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Molecular detection of haemoprotozoan parasites in sheep vectors in Karnataka

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Abstract
The increase in prevalence of tick borne parasites and their economic impact on livestock production have given way for better diagnostic tools and treatment. This study was conducted to study the prevalence and its diagnosis of haemoprotozoan parasites from sheep ticks. The salivarygl and of different tick spp. was subjected for PCR, in which about 13 percent of Haemaphysalis species of ticks amplified for *Theileria wenshui* by nested PCR and *Hyalomma* spp. amplified for *T. ovis* in organised farms. Whereas in unorganised farms 17 percent of Haemaphysalis spp. of ticks *viz*., *H. kutchensis* showed amplification at 388bp for *T. luwenshuni* by nested PCR and *Hyalommaantoli cumanatolicum* showed amplification of 237bp for *T. ovis.* Other species of ticks in the study *viz*., *Rhipicephalus* and *Hyalomma marginatum sasi* did not show amplification for haemoprotozoan parasites. The highest number of *H. kutchensis* showing amplification for *T. luwenshuni* and *H. a. anatolicum* showing amplification for *T. ovis* was considered as potential vector in disease transmission of *T. luwenshuni* and *T. ovis* in sheep in Karnataka.

Keywords: Haemoprotozoan parasites, vectors, sheep

Introduction
Tick - borne hemoprotozoan parasites including *Theileria* spp. and *Babesia* spp. is a major problem in small ruminants especially in tropical and sub-tropical regions of the world. They cause significant economic losses affecting the international trade of animals. Generally, the diagnosis of ovine piroplasmosis is based on morphological examination of blood smears and clinical symptoms, however a negative result does not rule out the possibility of infection. Besides, animals which recover from acute infection become carriers of the haemo-parasite in course of time making diagnosis difficult. The detection of haemoprotozoan parasites in the vector for assessing the infection rate in vectors will help to curtail the risk of *Theileriosis* and *Babesiosis* in small ruminants. Hence in this study the tick tissues *viz.*, salivary gland, mid gut and ovaries were stained by methyl green Pyronin and Giemsa stain to detect parasite infection. Apart from staining techniques in diagnosis of the haemoprotozoan diseases, the application of molecular techniques including conventional PCR, Reverse Transcriptase – PCR, Nested PCR, Semi nested or Reverse line blot would allow direct, specific and sensitive detection and simultaneous detection and differentiation of different pathogens.

Materials and Methods
1. **Study Area:** Different organised and unorganised sheep farms in Karnataka were screened representing eight districts from seven agro climatic zones during 2015-16.
2. **Sample collection:** Fully engorged ticks were collected randomly were collected in clean glass vials covered with muslin cloth and identified before dissection as per the standard keys given by Shariff 1928 [11]; Walker 1994 [13]. About 300 ticks were collected, washed with distilled water and cleaned with an absorbent paper then it was dissected to remove the salivary glands, mid gut and ovaries. The collected ticks were grouped into pool of five ticks according to their species. Then, the salivary glands of each tick pool were dissected out in 0.8% saline solution under stereo microscope. Then, the salivary gland samples were kept at -20 ºC until they were used for PCR. The total DNA was extracted from the EDTA blood and tick samples using Qiagen blood tissue DNA mini kit according to the manufacturer's protocol and it was stored at -20 ºC till subjected for PCR. The published primers as mentioned in table 1 and 2 were used in this study. Compositions of PCR mix for species specific amplification of Theileria and Babesia species is MM-12µl, red dye-2.5 µl, FP & RP-2 µl, template: 2µl, NFW-4µl. The amplification reactions were carried out in 0.2ml PCR tubes using a programmable thermal cycler using the following cycling conditions with slight modification as mentioned in table 3.

Table 1: Primer sequence of SSUrRNA of Babesia species

Target parasite	Nucleotide sequence	Product size	Reference
Babesia genus specific	F1: GTCTTGTAATTGGAATGATGG R1: CCAAAGACTTTGATTTCTCTC	350bp	Aktas *et al.* (2005) [1]
Babesia ovis	F1: TGGGCAGGACCTTGGTTCTTCT R2: CCGCGTAGCGCCGGCTAAATA	549bp	Aktas *et al.* (2005) [1]
Babesia motasi	F1: TAAACCAATTTGTTGGT R2: TCTGCCCAGGGTTTAAGTCGG	294bp	Peng *et al*. (2015)

Table 2: Primer sequence of 18 S rRNA gene of Theileria species

Target Parasite	Nucleotide sequence	Product size (bp)	Reference
Theileria genus specific	F: AGTTTCTGACCTATCAG R: TTGCCTTAAACTTCCTTG	1098bp	Allsopp *et al*. (1993) [2]
Theileriosis	F1: CACAGGGAGGTAGTGACAAG R2: AAGAATTTCACCTATGACAG F2: AAGAATTTCACCTATGACAG R2: TTGCTTTTGCTCCTTTACGAG	426bp 237bp	Razmi *et al*. (2013) [8]
Theileria luwenshuni	F1: CATGGATAACCGTGCTAATT R1: ATCGTCTCGATCCCCTAACT F2: GGTAGGGTATTGGCCTACCGG R2: TCATCCGGATAATACAAGT	388bp	Peng *et al*. (2015)
Theileria lestoquardi	F1: CACAGGGAGGTAGTGACAAG R2: AAGAATTTCACCTATGACAG F2: AAGAATTTCACCTATGACAG R2: ATTGCTTGTGTCCCTCCG	426bp 235bp	Razmi *et al*. (2013) [8]

Table 3: PCR conditions for the amplification of 18S rRNA gene of Theileria and SSUrRNA Babesia

Target parasite	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
Theileria genus	94 °C -3 min	94 °C - 30secs.	56 °C -1 min.	72 °C - 1min	72 °C-5min	40
T. luwenshuni 1set primers 2set primers	94 °C - 5 min 94 °C - 3 min	94 °C - 30secs 94 °C - 30secs	55 °C -1 min 55 °C -1 min	72 °C-10min 72 °C-10min	72 °C-1min 72 °C-1min	35
T. ovis 1set primers 2set primers	94 °C - 5 min 94 °C - 5 min	94 °C -45secs 94 °C -45secs	55 °C -45sec 55 °C -45 sec	72 °C - 5min 72 °C - 5min	72 °C-1min 72 °C-1min	36
T. lestoquardi 1set primers 2set primers	94 °C - 5 min 94 °C - 5 min	94 °C -45secs 94 °C - 45secs	55 °C -45 sec 55 °C -45 sec	72 °C - 5min 72 °C -5min	72 °C-1min 72 °C-1min	35
Babesia genus	94 °C - 5 min	94 °C –1 min	55 °C – 1min	72 °C - 1min	72 °C- 10min	30
B. ovis	94 °C - 3 min	94 °C –1 min	60 °C – 1min	72 °C - 5min	72 °C - 1min	30
B. motasi	94 °C - 3 min	94 °C –30 sec	55 °C – 1min	72 °C - 1min	72 °C- 10min	35

Results and Discussion

The salivary gland of tick spp. *Haemaphysalis, Rhipicephalus* and *Hyalomma* was dissected and the DNA was extracted from it by using Qiagen blood-tissue DNA mini kit and subjected for PCR as per the cycling conditions mentioned in the table 3, in which from organised farms, 13% of Haemaphysalis species of ticks *H. kutchensis* (9) amplified at 388bp for *Theileria luwenshuni* by nested PCR (Fig:2) and *H. a. anatolicum* amplified for *T. ovis* whereas other none of the other species of ticks in the study *viz., Haemaphysalis intermedia, Haemaphysalis bispinosa, Rhipicephalus sanguineus, Rhipicephalus haemaphysaloides* and *Hyalomma marginatum issaci* spp. did not amplify for haemoprotozoan parasites. Whereas in unorganised farm animals 17 percent of *Haemaphysalis* spp. of ticks *viz., H. kutchensis* (34) showed amplification at 388bp for *T. luwenshuni* by nested PCR and *Hyalomma anatolicum anatolicum* (20) showed amplification of 237bp for *T. ovis* by semi nested PCR.

The highest number of *H. kutchensis* ticks showing amplification for *T. liwenshunii* was considered as vector for disease tranasmission of *T. luwenshunii* in sheep in this study. Some of the authors from India have reported prevalence of *Haemaphysalis* and *Rhipicephalus* ticks in theileria infected sheep flock and are in accordance with findings of Ramanujachari and Alwar (1954) who has reported the high prevalence of *H. bispinosa* (90%) followed by *Hyalomma aegyptium* in sheep infected with *T. hirci* in Madras state. Hiregoudar and Prabhakar (1977) [6] reported the prevalence of *H. intermedia* followed by *R. haemaphysaloides* in *Theileria* carrier sheep from Karnataka. Jagannath and Lokesh (1988) has recorded prevalence of *H. intermedia* (>70%) along with *R. haemaphysaloides* on examination of ticks in sheep (1164) and goats (372) from different taluks of Kolar districts in Karnataka. In a recent study in Karnataka by Mamatha *et al.*, (2017) out of five tick species identified during their study, *H. kutchensis* was found to be the most predominant tick in sheep (51.44%) followed by *R. haemaphysaloides*, suggested that these ticks may play a important role as a vector in transmission of *T. luwenshunii.*

Previously the vectors for *T. ovis* has been reported as *H. anatolicum* (Bhattacharyulu *et al.*, 1972) [3]; *Rhipicephalus* spp. and *H. anatolicum* in India (Sisodia, 1981) [12]; *Rhipicephalus evertsi* in South Africa (Jansen and Neitz, 1956), *R. bursa* in Turkey (Sayin *et al*., 2009) [10], *Rhipicephalus spp.*, in Pakistan (Durrani *et al*., 2011) [4], *R. sanguineus* and *Rhipicephalus turanicus* in Iran (Zakkyeh *et al*., 2012; Razmi and Yaghfoori, 2013) [8, 14]. Whereas in this study *Hyalomma anatolicum anatolicum* showed

amplification of 237bp for *T. ovis* (Fig:4) by semi nested PCR suggesting *H. a. anatolicum* as vector for disease tranasmission of *T. ovis* in sheep in this study. In this study only larval and nymphal stage of ticks of species *Rhipicephalus sanguineus*, *Rhipicephalus haemaphysaloides* and *Haemaphysalis intermedia* were found on babesia infected sheep where the salivary gland DNA from nymphal stages did not show amplification for babesia parasites.

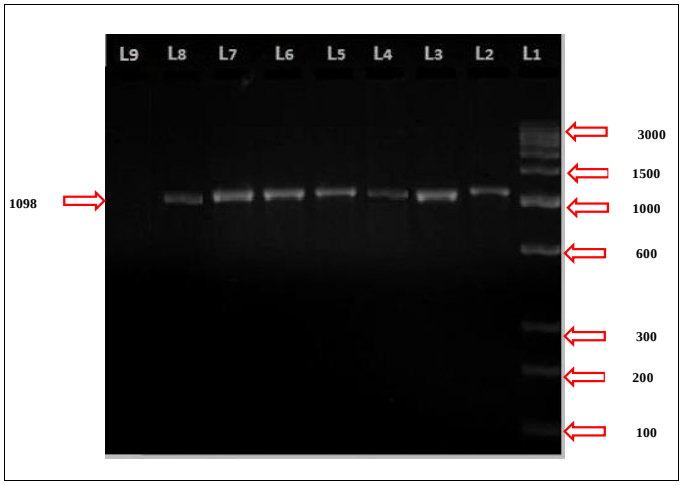

Fig 1: Amplification of *Theileria* genus specific Amplicon at 1098 bp

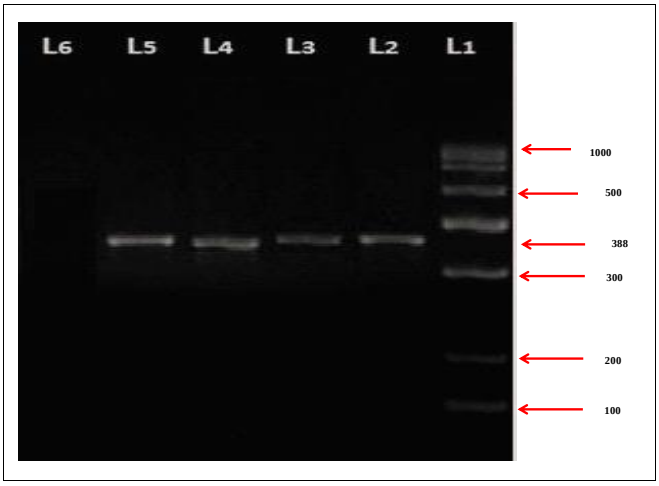

Fig 2: Amplification of *Theileria luwenshuni* species

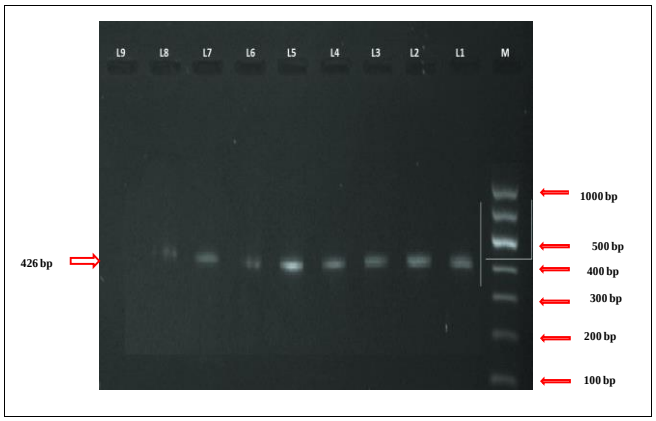

Fig 3: Amplification of *Theileria ovis* species specific gene of 426bp gene by using 1st of primers by Semi nested PCR

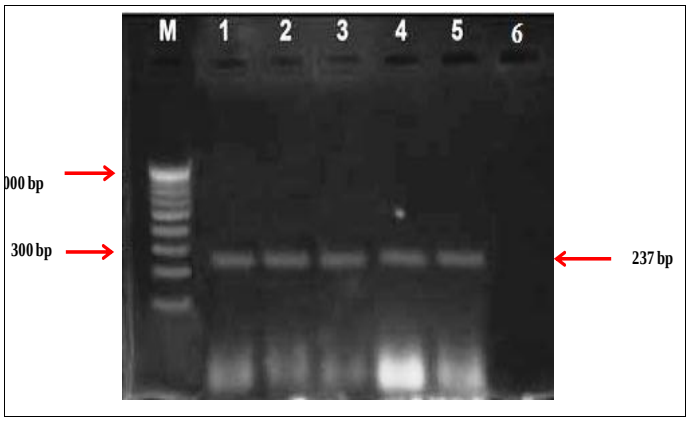

Fig 4: Amplification of *Theileria ovis* species at 237bpspecific by using 2nd set of primers amplicon by Semi nested PCR

Phylogenetic analysis

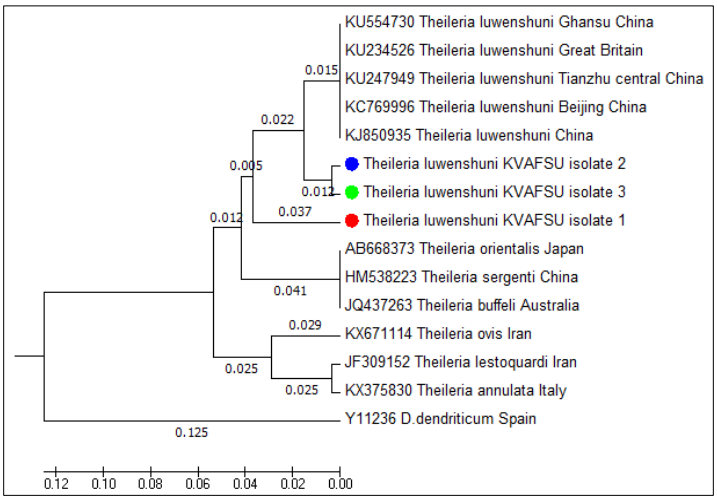

Fig 5: Phylogenetic tree of *Theileria luwenshuni* isolates

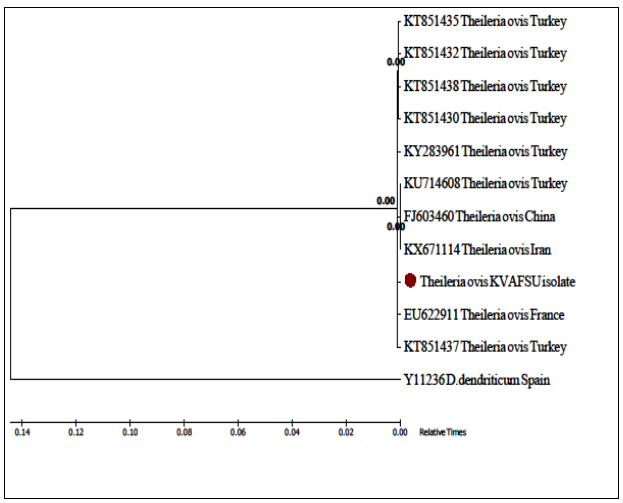

Fig 6: Phylogenetic tree of *Theileria ovis* isolates

In the present study, the phylogenetic analysis results showed that *T. luwenshunii* (Karnataka) isolates obtained during this study were genetically similar amongst each other and were in the same clade as *T. luwenshuni* isolates deposited in the Gen Bank (Accession numbers KU247949, KJ850935, KC769996, KU554730, KU234526). Therefore, the nucleotide sequence analysis results further confirmed that the isolates of the present study as *T. luwenshunii.* Whereas the Phylogenetic analysis results of *T. ovis* (Karnataka) isolates obtained during this study were genetically similar amongst each other and were in the same clade as *T. ovis* isolates deposited in the Gen Bank (Accession numbers KT 851435, KT851432, KT851438, KT851430, KT283961, KT14608, FJ603460 and KX671114).Therefore, the nucleotide sequence analysis results further confirmed that the isolates of the present study as *T. ovis.*

Conclusion

In this study the highest number of *H. kutchensis* showing amplification for *T. luwenshunii* and *H. a. anatolicum* showing amplification for *T. ovis* was considered as potential vector in disease transmission of *T. luwenshunii* and *T. ovis* in sheep in Karnataka.

Acknowledgments

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Conflict of Interest

All the authors declare that there is no actual or potential conflict of interest.

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