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Molecular investigations on outbreaks of ovine theileriosis among sheep and goats in Haryana, India

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Abstract

During February 2020 – October 2020, four outbreaks of theileriosis in small ruminants were recorded with overall morbidity, mortality and case fatality rates of 27.95 %, 17.46 % and 62.5 % respectively. The disease was characterized by high fever (up to 106°F), superficial lymphadenopathy, anaemia, anorexia, lethargy, respiratory distress and death. The presence of pleomorphic intra-erythrocytic piroplasms of *Theileria* species in Giemsa's stained blood smears was a common finding in all the episodes. Significant haematological alterations including high total leucocyte count, and low haemoglobin and packed cell volume were characteristic. Necropsy findings of the icteric liver, enlarged spleen, pulmonary oedema and abomasal ulcerations were observed in two flocks. *Theileria* spp in all four episodes was detected in smear-positive cases by PCR using genus-specific 18S rRNA primers that yielded a 1098 bp product. Further *Theileria* species was confirmed by using specific primer sets for *T. lestoquardi, T. luwenshuni, T. uilenbergi* and *T. ovis. T. lestoquardi* was detected in all four flocks while there was co-infection of *T. ovis* in two flocks. Phylogenetic analysis revealed that *T. ovis* and *T. lestoquardi* identified in this study had 100 % and ~99.86 % homology, respectively with the published sequences used for comparison. This is the first confirmed report of outbreaks of malignant ovine theileriosis in the Haryana state of India which caused high morbidity, mortality and case fatality among sheep and goats. Further studies on theileriosis in small ruminants are required to understand epidemiology better.

Introduction

Small ruminants play a significant role in the rural economy and livelihoods of millions of rural people in developing countries, including India. Mainly small and marginal farmers rear these animals as their sole source of income. According to 20th Livestock Census 2019, number of sheep and goats in India are 233.14 million, forming 4.16 per cent of the total livestock population (http://dahd.nic.in). Parasitic infections in sheep and goats are considered as one of the major obstacles in animal productivity. Amongst the different parasitic diseases, vector-borne diseases are responsible for serious health concerns.

Ovine theileriosis causes great economic losses to the farmers due to high morbidity and mortality. The disease also has a significant impact on the international trade of animals and animal products (El Imam et al. 2015). It is a tick-borne haemo-protozoan disease caused by various species of *Theileria* (Uilenberg 1995). *T. lestoquardi*, *T. uilenbergi* and *T. luwenshuni* are the most virulent species in sheep and goats. *T. lestoquardi* causes malignant ovine (or Small Ruminant) theileriosis, whereas, the disease caused by *T. luwenshuni* and *T. uilenbergi* is known as Cervine theileriosis as these agents also infect some cervids. Other species of *Theileria* including *T. ovis* also infect small ruminants, but without typical clinical signs, (Spickler 2019). The disease has been reported from many countries including China (Yin et al. 2008; Ge et al. 2012), Britain (Phipps et al. 2016), Turkey (Altay et al. 2007; Karatepe et al. 2019), Oman (Tageldin et al. 2005), Sudan (Taha et al. 2011), Iran (Hakimi et al. 2019) and India (Shruthi et al. 2017; Dhaygude et al. 2020). The disease is transmitted mainly by *Hyalomma anatolicum* ticks but *Haemaphysalis* and *Rhipicephalus* ticks have also been reported as vectors (OIE 2019; Shruthi et al. 2017). Bovine theileriosis has been extensively studied but there is a dearth of information on epidemiological and clinical aspects of the disease in small ruminants, sheep and goats in particular. Ovine theileriosis has been reported from India (Harish et al. 2006; Anumol et al. 2011; Tayo et al. 2011; Velusamy et al. 2015; Sahu et al. 2016 and Das 2017). These reports are based on microscopic examination of thin blood smears. Carrier status and subclinical infections cannot be detected by microscopic examination of blood smears, and this technique is even insufficient to differentiate between different species due to morphological similarities. These issues can be addressed with molecular approaches and have been used to study the disease (Shruthi et al. 2017; Mamatha et al. 2017; Begam et al. 2019; Nagaraj et al. 2019; Dhaygude et al. 2020). The present manuscript includes the investigation of outbreaks of malignant theileriosis in sheep and goats with both conventional blood smear microscopy and PCR technique, and confirm its identity by nucleotide sequencing analysis.

Material And Methods

The epidemiological investigation, study area and time

Haryana is a northwestern state of India that spanning from 27.65° N, 74.47° E, to 30.93° N, 77.60° E. This region has tropical monsoonal climate with four distinct seasons. During February 2020 – October 2020 four flocks of sheep and goats at different locations in Hisar district of Haryana state showed high morbidities and mortalities (Fig. 1). The outbreaks were named as A, B, C and D for further reference in the manuscript. The breed of sheep and goat were local and crosses (Rambouillet and Nali). The affected animals at all four units were clinically examined and the data regarding population at risk, morbidity and mortality was collected. Peripheral blood samples were collected in EDTA coated vials from 11 ailing cases recorded at different outbreaks. Post-mortem examinations of fallen sheep from outbreaks A and D were also conducted, gross lesions recorded and various samples collected which include heart blood, liver, lung, abomasum, intestine and lymph nodes.

Laboratory investigation

Haematological Analysis: Giema's stained blood smears prepared from the affected animals were examined microscopically for blood parasites. Haematological analysis was carried out using an automatic haematological analyzer (MS4Se-Melet Schloesing Laboratories, France).

Histopathological examination: The samples collected in 10% buffered formalin from fallen animals were processed for histopathology using standard histopathological techniques as described by Luna (1968).

Molecular detection of pathogen

Deoxyribonucleic acid extraction: Deoxyribonucleic acid (DNA) was extracted from the clinical samples (blood samples, n = 11) using DNeasy® mini kit (Qiagen, Germany) following the manufacturer's instructions. DNA concentration was estimated with BioSpectrometer® (Eppendorf, Germany).

Polymerase chain reaction: A polymerase chain reaction (PCR) was carried out on the extracted DNA using *Theileria* 18S rRNA genus-specific primers. Further, to confirm *Theileria* species, *Theileria* species-specific primers were used. Various primers used in the study along with their amplification conditions and expected amplicon sizes are listed in Table 1. The PCR reaction comprised of 2X TopTaq master mix (12.5 µl, Qiagen), 10 pmol of each forward and reverse primers, template DNA (~ 150 ng) and nuclease-free water to make a total volume of 25 µl. All the amplifications were carried out in BioRad® Thermocycler. The amplified products were separated by electrophoresis on 1.2% agarose gel. DNA ladders of 100 bp and/or 1000 bp were also run with the products and visualized in Gel Documentation apparatus (Biozen, India).

Controls: Five samples from sheep and goats affected with diseases other than theileriosis were taken as control, Control 1: PPR; Control 2: CCPP; Control 3: Enterotoxaemia; Control 4: Sheeppox; and Control 5: Pasteurellosis.

Table 1
Primers and amplification conditions used in the study.

Target gene	5'-3' Sequence	Product size (bp)	Amplification conditions Initial denaturation-(denaturation-annealing-extension)×no. of cycles-Final extension	Reference for Primers used
Theileria sp. 18S rRNA gene	F-AGTTTCTGACCTATCAG R-TTGCCTTAAACTTCCTTG	1098	95°C/5 min-(95°C/30 sec-60°C/30 sec-72°C/1 min) ×35–72°C/10 min	Allsopp et al. 1993
Theileria lestoquardi small subunit ribosomal RNA gene	F-CTGCTGCATTGCTTGTGTCC R-TCTAAGGGCATCACAGACCT	787	95°C/5 min-(95°C/30 sec-61°C/35 sec-72°C/1 min) ×35–72°C/7 min	Based on Genbank Acc. No. AF081135.1
Theileria luwenshuni small subunit ribosomal RNA gene	F-ATTGGAGGGCAAGTCTGGTG R-CGATCACGGGACAGCAAAAG	812	95°C/5 min-(95°C/30 sec-62°C/35 sec-72°C/1 min) ×35–72°C/7 min	Based on Genbank Acc. No. MG930124.1
Theileria uilenbergi 18S ribosomal RNA	F-TGACACAGGGAGGTAGTGAC R-CTCCCGCACCCTATTTAGCA	878	95°C/5 min-(95°C/30 sec-60°C/35 sec-72°C/1 min) ×35–72°C/7 min	Based on Genbank Acc. No. AY262121.1
Theileria ovis small subunit ribosomal RNA gene	F-TCGAGACCTTCGGGT R-TCCGGACATTGTAAAACAAA	520	95°C/5 min-(95°C/30 sec-57°C/35 sec-72°C/1 min) ×35–72°C/7 min	Altay *et al.*, 2005

Sequencing: Representative PCR purified products were got sequenced. The Blast analysis of the obtained sequences was performed on the NCBI GenBank database with other published sequences. Sequences were accessioned in NCBI with accession numbers MZ604318, MZ604319, MZ604320, MZ604321, MZ604123 and MZ604124.

Phylogenetic analysis: The obtained sequences were aligned to the published sequences deposited in the GenBank database and phylogenetic analysis was carried out using MEGA 11 software. The phylogenetic tree was constructed by using the Maximum Composite Likelihood method with 1000 bootstrap replicates.

Results

Epidemiological description

Outbreaks of suspected theileriosis were recorded in sheep and goat flocks during February – October 2020 at four different locations of the Haryana state of India. The first outbreak occurred at an organized sheep farm while the remaining three were recorded in backyard sheep and goat flocks. Outbreak A occurred in a farm which had a total of 4500 sheep but the disease was recorded in a particular lot of pregnant ewes only which were maintained at a distance from other flocks. The characteristics of outbreaks *viz.* total number of animals, number affected, died etc. are detailed in Tables 2 and 3. The overall morbidity rate, cumulative mortality and case fatality rate due to theileriosis at all four places were 27.95%, 17.46%, 62.5%, respectively.

Table 2
Characteristics of the outbreaks of theileriosis in small ruminants investigated

Outbreak	Month of occurrence of outbreaks	Place	Lat-Long	Species	Total no. of susceptible animals	No. of animals affected	No. of animals Died
A	Feb. 2020	Bir, Hisar	29.26N-75.72E	Sheep	70	47	25
B	Aug 2020	Budana, Hisar	29.24 N-76.11E	Goat	32	5	5
C	Aug 2020	Lohari Ragho, Hisar	29.24N-76.13E	Sheep/Goat	49 (39-Sheep; 10-Goat)	3 (Sheep)	1 (Sheep)
D	Oct 2020	Matarsham, Hisar	29.18N-75.59E	Sheep	78	9	9

Table 3
Morbidity, mortality and case fatality rates in outbreaks

Outbreaks	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
A	2.22	0.5	25
B	15.62	15.62	100
C	6.12	2.0	33.33
D	11.5	11.5	100
Cumulative	27.95	17.46	62.5

Clinical findings, haematology and gross pathology

In all the outbreaks, animals either were infested by ticks or had history of tick infestation. Ticks collected from affected animals of Outbreak A were identified as *Hyalomma anatolicum*. Affected animals (n = 64) in all the four outbreaks under investigation exhibited high fever (up to 106°F), dullness, anaemia, anorexia, weakness, emaciation, respiratory distress, occasional diarrhoea, recumbency and death. The course of disease observed was 4–5 days. The presence of intra-erythrocytic piroplasms of *Theileria* species in Giemsa's-stained blood smears was a common finding in all the farms under study (Fig. 2). Piroplasms observed were pleomorphic, appearing as ring, oval, round or rod-shaped. Impression smears prepared from superficial lymph nodes collected during postmortem examination (3 animals from Outbreak A and one each from Outbreaks C and D) did not reveal sporozoite/schizont. Animals that were tested positive for *Theileria* organisms had low haemoglobin concentration (ranging from 4.9 to 8.2 g/dl), low PCV (17.7 to 28.3%) and high total leukocyte count ($17.7 \times 10^3 - 54.7 \times 10^3$) (Table 4).

Table 4
Haematological findings in different outbreaks

Outbreak	Sample ID	Blood Profile						
		Hb (g/dL)	PCV (%)	TEC (millions/cumm)	TLC (/cumm)	G (%)	L (%)	M (%)
A	A1	6.1	21.5	5.38	54720	52	44	4
	A2	4.9	17.7	5.45	52480	58	39	3
	A3	6.6	24.6	5.58	33940	65	34	1
	A4	5.6	19.7	4.97	35890	42	55	3
	A5	6.3	23	5.53	53410	41	54	5
B	B1	8.2	28.3	6.24	19210	61	37	2
	B2	5.8	27.1	5.92	18870	58	39	3
	B3	7.6	26.4	5.83	21830	66	32	2
C	C1	5.4	ND	ND	18400	27	69	4
	C2	6.3	ND	ND	20040	23	73	4
D	D1	7.4	22.1	5.49	17750	47	52	1
Mean ± S.D.		6.38 ± 1.00	23.38 ± 3.53	5.60 ± 0.36	31.50 ± 15.43	49.09 ± 14.62	48.00 ± 13.85	2.91 ± 1.30

G = Granulocyte, L = Lymphocyte, M = Monocyte, ND = Note determined

Post-mortem findings in fallen animals (3 animals from Farm A and one each from Farm C and D) were enlarged superficial lymph nodes, deep yellow coloured omentum, icteric liver, enlarged spleen, congested tracheal mucosa, oedematous and consolidated lungs. Punched ulceration of abomasal mucosa was also noticed in Farm C.

Histopathological examination of tissues collected during post-mortem examination revealed focal areas of necrotic foci in the liver with mild mononuclear cells infiltration in the portal triad region while lungs showed severe congestion, interlobular oedema and mild mononuclear infiltration in the interstitial tissue. Intestinal mucosa had mild polymorphic cells (neutrophils/eosinophils) in the mucosa and mesenteric lymph nodes had congestion, neutrophilic infiltration and reticuloendothelial cell proliferation.

Polymerase chain reaction analysis

All the blood samples, which were diagnosed as *Theileria* positive by microscopic examination, yielded a product of 1098 bp size by PCR using *Theileria* 18S rRNA genus-specific primers (Fig. 3). No amplification was observed in negative controls. For species confirmation, primer sets specific for *T. lestoquardi, T. luwenshuni, T. uilenbergi* and *T. ovis* were used. *T. lestoquardi* was detected in all four outbreaks while concurrent infection of *T. ovis* was found in outbreaks A and B (Fig. 4 and Fig. 5).

Sequencing and phylogenetic analysis: Phylogenetic analysis of the partial sequences obtained in the present study, revealed that the detected sequences were homologous to the corresponding published sequences of *T. lestoquardi* and *T. ovis* (Fig. 6 and Fig. 7). The sequences on 16S rRNA of the four strains of *T. ovis* of this study were identical to each

other and shared 100% nucleotide sequence similarity with *T. ovis* isolates from China (Genbank Accession nos. FJ603460.1 and MF769699.1), Tunisia (Genbank Accession no. KM924444.1), Iraq (Genbank Accession nos. KR094863.1, MW735694.1 and MN544931.1), Turkey (Genbank Accession no. MN493111.1 and KT851437.1) and Saudi Arabia (Genbank Accession nos. - MG738322.1 and MG738322.1) and 99.84% similarity with Spanish isolate (accession no. AY533144.1). One of the study sequences of *T. lestoquardi* (Genbank Accession no. MZ604320.1) showed 100% homology with the published sequences from India (Accession nos. MZ220437.1, MZ220436.1 and MZ220435.1) and abroad (Accession nos. MT318171.1, MN544936.1, AF081135.1 etc.). Whereas, other obtained sequence of *T. lestoquardi* (Genbank Accession no. MZ604321.1) shared ~ 99.66 to 99.86% sequence identity with other published sequences of *T. lestoquardi* both from India and abroad. The identified sequences in this study did not share homology with published sequences of *T. uilenbergi* and *T. luwenshuni*. Therefore, the *Theileria* sp. identified in this study may be classified as *T. lestoquardi* and *T. ovis*.

Response to treatment

Once the disease was diagnosed, the ailing animals were treated with Buparvaquone @ 3.5 mg/kg body weight, Ivermectin @ 200 μg/kg body weight, antipyretics and hematinic. Animal responded favourably to the treatment and recovery was fast. Mamatha et al. (2017) also successfully treated clinical theileriosis in sheep with Buparvaquone.

Discussion

Conducive climatic conditions favour the tick population and increase the risk of tick-borne diseases in tropical countries like India. Extensive grazing practices further enhance the risk among sheep and goats. Several workers have reported theileriosis in small ruminants based on microscopic examination of blood smears from many countries (Altay et al. 2007; Fatima et al. 2015; Osman et al. 2017) and a few from India (Anumol et al. 2011; Tayo et al. 2011; Velusamy et al. 2015; Sahu et al. 2016; Das 2017; Begam et al. 2019 and Jayaram et al. 2019). Reports indicated the microscopy-based prevalence of theileriosis was as high as 20% in sheep and 68% in goats (Jayaram et al. 2019; Shruthi et al. 2018). However, reports on species identification are scarce in India. Hence, data on prevalent pathogenic species are less. Six different species have been reported from sheep and goat viz. *T. lestoquardi, T. uilenbergi, T. luwenshuni, T. ovis, T. separata* and *T. recondite*, among which *T. lestoquardi*, *T. uilenbergi* and *T. luwenshuni* have been described as highly pathogenic (Friedhoff, 1997; Ahmed et al. 2006; Yin et al. 2008). Recently, Nagaraj et al. (2019) reported prevalence of *T. ovis, T. luwenshuni* and *T. lestoquardi as* 75.28%, 32.58% and 7.86%, respectively in apparently healthy goats in Kerala, India indicating carrier status of infection. There are no documented reports on clinical disease due to *T. lestoquardi* from India.

In the present study, the overall morbidity rate, cumulative mortality and case fatality rate was around 27.95%, 17.46%, 62.5%, respectively. High morbidity and mortality rates in this report could be attributed to the more deaths in Farm A which may be due to pregnancy stress (Osman et al. 2017). Morbidity, mortality and CFR were more in sheep in comparison to goats in the present study. Moreover, *T. Lestoquardi* infection has been reported to cause heavy loss with high morbidity and mortality in sheep (Ghali and Hussein 1995; Tageldin et al. 1992) and low susceptibility of goats to the pathogen (Brown et al. 1998). However, in Sudan, Taha et al. (2011) reported high mortality of 72.7% in goats suffering with malignant ovine theileriosis due to *T. lestoquardi*. A previous study by Al-Hamidhi et al. (2021) indicated that the lone infection of *T. lestoquardi* (pathogenic) resulted in higher lethalities in comparison to coinfection with *T. ovis* (non-pathogenic) which might be due to possible competitive interaction between the parasites in mixed-parasite infection. It reflected that *T. ovis* guards against the pathogenicity of *T. lestoquardi* infection.

Clinical presentation of high fever, anorexia, lymphadenopathy, anaemia with pale mucus membrane and weakness in affected animals was consistent in all the farms. Similar clinical observations were made in small ruminants by Taha et

al. (2011). The affected animals were anaemic with Low haemoglobin, TEC, PCV values and high TLC count. Nazifi et al. (2012) and El Imam et al. (2015) also reported anaemia and low PCV due to erythrocyte destruction in sheep infected with *T. lestoquardi*, whereas marked leucopenia was reported in contrast to our findings. ME detected intraerythrocytic piroplasms which were pleomorphic under microscopy. Mamatha et al. (2017) and Dhaygude et al. (2020) also observed pleomorphism in piroplasms. The predominance of piroplasms over Koch blue bodies may be due to the late stage of the disease (Dhaygude et al. 2020).

At necropsy, some affected sheep showed enlargement of lymph nodes, enlarged and icteric liver, mucosal ulceration of abomasum and lung oedema. Histopathological findings of focal necrotic foci in the liver, interstitial oedema of the lung, enteritis with mononuclear infiltration and lymphadenopathy were in agreement with gross pathology. Tageldin et al. (2005) also reported similar necropsy and histopathological findings in affected sheep.

All the samples which were positive by ME were found positive by PCR for *Theileria* spp. targeting 18s rRNA gene. In order to differentiate the species of *Theileria* organism, species-specific PCR was used which confirmed the presence of *T. lestoquardi* in all the episodes while mixed infection *T. lestoquardi* with *T. ovis* in two outbreaks (farms C and D). Other *Theileria* species infective to small ruminants viz. *T. luwenshuni* and *T. uilenbergi* were not detected. Sequence analysis of PCR products further confirmed and revealed 100% homology with *T. lestoquardi* and *T. ovis*. This is the first confirmed report of outbreaks of malignant ovine theileriosis in the Haryana state of India which caused high morbidity, mortality and case fatality among sheep and goats. Most of the previous studies in India concluded that small ruminant theileriosis was due to *T. hirci*, merely based on microscopic examination of blood smears without any species level differentiation studies (Rao et al. 1991; Sasmal et al. 1982; Gautham et al. 1975).

The phylogenetic analysis of partial sequences of the small subunit of ribosomal RNA revealed that the identified sequences of *T. ovis* and *T. lestoquardi* shared the closest evolutionary relationship with the Asian isolates. The occurrence of theileriosis caused by *T. hirci* in sheep and goats in India has been documented in previous reports (Ramanujachari and Alwar 1954; Rao et al. 1991; Sasmal et al. 1982). This study confirms the conclusions which were drawn in previous findings based on microscopy.

During the study. *H. anatolicum anatolicum* was identified as tick in affected animals. However, further research is required to study the possibility of other ticks in the transmission of *T. lestoquardi* and *T. ovis*.

Despite the apparent clinical presentation of theileriosis in affected animals *viz*. anaemia, fever, superficial lymph node enlargement and unresponsive to antibiotic therapy, the disease went undiagnosed by the field veterinarian because theileriosis in small ruminants was not considered while making a differential diagnosis. The present investigation underscores the requirement of systemic studies to know the prevalence and vectors. In conclusion, this study improves our understanding of theileriosis disease in small ruminants.

Declarations

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All authors read and approved the final manuscript.

Conflict of interest

All the authors declare that we have no conflict of interest.

Ethical approval

The study was undertaken on the affected animal brought for disease investigation to the Disease Investigation laboratory at LUVAS, Hisar and did not require ethical approval.

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Figures

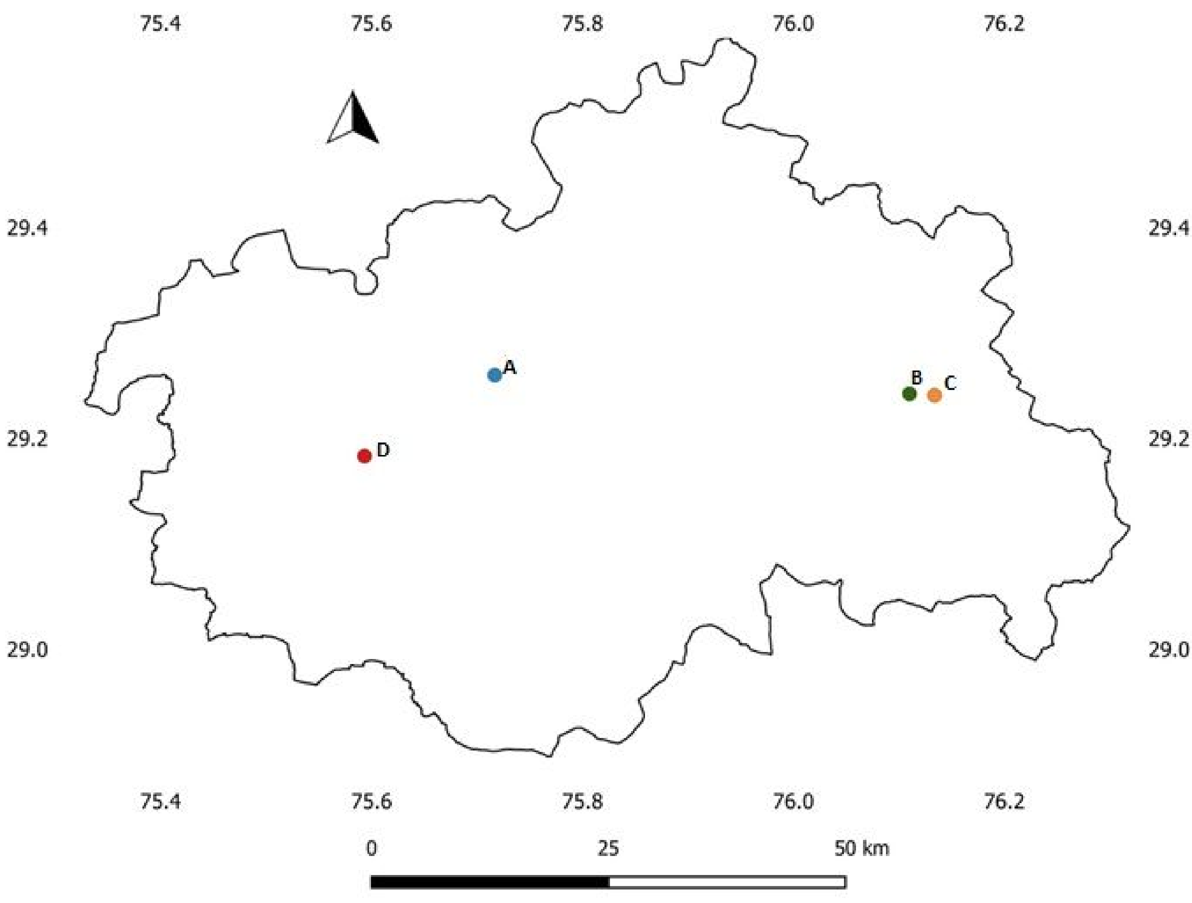

Figure 1

Map of district Hisar, Haryana and location of different outbreaks

Figure 2

Ring shaped intra-erythrocytic piroplasm of Theileria spp. (arrow) in blood smear of a sheep (Giemsa, 100×)

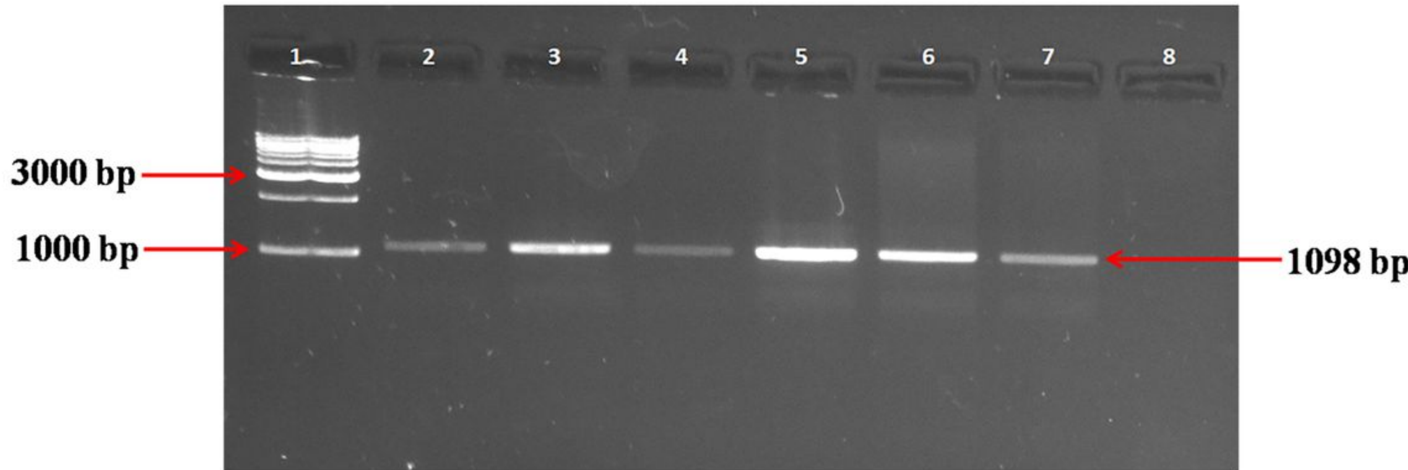

Figure 3

Gel electrophoresis showing amplification of *Theileria* genus specific amplicon at 1098 bp. Lane 1: 1Kb DNA ladder; Lanes 2-7: Samples from clinically affected animals; Lane 8: Negative control.

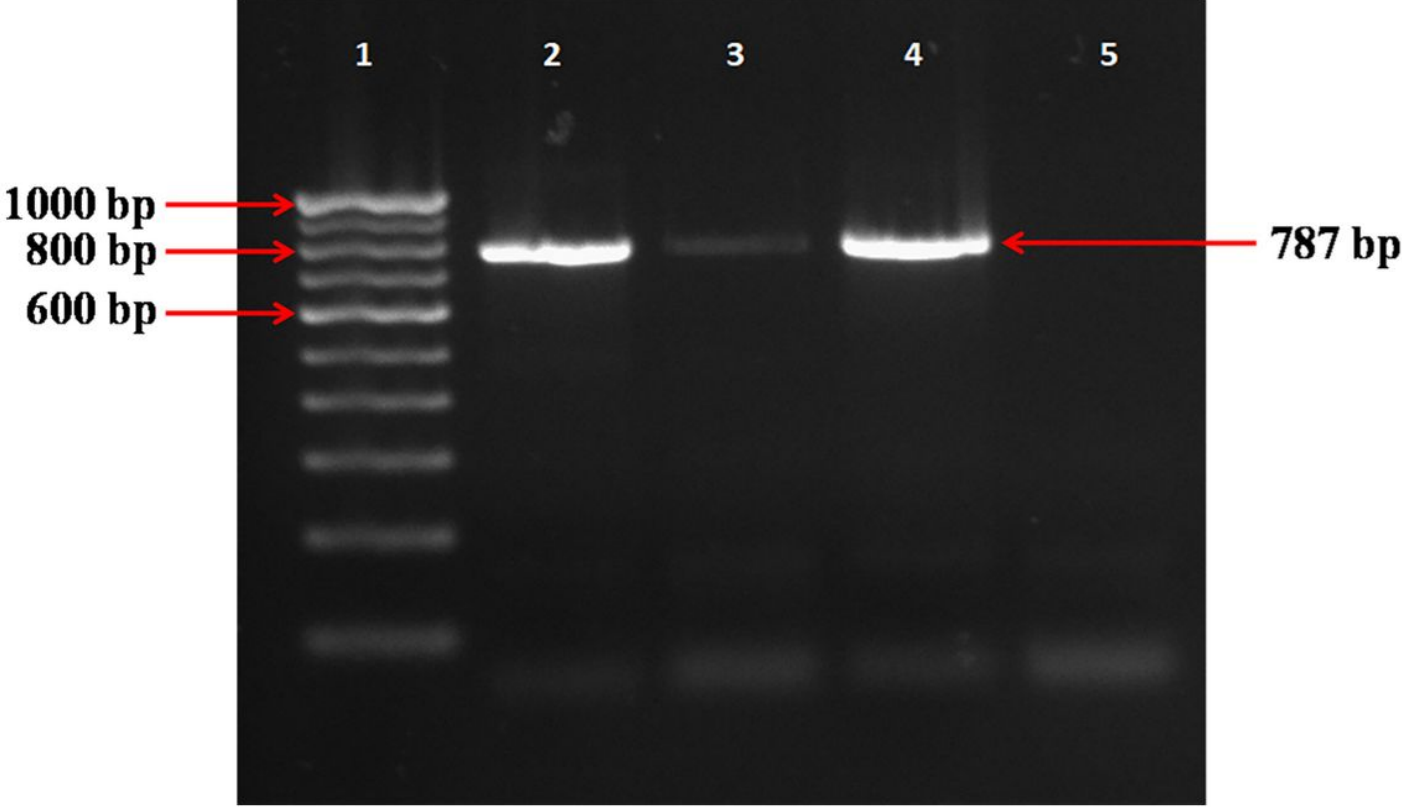

Figure 4

Gel electrophoresis showing amplification of *Theileria lestoquardi* specific amplicon at 787 bp. Lane 1: 100bp DNA ladder; Lanes 2-4: Samples from clinically affected animals; Lane 8: Negative control.

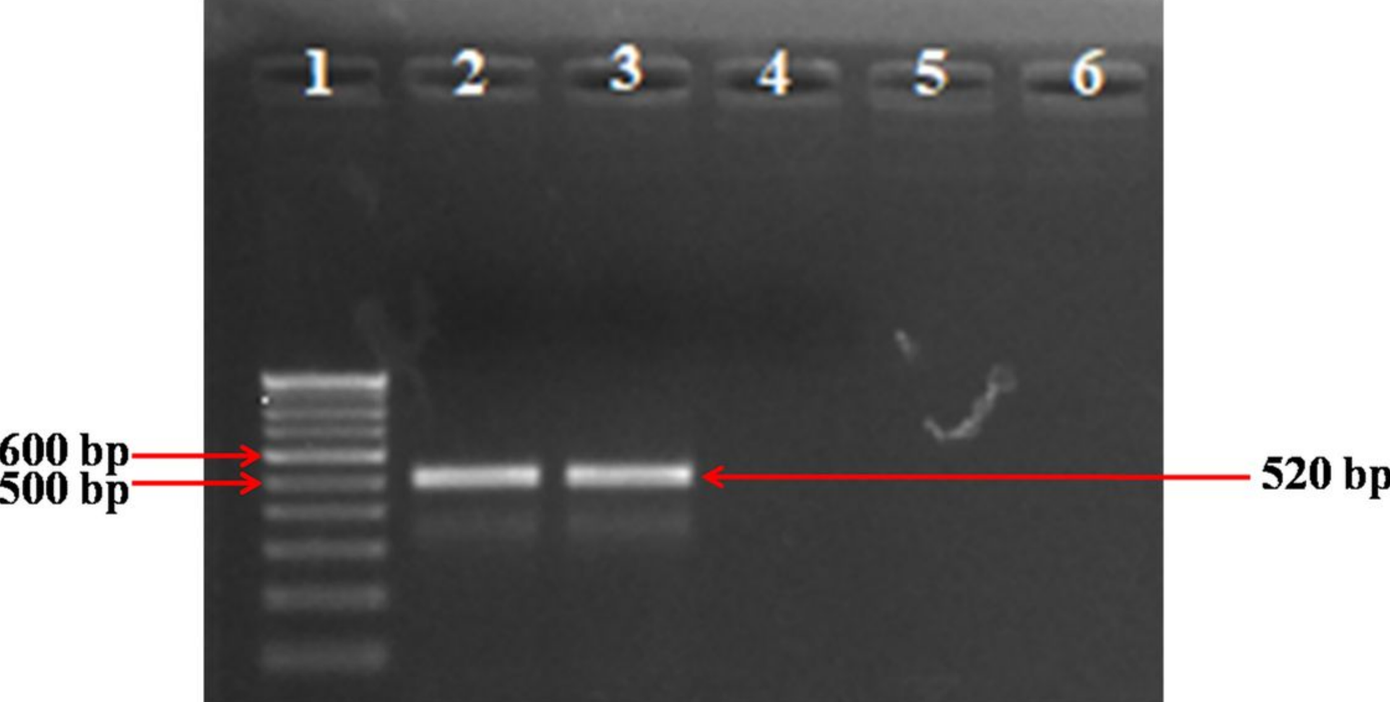

Figure 5

Gel electrophoresis showing amplification of *Theileria ovis* specific amplicon at 520 bp. Lane 1: 100bp DNA ladder; Lanes 2 and 3: Samples from clinically affected animals; Lanes 4-6: Negative controls.

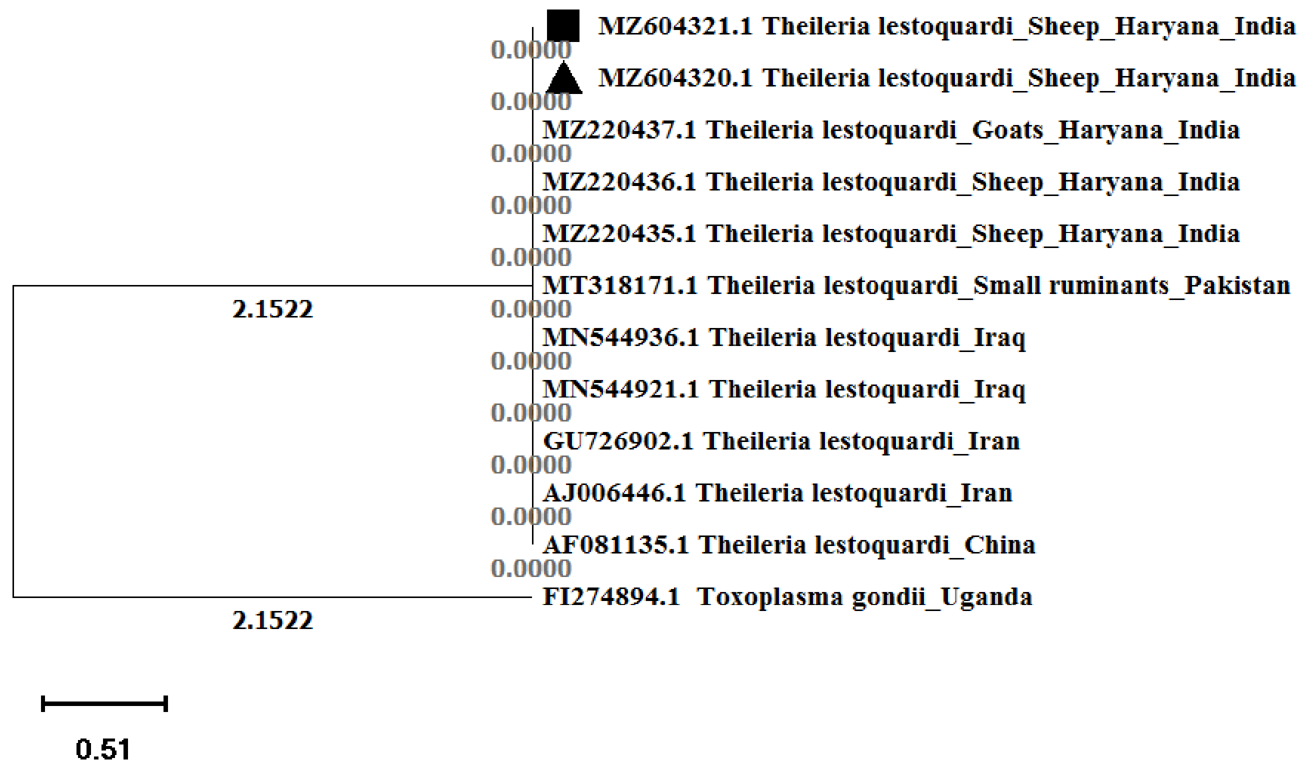

Figure 6

Phylogentic analysis of *Theileria lestoquardi* identified during this study.

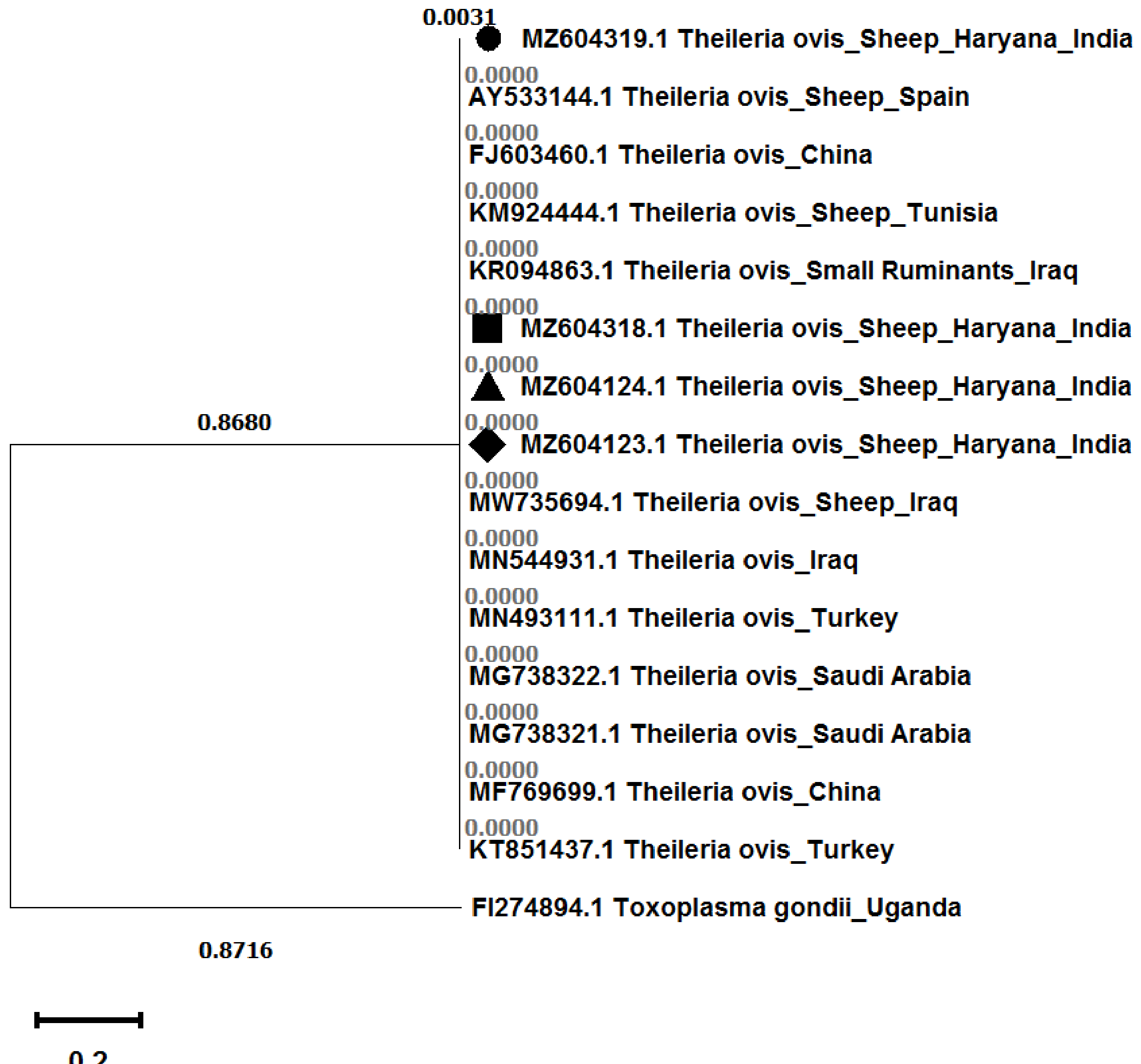

Figure 7

Phylogentic analysis of *Theileria ovis* identified during this study.