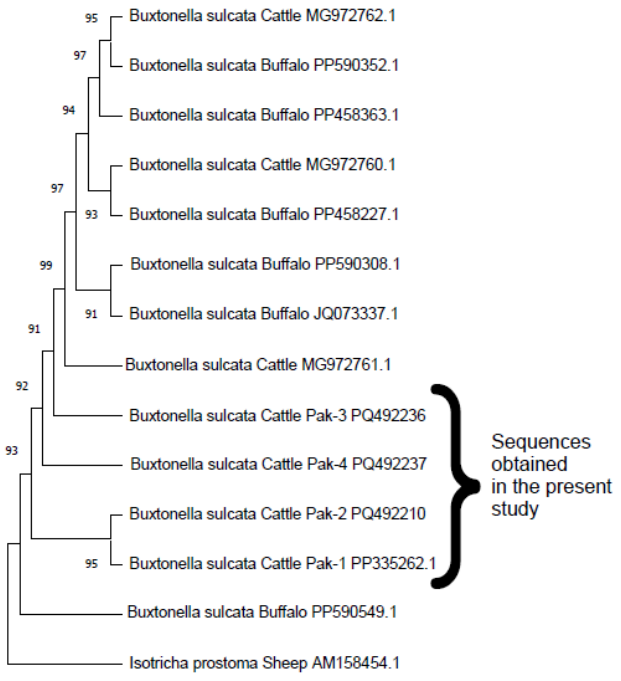

Fig. 2: Neighbor joining tree showing the phylogenetic relationships among the obtained sequences of *B. sulcata* (18S rRNA gene) and closely related sequences.

in Faisalabad, Pakistan to describe the data related to the prevalence of *Buxtonella* spp. and its molecular detection in bovines. The occurrence of infection is influenced by various risk factors like feeding and drinking patterns, host species, age and gender. In this study, the overall prevalence of *Buxtonella* spp. was found to be 20.31%. However, higher prevalence rates have been documented in previous studies, such as 30.15% in cattle and buffaloes in various localities of El-Minia Province, Egypt, determined using floatation and modified Ziehl-Neelsen techniques (El-Ashram *et al.*, 2019), and 54.5% in buffaloes in the Hisar district, Haryana, India, identified through sedimentation technique (Kalkal and Sangwan, 2019). Alternatively, another study showed lower prevalence rate of 5% by microscopy and 7% by molecular methods in cattle in Qazvin province, Iran (Baghaie *et al.*, 2021).

In the current study, the infection rate was significantly (P<0.05) higher in female animals (25.21%) compared to male animals (12.33%). This difference may be related to immunocompromised issues in female animals, especially during pregnancy (Chauhan *et al.*, 2024). Similarly, Hasheminasab *et al.* (2015) found a higher infection rate of buxtonellosis in female animals (47.32%) compared to male animals (38.46%) in Snandaj province, Iran. In contrast, Al-Zubaidi and Al-Mayah (2011) reported a slightly higher rate of *Buxtonella* infection in male animals (43.6%) compared to females (42.8%) in Baghdad.

In the present study, significantly (P<0.05) higher infection rates were detected in younger animals having age less than 1 year (29.82%) compared to other age categories, and in cattle (25.52%) compared to buffaloes. Similarly, Ganai *et al.* (2015) also found a higher infection 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 exhibited a more significant infection rate (33.1%) than adults (13.9%). In Kaluga region of Russian Federation, the cattle having less than 1-year of age exhibited a higher prevalence of buxtonellosis, especially during autumn season with prevalence peak up to 95%, as determined through sedimentation and McMaster techniques (Shibitov and Abdelhamid, 2022). The infection rate tends to decrease with advancing age due to increased resistance, immunity and the relocation of animals to less contaminated environment after weaning (Al-Saffar *et al.*, 2013).

BLAST analysis of obtained sequence showed 97.48% similarity with *B. sulcata* from cattle (MG972760.1 & MG972761.1) and 97.28% similarity with *B. sulcata* from buffalo (JQ073337.1). 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 to other protozoan parasites registered on GenBank.

Conclusions: In conclusion, *Buxtonella* spp. is prevalent in bovines in District Faisalabad, Pakistan. Various risk factors (gender, age, drinking water, species and feeding pattern) may influence the occurrence of infection in the study area. Moreover, detection of infection through PCR by amplifying 18S rRNA gene was found more suitable as compared to microscopy. Further research is suggested in the form of a comparative analysis of different genes and their different regions using different primer pairs to find more suitable markers for detection and differentiation of *Buxtonella* spp. in hosts.

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Authors contribution: MASM and AKC have completed the research work under supervision of MKK, MSM and AR. MASM, AA and ZA have completed the write up process and AK and SU proofread the paper. MSM and SA helped in the phylogenetic analysis of positive samples after sequencing.

Conflict of interest: On behalf of all authors, it is declared that all authors have no known conflict of interests, including competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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