

Molecular Detection of Haemoprotozoan Infections in Hard Ticks of Cattle in Maharashtra

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ABSTRACT:

Detection of haemoprotozoan infections by microscopy is usually adequate for detection of acute infections, but not for detection in case of carrier animals owing to low parasitemia. Therefore, a sensitive and highly specific method like polymerase chain reaction was used to detect haemoprotozoan infections in ticks of Maharashtra state. For this purpose, ticks were collected from 18 different sites of five regions of the state, viz. Marathwada, Konkan, Western Maharashtra, North Maharashtra and Vidarbha. Ticks were identified morphologically, the DNA was extracted from the four adult ticks of each species from each collection site and were pooled together and polymerase chain reaction was performed for detection of haemoprotozoan infections. The PCR analysis revealed the presence of haemoprotozoan (*Theileria* or *Babesia* spp.) in 7 and 5 pools of *Rhipicephalus microplus* and *Hyalomma anatolicum*, respectively. Whereas, no haemoprotozoan parasite was detected in *Haemaphysalis bispinosa* and *Rhipicephalus sanguineus*. The higher prevalence rate was observed in *H. anatolicum* compared to *R. microplus*. The overall prevalence of intra-acarine haemoprotozoans was highest in North Maharashtra (66.66%) followed by Western Maharashtra (57.14%), Marathwada (52.13%), Konkan (41.17%) and Vidarbha region (39.13%).

Keywords: Babesia, Hyalomma Anatolicum, Maharashtra, Rhipicephalus Microplus, And Theileria

INTRODUCTION

Ticks and tick-borne diseases (TTBDs) affect 80% of world cattle population, and their prevalence is throughout the world, particularly important in tropical and sub-tropical countries including India causing loss of production (De Castro *et al.*, 1997) and rank fourth among the major infections of livestock (Ghosh *et al.*, 2007). In developing countries like India, tick borne pathogens such as *Theileria* spp. and *Babesia* spp. are known for high mortality rates, reduced milk production and loss of body condition (Ghosh and Nagar, 2014). Among the haemoprotozoan diseases of livestock, babesiosis and tropical theileriosis are responsible for annual economic loss of more than 57.2 million (Ghosh and Nagar, 2014; Mcleod and Kristjanson, 1999) and US \$ 800 million, respectively. The information about the prevalence of tick-borne diseases, particularly, the role of vector ticks on the transmission dynamics of haemoprotozoan diseases is essential for the adoption of efficient control strategies. Additionally, the information on the epidemiology of haemoprotozoan diseases in ruminants, their isolation and infection rate of vector ticks is also important. However, only few sporadic reports are available in literature regarding the extent of tick infection with haemoprotozoan infections in Maharashtra state. Till date, the prevalence of different species of ticks and haemoprotozoan infections, particularly *Theileria* spp. and *Babesia* spp. have been generally investigated and these parasites were found prevalent in the Maharashtra state. However, most of the studies were based on examination of blood smears for the detection of haemoprotozoan infections in host animals. The traditional method of identifying the haemoprotozoan parasites in infected animals by microscopic examination of blood smears is usually adequate for detection of acute infections, but not for detection of carrier animals, where parasitemia may be low (Friedhoff and Bose, 1994). Serological methods are employed in diagnosing subclinical infections in epidemiological studies, but false-positive and false negative results, due to cross-reactions or weakening of specific immune responses, are common (Passos *et al.*, 1998). Therefore, a sensitive and highly specific method for the diagnosis of piroplasms is required. Recent developments in application of molecular techniques enable sensitive and specific detection of the parasites, particularly; PCR-based assays emerged as powerful tools for detection of infection both in the vertebrate hosts and ticks (Oliveira-Sequeira *et al.*, 2005; Smeenk *et al.*, 2000; Gayo *et al.*, 2003; Oliveira *et al.*, 2008; Tavassoli *et al.*, 2013). However, only few published reports regarding the use of molecular techniques for detection of tick-borne pathogens in vector tick are available (Ravindran *et al.*, 2006). Considering the available published literature, the present study represents the first molecular detection of haemoprotozoan parasites (*Theileria* and *Babesia* spp.) in ticks of Maharashtra state.

MATERIALS AND METHODS

Study area

The ticks were collected from cattle of Maharashtra state which is located in the heart of India covering an area of 307,713 km² and placed as third largest state. Geographically, Maharashtra state lies between 15°35' N to 22°02' N latitude and 72°36' E to 80°54' E longitude. The state has emblematic monsoon climate and tropical environment exist all over the state with hot, rainy and cold weather seasons. The ticks were collected from cattle of five regions of Maharashtra state, *viz.* Marathwada, Konkan, Western Maharashtra, North Maharashtra and Vidarbha. A total of 18 different sites comprising organised and non-organised/private cattle farms were selected for collection of ticks. Ticks were collected from 5 districts, *viz.* Parbhani,

Aurangabad, Latur, Beed, and Osmanabad of Marathwada region; from 4 districts, *viz.* Ratnagiri, Raigad, Mumbai, and Sindhudurga of Konkan region; 3 districts, *viz.* Solapur, Pune, and Satara of Western

Maharashtra region; from 2 districts, viz. Nasik and Nandurbar of North Maharashtra region; from 4 districts, viz. Akola, Nagpur, Yewatmal, and Gadchiroli of Vidarbha region.

Morphological identification of ticks

The gross examination of tick specimens was carried out using a stereoscope zoom microscope for morphological identification. The procedure detailed by Shirsikar *et al.* (2023) was followed for morphological identification of ticks based on the keys described by Sen and Fletcher (1962) and Walker *et al.* (2003) at Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, MAFSU, Parbhani.

Molecular detection of intra-acarine haemoprotozoan infections

DNA Extraction

The DNA was extracted from the four adult ticks of each species, recorded in each collection site and were pooled together using DNA extraction kit (DNeasy blood & Tissue Kit, QIAGEN (Germany)). For this purpose, the collected ticks were pooled and kept individually in 1.5 ml labelled microcentrifuge tubes. Using liquid nitrogen each tick isolate was crushed in porcelain mortar and pestle. Then PBS (80µl) was added to each tick lysate followed by 180 µl of tissue lysis buffer ATL, and 20 µl of proteinase K. After proper mixing by vortex, tubes were kept at -20°C for 30 minutes. For proper lysis, the samples were placed immediately in water bath for incubation at 56°C with intermittent shaking. After vortexing for 15 seconds, lysis buffer concentration 216 ml (AL) (200 µl) was added, again mixed by vortexing and incubated at 56°C for 10 minutes. Then 200µl of ethanol was added, mixed properly using vortex and mixture was then pipetted into DNeasy Mini Spin column in a 2 ml collection tube. After brief centrifugation at a maximum speed at 8000 rpm for 1 minute, the collection tube and flow through was discarded. The spin column was placed in a new 2 ml collection tube, 500µl of wash buffer concentration 242 ml (AW1) was added to the mixture and centrifuged at 8000 rpm for 1 minute and the flow-through and the collection tube were discarded. The repeated washing process was carried out by adding wash buffer 2, concentration 342 ml (AW2) (500 µl) and centrifuged at 14,000 rpm for 3 min and the flow-through and collection tube were discarded. Elution was done by keeping the spin column in a 2 ml microcentrifuge tube with buffer AE (200 µl) and incubation for 1 min at 37°C and then centrifuged at 8000 rpm for 1 minute. To increase the DNA yield, the procedure was repeated for 2-3 times. The obtained pellet of DNA was re-dissolved in 100µl TE by keeping at 65°C for 1 hr. Then isolated DNA was run on 0.8 % agarose gel to check and visualize under ultraviolet light, and or nanodrop spectrophotometry (Nanodrop 2000 UV-Vis, Thermo Scientific, USA) for quality. The DNA quantity was measured at 260 nm and obtained 260/280 ratio was 160 to 180 for purified DNA samples indicated free of any major protein. The quantified DNA samples were stored at -20°C till used for PCR analysis.

Polymerase Chain Reaction

The DNA extracted from the tick tissues was amplified by using a pair of primers designed for *Theileria* and *Babesia* Universal (Simuunza *et al.* 2011). The forward 5'GACACAGGGAGGTAAGTACAAAG3' and reverse 5'CTAAGAATTTCACCTCTGACAGT3' primers were used. The amplification of DNA was carried out in a final volume of 25µl reaction containing 12.5µl of master mix, 8.5µl of NH₂O, 2µl of genomic DNA, 1µl of each primer (forward and reverse). The PCR tubes were then placed into a programmed Lark cycler 96 well gradient thermal cycler where the reaction mixture was subjected to 4 min DNA denaturation at 95°C, 35 cycles of denaturation at 95°C for 20 sec, annealing at 54°C for 30 seconds and elongation at 72°C for 1min. The reaction was completed by a further 5 min final elongation

step at 72°C. The amplified products were analyzed by electrophoresis on 1% agarose gel by aliquoting 7µl of PCR products and 100 bp DNA ladder and run on the gel stained with ethidium bromide for 1 hour at 80 volts.

RESULTS AND DISCUSSION

Prevalence of intra-acarine haemoprotozoa by PCR

The DNA isolated from *Rhipicephalus microplus*, *Hyalomma anatolicum*, *Haemophysalis bispinosa*, and *Rhipicephalus sanguineus* collected from heavily infested cattle from Marathwada region were pooled. A total of 23 adult tick pools constituting 10 pools of *Rhipicephalus microplus*, 10 pools of *Hyalomma anatolicum*, 1 pool of *Haemophysalis bispinosa* and 2 pools of *Rhipicephalus sanguineus* ticks were prepared. The PCR analysis revealed 52.13% prevalence of haemoprotozoa (*Theileria* or *Babesia* spp.) in Marathwada region. Whereas, no haemoprotozoan parasite was detected in *Haemophysalis bispinosa* and *Rhipicephalus sanguineus*. The higher prevalence rate was observed in *H. anatolicum* compared to *R. microplus* (Table 1).

A total of 17 adult tick pools comprising of 08 pools of *Hyalomma anatolicum*, 08 pools of *Rhipicephalus microplus* and 01 pool of *Rhipicephalus sanguineus* from different geographical locations of Konkan region were examined. The PCR analysis showed the presence of 83.33% (5/6) and 33.33% (2/6) distribution of haemoprotozoan parasites, respectively. The intra-acarine prevalence rate of haemoprotozoan parasites in Konkan region was recorded as 41.17%. The higher prevalence of haemoprotozoan infections was revealed in *Hyalomma anatolicum*, while, haemoprotozoa were not found in *Rhipicephalus sanguineus* (Table 1).

For prevalence of intra-acarine haemoprotozoan parasites in Western Maharashtra region, 14 pools constituting each 07 pools of *Hyalomma anatolicum* and *Rhipicephalus microplus* were examined. The intra-acarine prevalence of haemoprotozoan parasites in Western Maharashtra was recorded as 57.14 % (Table 1).

A total of 9 pools comprising of 05 and 04 pools of *Hyalomma anatolicum* and *Rhipicephalus microplus* were prepared, respectively. Among which higher haemoprotozoan prevalence was revealed in *Hyalomma anatolicum* compared to *R. microplus*. The overall intra-acarine prevalence rate in North Maharashtra was observed as 66.66% (Table 1).

In Vidarbha region, the intra-acarine haemoprotozoan prevalence was recorded as 39.13%. The 66.66% prevalence of intra-acarine haemoprotozoa was recorded from Akola and Nagpur districts. While 50.00% prevalence rate was observed in both tick species collected from Yawatmal and Gadchiroli districts (Table 1).

Table 1: Prevalence of intra-acarine haemoprotozoa in Maharashtra by PCR

Sr. No.	Name of the region	Tick species	Pools	Positive	Prevalence (%)
1	Marathwada	<i>Hyalomma anatolicum</i> , <i>Rhipicephalus microplus</i> , <i>Haemophysalis bispinosa</i> , <i>Rhipicephalus sanguineus</i>	23	12	52.13
2	Konkan	<i>Hyalomma anatolicum</i> , <i>Rhipicephalus microplus</i> , <i>Rhipicephalus sanguineus</i>	17	07	41.17
3	Western	<i>Hyalomma anatolicum</i> , <i>Rhipicephalus</i>	14	08	57.14

	Maharashtra	<i>microplus</i>			
4	North Maharashtra	<i>Hyalomma anatolicum</i> , <i>Rhipicephalus microplus</i>	09	06	66.66
5	Vidarbha	<i>Hyalomma anatolicum</i> , <i>Rhipicephalus microplus</i> , <i>Haemophysalis bispinosa</i>	23	09	39.13

In the present study, two commonly encountered haemoprotozoan parasites in Maharashtra state, viz. *Theileria* spp. and *Babesia* spp. were detected only in *H. anatolicum* and *R. microplus*, respectively. In accordance with our findings, Ali *et al.* (2013) also reported the presence of *Theileria annulata* only in *H. anatolicum* and *H. dromedari* but not in *H. marginatum*, *B. annulatus* and *Amblyomma variegatum*. In another study, using reverse line blot hybridization, M'ghirbi *et al.* (2013) noted the 17.8% and 8.5% prevalence of *Theileria* spp. and *Babesia* spp. in *Hyalomma anatolicum* and *Rhipicephalus microplus* spp., respectively. However, Iori *et al.* (2005) detected the different species of *Babesia* (*B. bigemina*, *B. divergens*, *T. ovis*, *T. equi*) and *Theileria* (*T. annulata*) in *R. annulatus* and *R. bursa*, respectively. In the present study, the absence of haemoprotozoa in other prevalent tick species could be due to small sampling size until a larger molecular survey has been conducted.

The prevalence of different tick species and the diseases transmitted by them depends on the local climatic conditions like rainfall, vegetation, altitude and temperature as well as host availability. Whereas, M'ghirbi *et al.* (2013) mentioned that the host factors such as age, sex and ecotype may also play the important role in the intensity of occurrence of hard ticks and their associated piroplasmosis in ruminants. In the present study, the high prevalence of *Theileria* spp. in vector *H. anatolicum* as compared to *R. microplus* in regions of the state indicates that an epidemiology of haemoprotozoan parasites could be different as per regions. After the discovery of etiology of cattle fever and detection of *Babesia* in *R. microplus* by Smith and Kilborne (1893), the studies by Oliveira *et al.* (2005) and recently Storey- Lewis *et al.* (2018) confirmed the role of *R. microplus* as a potential vector in transmission of *Babesia* spp. Similarly, the presence of *Babesia* spp. in the present study was detected only in *R. microplus* and no parasites were detected in either in *R. turanicus* or *Haemophysalis bispinosa*.

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