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Case report

Detection of naturally occuring mixed infection of *Theileria lestoquardi* and *Theileria annulata* in buffalo, in Pakistan

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ABSTRACT. Theileriosis caused by *Theileria lestoquardi* (malignant ovine theileriosis) in sheep and *Theileria annulata* (topical theileriosis) in cattle, is a tick-borne hemoprotozoal disease that causes major economic losses in animal production caused by a tick-transmitted haemoprotozoan *Theileria* species, infecting various domestic and wild animals. It is a threat to the buffalo population in Pakistan due to high mortality rates and economic losses. Extensive mixed farming in Pakistan poses threats due to mixed infections and increased mortality rates. The current case report describes the mixed infection and characterization of *Theileria lestoquardi* with *Theileria annulata* in a buffalo raised in a mixed farming setup, in Pakistan. A 6.5-year-old female buffalo presented at the Outdoor Hospital exhibiting swollen sub-scapular lymph nodes, pale mucous membranes, a high fever (41.5°C), dullness, depression, shortness of breath, and a decrease in milk production. Investigation through microscopy, hematology, and PCR confirmed that the buffalo was suffering from theileriosis. A Giemsa-stained thin blood smear showed piroplasms indicating theileriosis. Hematological analysis of the blood revealed lower values for hemoglobin and red blood cells (RBCs), while higher counts of lymphocytes, neutrophils, and monocytes were observed during the infection. The PCR performed on the blood sample was positive for *Theileria* spp. using RLB universal primers and *T. annulata*-specific primers. The sequence revealed similarity with *T. lestoquardi* reported in Pakistan, Iran, Tunisia, and India. The buffalo was treated with Bupralex® and Oxtra LA® at a dosage of 1ml/20kg of body weight I/M, respectively, along with supportive treatment. Despite these interventions, the animal died probably due to damage to cardiac muscles. This study reports the first case of *T. annulata* mixed infection with *T. lestoquardi* in a buffalo in Punjab, Pakistan. It can be concluded that mixed farming exacerbates the epidemiology and spread of ovine malignant theileriosis and mortalities possibly due to cardiac arrest in mixed infections.

Keywords: *Theileria annulata*, *Theileria lestoquardi*, buffalo, mixed infection, Pakistan

Introduction

Buffalo (*Bubalus bubalis*) is known as the black gold of Pakistan. In Pakistan, 8 million rural families are deeply engaged in livestock production which makes 35–40% of their total income. Buffalo constitutes a livestock population of 46.3 million and contributes 59.78% of total milk production annually [1]. It is of prime importance for dairy industry of Pakistan [2].

Theileriosis is a non-contagious bovine tick-borne, hemoprotozoal disease caused by various species of *Theileria*, infecting domestic as well as wild animals [3]. Among *Theileria* spp., *T. annulata* cause tropical theileriosis while *T. lestoquardi* cause malignant ovine theileriosis [4]. In Pakistan, the pathogenic parasitic species for cattle is *Theileria annulata* [5], while that for small ruminants is *Theileria lestoquardi* [6]. Theileriosis is a threat to the buffalo population in Pakistan, and it is responsible for causing substantial economic losses to livestock due to high morbidity, mortality, and production losses [7]. It is characterized by a high fever of 41.5°C, petechial hemorrhages on mucus

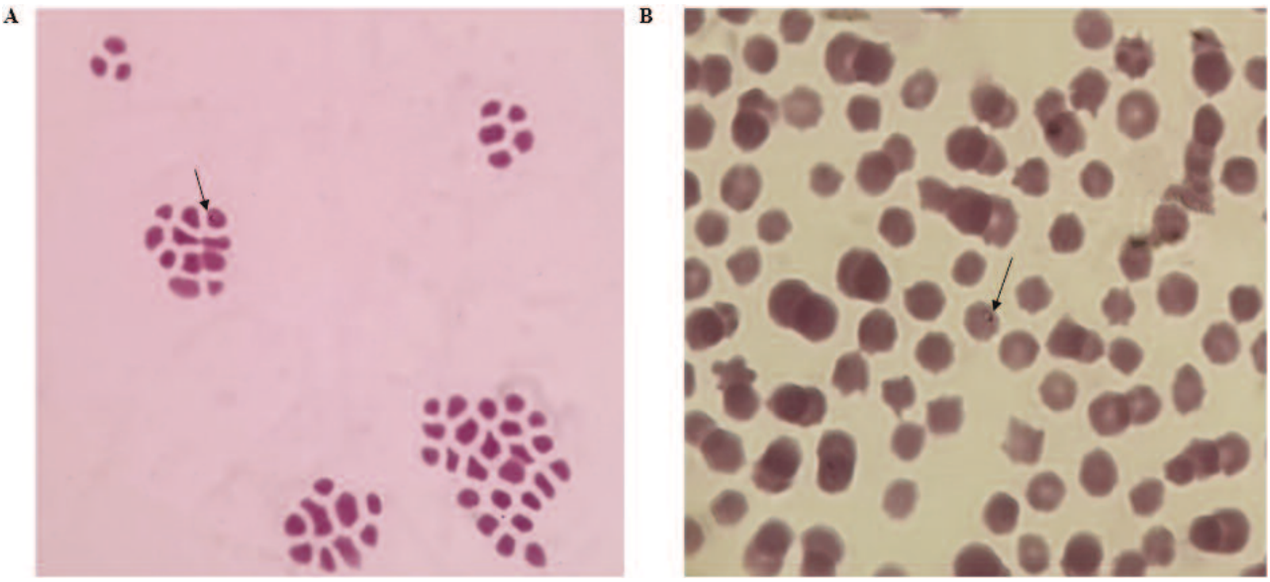

Figure 1. Microscopic images (A,B). Typical intracellular pleomorphic *Theileria* piroplasms (arrows) in a stained thin blood smear of the buffalo, 1000×

membranes and conjunctiva, lacrimation, nasal discharges, superficial lymph node swelling, anemia, loss of milk production, bulging eyes, nervous signs, and death [8].

In Pakistan, the mixed farming system comprises different livestock species such as buffalo, sheep, cattle, and goats [9]. *T. annulata, T. lestoquardi,* and their tick vectors are most prevalent in Pakistan [10]. Considering the shared vector between *T. annulata* and *T. lestoquardi* as well as the prevalence of mixed farming practices in Pakistan, there is a high likelihood of mixed infection with both *Theileria* species occurring in the same host as recently reported in Iran [11].

Reports have indicated that *T. annulata* and *T. lestoquardi* are antigenically related, suggesting cross-immunity between them. In ovines, *T. lestoquardi* results in endocarditis and necrosis of the cardiac cells leading to ultimate death [12]. Diagnosis of theileriosis depends on microscopic examination which is an age-golden method [13] but PCR-based tests have made it possible to define each *Theileria* species [14].

Naturally occurring infection of cattle with *Theileria lestoquardi* has been reported in Iran [15]. However, no published reports are available that

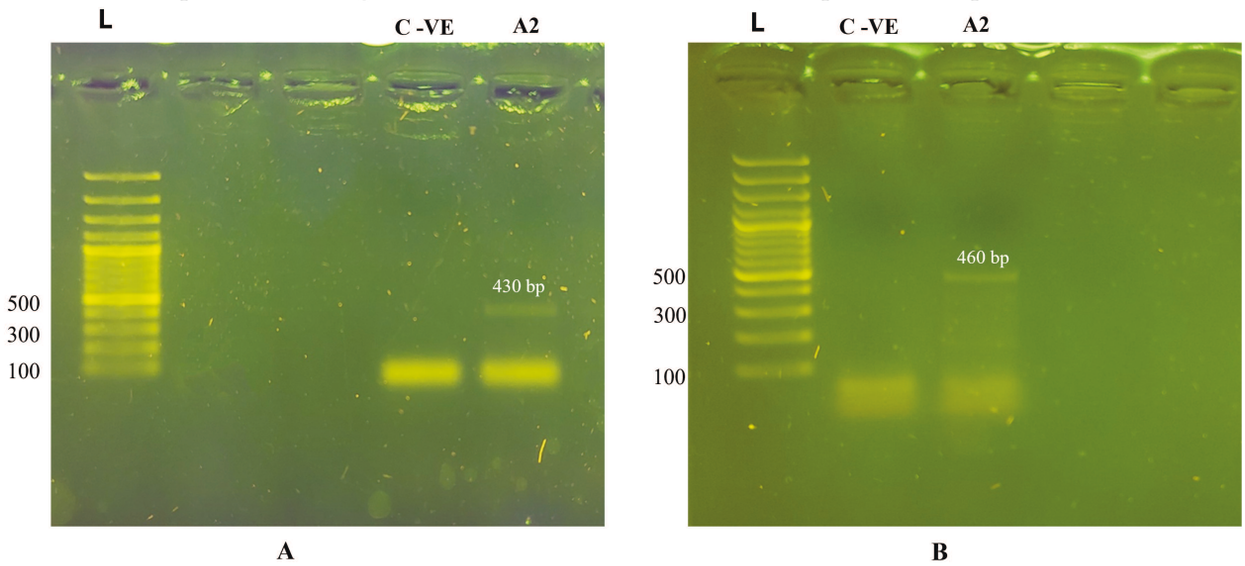

Figure 2. PCR results of infected buffalo sample with RLB universal primers (A) and *Theileria annulata*-specific primers (B). (A) L: DNA ladder 100bp, lane C-ve: control negative, lane A2: buffalo sample; (B) L: DNA ladder 100bp, lane A2: buffalo sample

Table 1. Hematology results of infected buffalo and normal reference values are as per [16]

Hematological parameters	Results	Unit	Normal values
Hemoglobin	63	g/l	95.3–145.0
T.L.C	12.4	$\times 10^3$/ul	6.0–12.8
RBC	4.60	M/cmm	5.2–8.4
MCV	48.1	fL	46.9–61.7
MCH	13.7	Pg	15.1–21.3
MCHC	28.5	g/dl	31.0–35.8
HCT	22.1	%	27.2–44.2
Platelets	150	$\times 10^3$/ul	68.3–281.0
Neutrophils	83	%	1.9–6.9
Lymphocytes	13	%	2.5–7.1
Monocytes	02	%	0.0–1.0
Eosinophils	02	%	0.0–10.0

indicate naturally occurring mixed infection of buffalo with *T. lestoquardi* and *T. annulata* in Pakistan. This case report describes the natural mixed infection of *T. lestoquardi* and *T. annulata* in buffalo raised with sheep in mixed farming premises, in Pakistan through molecular analysis which highlights the importance of mixed farming in epidemiology and control of theileriosis.

Case report

This is a case involving a naturally infected female buffalo of a local breed, weighing approximately 300 kg and aged 6.5 years. Buffalo was held with sheep at a mixed farming setup in Lahore. The buffalo was presented at the Outdoor Hospital, Department of Veterinary Medicine, University of Veterinary and Animal Sciences (UVAS) Lahore, exhibiting several clinical signs of theileriosis including swollen sub-scapular lymph nodes, pale mucous membranes, high fever (41.5°C), dull, depression, shortness of breath and decrease in milk production. For Laboratory investigations, 2 ml blood sample was taken from the jugular vein into EDTA (ethylenediamine tetra acetic acid) coated tube (LABOVAC Italiano) and dispatched to the Department of Parasitology UVAS Lahore Pakistan, for screening of hemoparasites through microscopy, hematology, and PCR analysis

Microscopic examination

A thin blood smear was formed and fixed with absolute alcohol, air-dried, and stained with Giemsa. The blood smear was then examined under an oil-immersion lens at 1000× (Fig. 1) for the identification of hemoparasites.

Hematological analysis

Hematological analysis using a hematology analyzer revealed neutrophilia, lymphocytosis, monocytosis, and regenerative anemia (Table 1).

Dna extraction and PCR

The collected blood was used to extract the DNA using a DNA extraction kit (Quick-DNA™ Miniprep Plus Kit, USA) as following by the manufacturer's protocol. DNA was quantified using the OD260 value with a NanoDrop 2000 spectrophotometer and was kept at –20 for further examination. The extracted DNA was amplified using a thermocycler by Thermo Fischer Scientific (Applied BioSystems, USA). For PCR, RLB

Table 2. Target region, primers sequence and the product size for the mentioned RLB and *T. annulata* specific primers

Primers	Primer's sequence	Target gene	PCR condition	Product size	Reference
RLB* Primers	F=5'-GACACAGGGAGGTA R=5'-CTAAGAATTTCACCT CTGACAGT-3'	18S rRNA	Denaturation: 95°C, at 4 min ` Cycles: 40 times Annealing: 58°C at 30 sec Extension: 72°C at 1 min Final extension: 72°C at 10 min	430 bp	[17]
Theileria annulata-specific primers	F=5´-CAAATGAGC TTCTGGOGAGC-3´ R=5´-TTCCTGCCA TTGCCAAAAGTC-3´	Cytochrome b l Gene	Denaturation: 95°C at 4 min Cycles: 35 times final denaturation: 95° C at 30 sec Annealing: 58°C at 30 sec Extension: 72°C at 1 min Final extension 72°C at 10 min	460 bp	[18]

universal primers and specific-*T. annulata* primers were used. The primer sequence, target region, and product size of these primers are given in Table 2. The PCR reaction mixture was used with a final volume of 20 µl and was performed using conditions as reported in Table 2. After PCR, the amplified DNA was run on 1.5% agarose gel electrophoresis using the Gel Documentation System (Biobase, USA). The amplicons were purified by the Gel Purification Kit (WizPrep, Korea Ref no: W70150-300).

Phylogenetic analysis

In order to confirm specificity of PCR analysis, the 430 bp amplicons obtained from RLB universal primers and the 460 bp amplicons from *Theileria annulata*-specific primers were sequenced by Sanger Sequencing Method [19]. The Chromas Pro software (version 1.7.4) was used to analyze the chromatograms, and the results of sequencing were compared with the database of Gene Bank to identify the homology of the nucleotide sequence. For the BLAST algorithm, the data was analyzed via National Centre for Biotechnology Information (NCBI) network. All the obtained data were aligned by CLUSTAL W [20] whereas Mega 7.0 software was used to analyze the alignment of sequence. A phylogenetic tree was built by a maximum-likelihood method via Mega 7.0 software [21]. The products of PCR were confirmed by sequencing method. BLAST analysis indicated the close homology to 18S rRNA or DNA of *T. lestoquardi* and to the sequence related to various parts of the world. Phylogenetic analysis related to our obtained sequence (Accession no. OR069482) was compared to other relevant sequences, as shown in Figure 3.

The infected buffalo sequence (Accession no. OR069482) was closely related to the sequence from Pakistan, Iran, Tunisia, and India (OP950282, KC599235, KM117212, and MG564223), respectively. DQ341370 was taken as an out-group from *Anaplasma marginale*.

Treatment

Bupralex® (Star Laboratories Pvt. Ltd.) and Oxtra LA® (FATRO) at 1ml/20kg and 1ml/20 kg, respectively, were injected I/M into buffalo, along with supportive treatment. Since the animal was brought very late for clinical intervention, it did not survive and died.

Discussion

Based on the clinical signs, microscopy, hematology, PCR, and sequencing results, the current report confirmed mixed infection of *T. lestoquardi* and *T. annulata* in buffalo. Infected buffalo was positive for theileriosis because typical intracellular pleomorphic piroplasms related to *Theileria* spp. were seen on microscopic examination. Buffalo showed 12.5% parasitemia, calculated following [22] in which they prepared 452 blood smears, observed 10,000 RBCs (40 fields) per slide, and applied the Ducan multiple range test in statistical analysis to determine the level of parasitemia in samples. Studies have shown

Figure 3**.** Phylogenetic investigation of *T. lestoquardi* based on 18S rRNA sequence

the level of parasitemia may range from 1% to more than 15% [23].

Complete blood count (CBC) values for hemoglobin (Hb), red blood cells (RBCs), hematocrit (Hct), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) were found to be lower than reference values, whereas the lymphocytes, neutrophils, and monocytes were observed higher during infection. Similar findings have been reported in buffalo by [23], they selected 600 buffaloes, declared 107 positive for theileriosis via blood smear examination, and performed a hematological analysis of *Theileria*-positive samples.

According to [25], the protozoan is responsible for the occurrence of anemia since the lysis of RBCs due to the multiplication of piroplasms in RBCs is one of the main causes of red cell injury, resulting in cell destruction.

Opting for cost-effectiveness, we first utilized RLB universal primers used to amplify the V4 region of the 18S rRNA gene and sequenced PCR product to define *Theileria* species infecting buffalo. Similar work has been done by [26] in which they first used RLB universal primers and then performed sequencing. After this, we used *Theileria annulata*-specific primers. The results of PCR and sequencing revealed mixed infection with both *T. lestoquardi* and *T. annulata*. Similar findings have been reported in Iran [27], in which mixed infection with *T. annulata* and *T. lestoquardi* in cattle was found. The obtained sequence in this study revealed a close identity to previously

deposited *T. lestoquardi* sequences from Pakistan, Iran, and India at GenBank database of NCBI. This highlights the need to understand the spread of transboundary animal diseases as elaborated by [28] in which they reported transboundary infection with *Theileria* spp. in imported cattle using PCR and sequencing.

In the present study, the buffalo was raised with sheep and probably contracted *T. lestoquardi* as they share common tick vectors [10]. There is a possibility of sheep acting as a reservoir of *T. annulata* and cattle acting as a reservoir of *T. lestoquardi* under field conditions. Also, the successful experimental transmission of *T. lestoquardi* to cattle and *T. annulata* to sheep using infected ticks suggests susceptibility of cattle to *T. lestoquardi* [15]. Buffalo might die of cardiac arrest caused by *T. lestoquardi* infection as previously reported in sheep [12]. These authors infected sheep with *T. lestoquardi* and reported its effects on the cardiovascular system under experimental conditions that *T. lestoquardi* causes degeneration of cardiac muscles. This may highlight the need to investigate the role played by dual infections in cases of frank clinical diseases of malignant ovine theileriosis and tropical theileriosis, both experimentally and under field conditions.

In conclusion, the buffalo was infected with two pathogenic *Theileria* species; *T. lestoquardi* and *T. annulata.* The buffalo later died because of the non-responsiveness of the treatment. The clinical importance of mixed *Theileria* infections in ruminants, whether natural single infections with *T. lestoquardi* can occur in buffalo. There is a need to find out the epidemiology and spread of ovine malignant theileriosis in the bovine population.

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