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Differential diagnosis of theileriosis through blood smear examination and polymerase chain reaction in small ruminants from Pakistan

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Abstract

Background: Ovine and caprine theileriosis is a tick-borne hemoprotozoan disease, caused by *Theileria* spp., responsible for heavy economic losses in terms of high mortality and morbidity rates. Diagnosis of ovine theileriosis is primarily based on clinical symptoms, microscopic screening of stained blood smears, and lymph node biopsy smears, but the limitations of these detection methods against *Theileria* spp. infection limits their specificity.

Aim: To overcome these limitations, the current study reports the differential diagnosis of theileriosis through a blood smear examination and polymerase chain reaction (PCR) in small ruminants from Pakistan.

Methods: The study was conducted on 1,200 apparently healthy small ruminants (737 sheep and 463 goats). First, blood smears were screened for the presence of *Theileria* piroplasms in red blood cells. Second, PCR amplification based on 18S rRNA gene was performed by using primers specific to *Theileria* spp.

Results: Out of the 1,200 samples of examined blood smears, 100 animals (8.33%) were found positive for *Theileria* species, which showed intra-erythrocytic bodies in the form of dot and comma shapes. Amplification of the isolated DNA from randomly collected blood samples of 737 sheep and 463 goats showed that an amplicon size of 1,098 bp was positive for *Theileria* spp. In total, 315 out of the 1,200 small ruminants examined in this study were found positive for *Theileria* spp. DNA through PCR amplification. Notably, out of the 885 blood samples negative by PCR amplification, only 15 blood samples were found positive by the blood smear test. Conversely, 230 blood samples that tested negative in the smear technique produced a specific band through PCR amplification. Overall, the sensitivity and specificity rates were 26.98% and 98.31% for the blood smear method and 73.01% and 100% for the PCR assay, respectively.

Conclusion: Our finding suggests that PCR is the gold standard method compared to the conventional method of smear examination for the diagnosis of ovine and caprine theileriosis in Pakistan.

Keywords: Ovine and caprine theileriosis, Differential diagnosis, Microscopic screening, PCR assay, Sheep and goats, Pakistan.

Introduction

Hemoprotozoan diseases are a significant threat to the livestock sector, affecting 80% of the world's ruminants and causing major losses in the dairy industry worldwide (Kohli *et al*., 2014). Among these diseases, Theileriosis is a tick-borne illness caused by the genus *Theileria* (Demessie and Derso, 2015) and characterized by distinctive organelles known as the apical complex (Gul *et al*., 2015). *Theileria* is obligate intracellular protozoan parasites belonging to the phylum Apicomplexa and order Piroplasmida, infecting both wild and domestic ruminants and spreading through ixodid ticks. The completion of their life cycle involves both vertebrate and invertebrate hosts, making it a significant problem in tropical and subtropical regions of the world (Sitotaw *et al*., 2014). Theileriosis continues to pose a significant constraint for small ruminants in Asia, as well as parts of Africa and Southern Europe (Mehlhorn and Schein, 1984). Recent studies have highlighted the continued prevalence and impact of this disease on domestic ruminant production, especially compared to cattle. In China, for example, a study by Yang *et al*. (2022) found that *Theileria* infections were common among goats,

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while a recent study by Rahravani *et al*. (2023) reported a high rate of *Theileria* spp. infection among small ruminants in Iran. These findings underscore the need for increased attention and management of Theileriosis in small ruminant populations.

Sheep and goats can be infected by six different species of *Theileria* (Razmi and Yghfoori, 2013). Among these, *Theileria lestoquardi*, *Theileria luwenshuni*, and *Theileria uilenbergi* are highly pathogenic, while *Theileria separata*, *Theileria ovis*, and *Theileria recondite* cause less severe disease in these animals (Razmi *et al*., 2003; Taha *et al*., 2013). *Theileria lestoquardi*, which causes malignant ovine theileriosis, can lead to several clinical signs in small ruminants, such as fever, anemia, lethargy, icterus, and hemoglobinurea (Sayin *et al*., 2009).

Theileriosis is mainly spread through the bites of ixodid ticks (Durrani *et al*., 2012). Countries like Pakistan, Bangladesh, and India have a suitable climate for the growth and spread of ixodid tick species that can transmit the disease (Irshad *et al*., 2010). For instance, *Rhipicephalus microplus*, *Rhipicephalus turanicus, Rhipicephalus annulatus*, and *Hyalomma anatolicum* are some of the tick species known to transmit *Theileria* parasites (Aung *et al*., 2022; Norouzi *et al*., 2022; Rahravani *et al*., 2023). The prevalence and distribution of these tick species in different regions can vary and affect the incidence of theileriosis in small ruminants (Kumar *et al*., 2023; Prajapati *et al*., 2023).

Theileriosis is typically diagnosed in acute cases through clinical symptoms and thin smears of blood and lymph nodes stained with Giemsa and Wright. Interestingly, Sallam *et al*. (2023) developed an enhanced methodology for classifying subtypes of acute lymphoblastic leukemia (ALL) using peripheral blood smear images. Their methodology includes image preprocessing, feature extraction, and the use of enhanced grey wolf pptimization algorithm to select the most important features characterizing the blood cells' histology. This algorithm achieves a high degree of accuracy, precision, and sensitivity in identifying the subtypes of ALL. However, clinical symptoms and thin smears are not useful for identifying carrier animals due to their low rate of parasitemia (Aktas *et al*., 2006). In cases of mixed infections, microscopy cannot confirm the presence of *Theileria* species, and therefore, the PCR assay has become the preferred technique for detecting ovine and caprine *Theileria* piroplasms for epidemiological analysis (Aktas *et al*., 2005).

The OIE recommends the use of serological tests, such as the indirect fluorescent antibody test (IFAT), to detect circulating antibodies against *Theileria* piroplasms. However, the IFAT test's specificity is limited due to the cross-reactivity of other piroplasms with the antibodies (Nourollahi-Fard *et al*., 2012). It is worth noting that long-term carrier animals, considered serologically negative, may be positive for *Theileria* species and can infect susceptible animals (Altay *et al*., 2012). This underscores the importance of using accurate and specific tests for detecting *Theileria* piroplasms to prevent their spread to susceptible animals.

Accurate and rapid detection of *Theileria* and *Babesia* species in carrier animals is crucial for controlling the spread of these pathogens. While traditional diagnostic methods such as microscopy and serology have limited sensitivity and specificity, a diagnostic method that offers higher accuracy and faster turnaround time is needed. Fortunately, the polymerase chain reaction (PCR) has emerged as a sensitive and specific molecular assay for detecting many pathogens, including several *Theileria* and *Babesia* species in carrier animals (Lew *et al*., 1997). PCR offers several advantages over traditional methods, including the ability to detect low levels of pathogens and the potential to identify multiple species in a single assay. Additionally, PCR can be performed in as little as one hour (Chauhan *et al*., 2015), making it a valuable tool for timely diagnosis and effective control of *Theileria* and *Babesia* infections.

In parasitological investigations, the conventional diagnosis method for identifying parasites is light microscopy, which relies on their morphological characteristics. However, this technique has limitations, particularly in differentiating between piroplasms, making it less practical for use (Quintao-Silva and Ribeiro, 2003; Shayan and Rahbari, 2005). Molecular techniques, such as PCR, have recently been employed for diagnosing hemoprotozoa that cause parasitic diseases. Unlike the conventional technique, PCR is not influenced by environmental conditions, and animals that recover from acute or primary *Theileria* infection remain positive for the protozoan and act as reservoirs, contributing to the transmission of theileriosis in other sheep and goats (Cacci *et al*., 2000). Thus, an accurate diagnosis of theileriosis in asymptomatic carrier animals is crucial for implementing effective control programs.

Despite the availability of molecular techniques, it is still important to compare their sensitivity and specificity with the conventional microscopy method for the detection of *Theileria* piroplasms in small ruminants in Pakistan. This study aims to assess the diagnostic performance of PCR amplification and microscopic smear techniques, as this will aid in developing more effective control strategies for theileriosis. The findings of this study are important for veterinarians, farmers, and policymakers as they provide critical information about the accuracy of different diagnostic methods for theileriosis. By identifying asymptomatic carrier animals, control measures can be implemented to prevent the transmission of the disease, ultimately reducing the economic impact of theileriosis on small ruminant production.

Materials and Methods

Blood sampling

About 1,200 blood samples were gathered from randomly chosen herds of seemingly healthy small ruminants in

Multan district, Pakistan (Fig. 1), for the purpose of detecting the presence of theileriosis. The blood samples were drawn into labeled glass tubes, with each tube having a reference number and the species of the corresponding small ruminant. Screening for *Theileria* infection was carried out using both blood smear microscopy and PCR amplification methods. The samples were then stored in a sterilized container and kept at −20°C in the laboratory for subsequent processing.

Microscopic examination

Blood smears were prepared in the field, following the guidelines outlined by the Tick Fever Research Center (TFRC, 1996). The smears were air-dried and fixed using methanol as a fixative. Subsequently, the smears were stained with Giemsa stain, diluted with water, and allowed to rest for approximately 30 minutes. To eliminate any excess stains, the smears were washed with water 3–4 times and air-dried. Detection of *Theileria* piroplasms in infected red blood cells was accomplished using a single drop of cedar wood oil, and the slides were examined under an oil immersion lens (Fig. 2). Morphological features, as defined by Soulsby (1982) and Urquhart *et al*. (1988), were used to identify *Theileria* piroplasms.

DNA extraction

For DNA extraction from blood samples, an inorganic method was used (Shaikh *et al*. 2004). Briefly, 750 µl of blood and 750 µl of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0) were taken in an Eppendorf tube, mixed by vortex, and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and a washing step was repeated 2–3 times to remove hemoglobin from the white blood cell pellet. A total of 600 ml of Tris-NaCl-EDTA (TNE) buffer (10 mM Tris HCl, 2 mM EDTA, 400 mM NaCl), 20 µl of 10% sodium dodecyl sulfate (SDS), and 1 µl Proteinase K was added for protein digestion after incubation overnight at 37°C. For the precipitation of digested proteins, 1 ml of 5 M NaCl was added, shaken vigorously, and chilled on ice for 15 minutes. An equal volume of chilled isopropanol was added to a new Eppendorf tube containing supernatant; the DNA fiber was appeared after shaking the mixture in the Eppendorf tube. The DNA fiber was pelleted after centrifugation at 13,000 rpm for 5 minutes. A washing step was performed on the DNA pellet by using 70% ethyl alcohol and finally, the DNA was dried and then dissolved in TE buffer for further use. The DNA quality was evaluated by analysis with a spectrophotometer at 260/280 nm density constant and gel electrophoresis method by using a 1% concentration agarose gel.

PCR amplification

Extracted DNA was used for PCR amplification, by using primer set 989F: 5′-AGTTTCTGACCTATCAG-3′ and 990R: 5′-TTGCCTTAAACTTCCTTG-3′, of a 1,098 bp fragment of the small subunit rRNA gene of *Theileria* genus as described by Allsopp *et al*. (1993). The PCR reaction mixture comprising 5 µl template DNA (50–150 ng), 5 µl of 10× PCR buffer (100 mM Tris–HCl (pH 9) 500 mM KCl, 1% Triton X-100), 5 µl 50 Mm $MgCl_2$, 6 µl of all 4 dNTPs, 4 µl each primer (Penicon) at a concentration of 10 pmol/µl, 2 U of Taq DNA polymerase (Vivantis) and 20.5 µl distilled water for total final reaction mixture of 50 µl. The

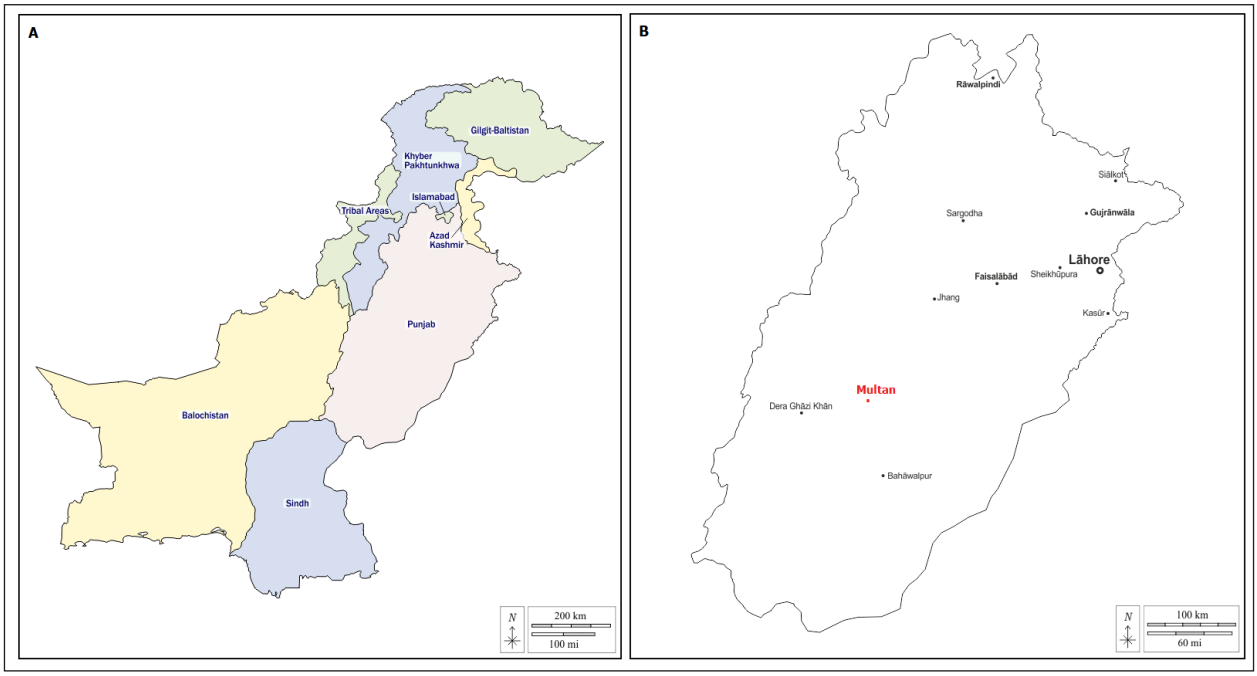

Fig. 1. Map of the Pakistani studied region. (A) Map of Pakistan showing Punjab province. (B) Map of Punjab showing the Multan district.

PCR amplification was done using BIORAD thermal cycler. PCR tubes were incubated at 94°C for 5 minutes to denature genomic DNA, followed by 45 cycles at 94°C for 1 minute for DNA denaturation, 60°C for 1 minute for primer annealing, and 72°C for 1 minute for elongation. The PCR reaction was ended with a final extension step at 72°C for 7 minutes. An agarose gel with a concentration of 1.5% was used for the separation of the amplified PCR product. The PCR product was envisioned by using ethidium bromide under a UV-illuminator and compared with a 100–2,000 bp DNA ladder (Vivantis) (Fig. 3).

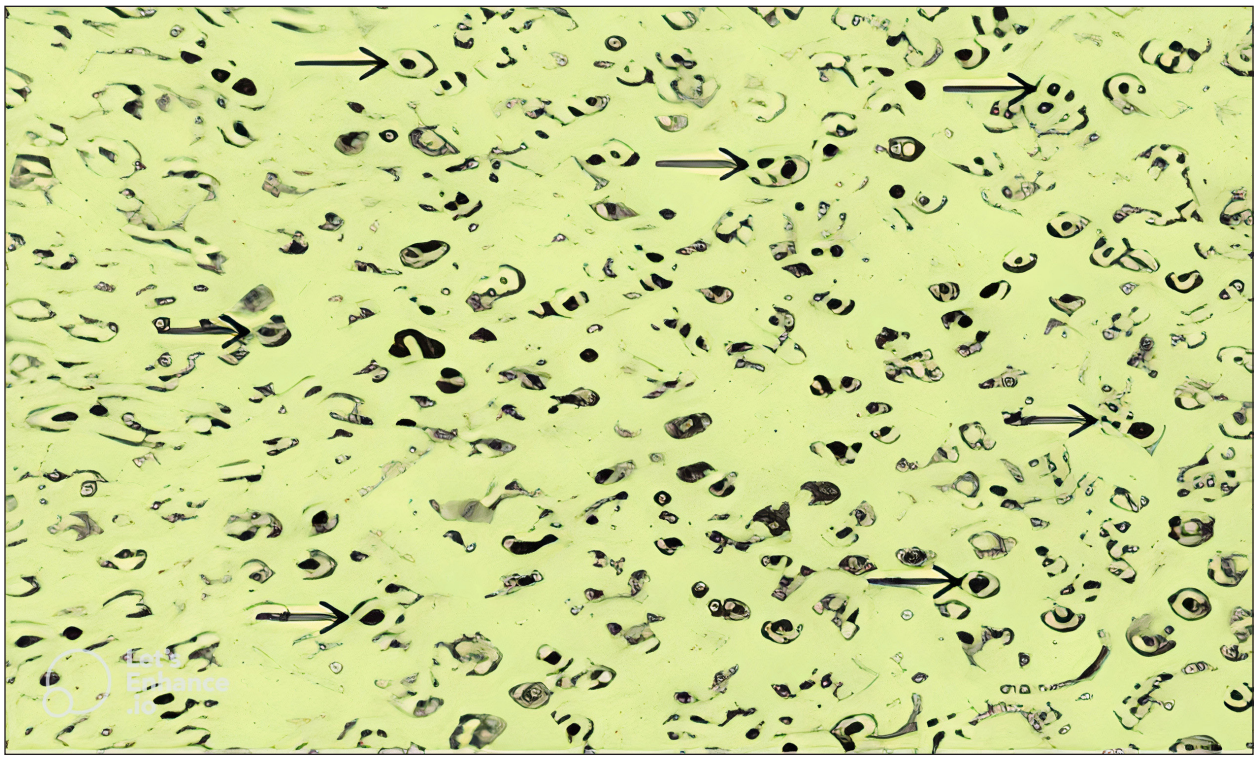

Fig. 2. Blood smears showing infection of red blood cells of sheep with *Theileria* schizonts.

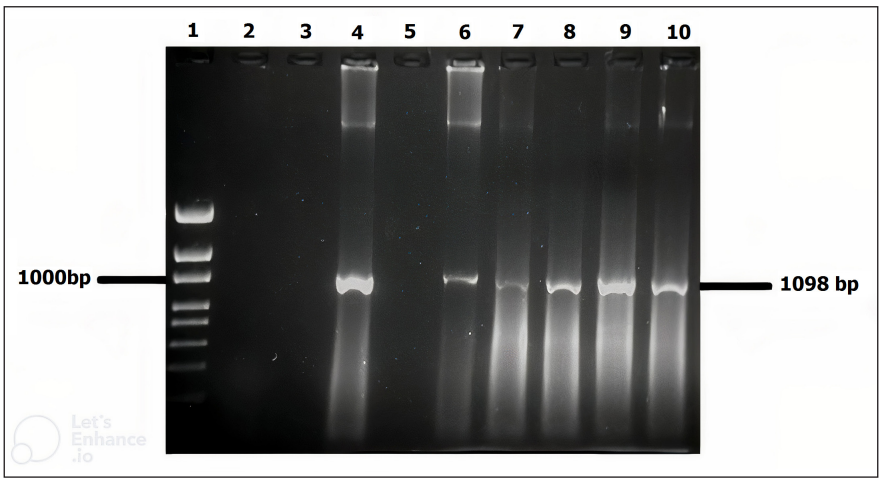

Fig. 3. Molecular detection of *Theileria* spp. by PCR amplification of a partial sequence (1,098 bp) of 18S rRNA gene. Lines 1: 1kb ladder; lines 2 and 3: PCR results of negative controls by using free nuclease water and *Theileria* spp. negative control, respectively, line 4: PCR result of positive control by using *Theileria* spp. positive control, line 5: PCR product of a field sample negative to *Theileria* spp., and lines 6–10: PCR products of field samples positive to *Theileria* spp.

Sensitivity and specificity of PCR and microscopy

The sensitivity and specificity values for the PCR amplification diagnosis method were estimated according to Anderson *et al*. (1980) by using the following correlations:

Sensitivity = true positive/true positive + false negative × 100.

Specificity = true negative/true negative + false positive × 100.

Ethical approval

Ethical Research Committee of the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan approved all the experimental procedures and protocols applied in this study.

Results

Infections caused by *Theileria* spp. were detected in the blood smears of animals, where intra-erythrocytic bodies with dot and comma shapes indicated the presence of *Theileria* schizonts (Fig. 2). Out of 1,200 samples of examined blood smears, 100 (8.33%) animals were positive for *Theileria* species (Fig. 2).

Amplifications of the isolated DNA from randomly collected blood samples of 737 sheep and 463 goats showed an amplicon size of 1,098 bp which is considered positive for *Theileria* spp. (Fig. 3). Out of the 1,200 small ruminants examined by PCR in this study, *Theileria* spp. DNA was detected in 315 animals (26.25%) (Table 1).

A total of 230 blood samples were detected negative in smear technique had produced specific band by PCR amplification while, out of 885 blood samples negative by PCR amplification, only 15 blood samples were found positive by a blood smear test. The sensitivity and specificity were 26.98% and 98.31% for the blood smear method, and 73.01% and 100% for the PCR test, respectively (Table 2).

Discussion

Ovine and caprine theileriosis is a tick-borne hemoprotozoan disease that is caused by *Theileria* spp. and is responsible for causing significant economic losses due to high mortality and morbidity rates. The diagnosis of ovine theileriosis is primarily based on clinical symptoms, microscopic screening of stained blood smears, and lymph node biopsy smears. However, these detection methods have limitations against *Theileria* spp. infection that restricts their specificity. Therefore, to overcome these limitations, the current study reports the differential diagnosis of theileriosis in small ruminants from Pakistan using both blood smear examination and PCR

A total of 1,200 blood samples from small ruminants were randomly examined to diagnose theileriosis. The prevalence rate was found to be 26.25% by PCR, compared to 8.33% by microscopy. Of the 230 blood samples that were negative in the smear technique, specific band production was observed by PCR amplification. Conversely, out of the 885 blood samples negative by PCR amplification, only 15 were found positive by blood smear test. The sensitivity and specificity of the blood smear method were 26.98% and 98.31%, respectively, while the PCR test had a sensitivity and specificity of 73.01% and 100%, respectively. These findings indicate that PCR amplification is a more sensitive method than microscopy for diagnosing *Theileria* piroplasms in infected small ruminants (Table 1).

Giemsa staining is a commonly used method for identifying ovine and caprine theileriosis worldwide due to its simplicity and affordability. However, microscopy is not sensitive enough to diagnose carrier animals with low parasitemia. Serological tests are also not reliable since *Theileria* antibodies disappear quickly, leading to negative results in carrier animals with low parasitemia (Aktas *et al*., 2006). These animals could potentially spread *Theileria* piroplasms through the animal-tick life cycle. Therefore, PCR-based molecular diagnosis is better for understanding the status of *Theileria* spp. infection. Several studies have reported that PCR assay is more specific and sensitive than microscopy (Oliveira-Sequeira *et al*., 1995; Kirvar *et al*., 1998; Martin-Sanchez *et al*., 1999; Nagore *et al*., 2004; Altay *et al*., 2005; Altay *et al*., 2008; Taha *et al*., 2010). This method allows for direct, accurate, and sensitive identification of piroplasms even in carrier small ruminants with a low

Table 1. Comparison between blood smear examination and PCR amplification.

Methods	Screened animals (%)	Positive for theileriosis (%)	Negative for theileriosis (%)
Blood smear	1,200 (100)	100 (8.33)	1,100 (91.66)
PCR	1,200 (100)	315 (26.25)	885 (73.75)[a]

[a]Variability in the prevalence of theileriosis between the two diagnostic methods was statistically significant ($p < 0.05$). The chi-square value is 298.2.

Table 2. 2 × 2 contingency for blood smear examination and PCR.

Blood smear	PCR		Total
	Positive (Rate, %)	Negative (Rate, %)	
Positive	85[a] (26.98)	15[b] (98.31)	100
Negative	230[c] (73.01)	870[d] (99)	1,100
Total	315 (100)	885 (100)	1,200

[a] True positive.
[b] False positive.
[c] False negative.
[d] True negative.

parasitemia level that show no symptoms of *Theileria* infection.

Previous studies on ovine and caprine *Theileria* spp. infection have shown higher prevalence rates when diagnosed by PCR compared to microscopy. For example, in Turkey, Altay *et al*. (2005) reported rates of 54.03% and 19.35%, while in Iran, Heidarpour *et al*. (2009) reported rates of 56% and 21%, Heidarpour *et al*. (2010) reported rates of 60% and 22.27%, Yaghfoori *et al*. (2013) reported rates of 76% and 46%, and Jalali *et al*. (2014) reported rates of 89% and 69.7%. However, differences in prevalence rates could be due to various factors such as differences in diagnostic methods, the number of animals tested, sampling procedures, parasitemia and immunity levels in infected animals, as well as abiotic factors such as bioclimatic and ecological conditions, and host management. These factors can influence the spread of ticks and tick-borne diseases and therefore cause variations in prevalence rates (Kivaria, 2006; Zangana and Naqid, 2011).

The difference in *Theileria* infection rates found using microscopy and PCR can be attributed to the limited sensitivity of microscopy to detect low levels of parasitemia in carrier animals (Aktas *et al*., 2007; Altay *et al*., 2007). Microscopy can only diagnose up to 1 infected cell per 10,000 cells by investigating at least 100–200 fields, and at least 0.5 µl of blood is required for such examination (Mosqueda *et al*., 2012). On the other hand, PCR is more sensitive and can detect even a single infected cell in 107 erythrocytes, which is equivalent to a blood parasitemia of 0.00001% (Altay *et al*., 2007). Due to this limitation, carrier animals with low levels of parasitemia may not be detected by microscopy, whereas PCR can detect the presence of the parasite even in carrier animals without any symptoms of *Theileria* infection.

Results of this study indicate that small ruminants like sheep and goats could act as a host for *Theileria* spp. infection and they may have a lower level of parasitemia that could go unnoticed by the traditional microscopy technique. If left untreated, the infection can cause anemia, weight loss, reduced milk production, and even death in the infected small ruminants. However, treating the suspected animals with unrelated drugs, especially antibiotics, can lead to unnecessary medication and residues in milk and dairy products that may ultimately result in the rejection of their products in the international market (Chauhan *et al*., 2015).

During this study, specific primers were used to identify the *Theileria* genus, which amplified a 1,098 bp fragment of the 18S rRNA gene in 315 blood samples obtained from sheep and goats (Fig. 3). The PCR technique used for the molecular diagnosis of blood samples proved to be more sensitive and specific compared to the traditional smear screening method. This technique is especially useful for identifying subclinical infections of theileriosis in small ruminants. In field conditions, subclinical theileriosis infections in small ruminants were not detected by microscopy but were picked up by PCR, indicating its effectiveness as a molecular diagnostic tool for carrier small ruminants.

Conclusion

In conclusion, the study highlights that the diagnosis of ovine and caprine theileriosis in Pakistan should shift from symptom-based detection to DNA-based identification. The use of PCR amplification based on the 18S rRNA gene is a more reliable and accurate method for detecting *Theileria* spp. compared to the conventional method of blood smear examination. This shift from symptom-based detection to DNA-based identification can provide a significant improvement in the diagnosis of *Theileria* spp., reduce drug wastage, and curtail the development of drug resistance. Therefore, the use of PCR should be implemented as the gold standard method for the diagnosis of ovine and caprine theileriosis in Pakistan.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

M.R. designed this study, collected samples and epidemiological data, and performed the molecular and microscopic diagnosis. M.R. and M.B.S performed the statistical analysis. M.R. and M.B.S. wrote the manuscript, and N.N., A.K., and MBS edited it. M.B.S finalized the manuscript and all the authors approved the final version.

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