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Research Article

MOLECULAR INVESTIGATION ON CROSS INFECTION OF *BABESIA BIGEMINA* AND *THEILERIA ANNULATA* IN *HYALOMMA ANATOLICUM* AND *RHIPICEPHALUS* (*BOOPHILUS*) *MICROPLUS* TICKS

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ABSTRACT

Tropical bovine theileriosis caused by *Theileria annulata* and babesiosis caused by *Babesia bigemina* in cattle are important tick-borne haemoprotozoan diseases in India. The ticks, *Hyalomma anatolicum* and *Rhipicephalus (B.) microplus* are considered as main vectors for *T. annulata* and *B. bigemina*, respectively. Due to common host and multi-genera tick infestation on cattle in Indian conditions, there are possibilities for co-infection of ticks with both the haemoprotozoa species. However, there is dearth of reports on molecular investigation of *B. bigemina* and *T. annulata* parasites in *H. anatolicum* and *R. (B.) microplus* ticks, respectively. In the present study, molecular investigation by PCR method on *R. (B.) microplus* ticks collected from three districts (Hisar, Jhajjar and Sirsa) of Haryana revealed presence of *T. annulata* infection. Data generated showed 16% of Hisar isolates, 14.6% of Jhajjar isolates and 28.6% of Sirsa isolates positive for *T. annulata*. Similarly, in *H. anatolicum* ticks, 10% of Hisar isolates, 12.5% of Jhajjar isolates and 16.7% of Sirsa isolates were found positive for *B. bigemina*. The study provides molecular evidence of the vector potential and intricacy of tick-haemoprotozoa relationship.

Keywords: *Babesia bigemina*, Cattle Tick, Haryana, PCR, *Theileria annulata*

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Tick and tick-borne diseases pose a serious threat on the growth and productivity of livestock sector. At present, 931 valid species of ticks are known to be prevalent throughout the world (Nava *et al.*, 2017). Of these, *Hyalomma anatolicum* and *Rhipicephalus* (*Boophilus*) *microplus* are the most commonly found cattle ticks in North India (Haque *et al.*, 2011; Surbhi *et al.*, 2019; Gupta *et al.*, 2020). *H. anatolicum* ticks play a major role in the epidemiology of *Theileria annulata* infection in Haryana (Sangwan *et al.*, 1989). Similarly, *R. (B.) microplus* serves as major vector for *Babesia bigemina* infection in Indian cattle (Bhat *et al.*, 2017). Global impact of economic loss due to ticks and tick borne diseases is estimated at the tune of US$ 22–30 billion annually (Lew-Tabor and Valle, 2015). In India, the control cost of tick and tick borne diseases has been roughly estimated to be around 2500 crore rupees per annum (Minjauw and McLeod, 2003). Heavy tick infestation on cattle in a tropical country like India has resulted into endemicity of bovine theileriosis and babesiosis in cattle farms leading to huge economic losses.

Due to common large ruminant host and multi-genera tick infestation on cattle in Indian conditions, there are all possibilities for co-infection of ticks with both the haemoprotozoan species. However, there is dearth of reports on attempts of molecular investigation of *B. bigemina* in *H. anatolicum* and *T. annulata* in *R. (B.) microplus* ticks. Therefore, in the present study, cross-infections of *B. bigemina* and *T. annulata* in *H. anatolicum* and *R.(B.) microplus* ticks collected from three districts (Hisar, Jhajjar and Sirsa) of Haryana were detected using PCR.

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MATERIAL AND METHODS

Selection of animals: A total of 140 cattle (indigenous cattle of Haryana breed) from 10 gaushalas situated in Hisar, Jhajjar and Sirsa districts of Haryana were included in the present study. Seventy cattle from five gaushalas (14 cattle per *gaushala*) of Hisar district, 57 cattle from four gaushalas (14 cattle from three gaushala and 15 cattle from one gaushala) of Jhajjar district and 13 cattle from one gaushala of Sirsa district were included in the study for the collection of ticks.

Collection of ticks: The body of the animals was thoroughly examined and engorged female ticks were collected from infested cattle by hand picking. Five ticks were collected from each animal and placed as one group of pooled tick sample in properly labeled plastic tubes and brought to the laboratory of Department of Veterinary Parasitology, College of Veterinary Science, LUVAS, Hisar for further identification and molecular examination of haemoprotozoan infection.

Identification and maintenance of ticks in the laboratory: Ticks were identified as *Rhipicephalus* (*Boophilus*) *microplus* based on brevirostrate mouth parts, triangular coxa I, caudal appendages and roughly hexagonal basis capitulum. Similarly, *Hyalomma*

anatolicum was identified based on morphological characters such as eyes, longirostrate, bifid Coxa I, ventral plates and pale rings in the legs (Walker, 2003). For molecular examination, fully engorged female ticks were preserved in 70% alcohol in clean, well-stoppered glass vials and stored at -20 °C. Other ticks were placed at BOD incubator in a dessicator at 85 ± 5% relative humiditity and 28±1 °C temperature. Eggs and salivary gland of tick isolates found positive for cross infection were later investigated for presence of protozoan infection.

Tick dissection for salivary gland: Ticks were embedded dorsal side up on low melting point paraffin in a petri dish and dissection was performed under a dissecting microscope (Purnell and Joyner, 1968). The salivary gland was transferred in 60 µl of lysis buffer in an eppendorf tube and stored at -20 ºC until DNA extraction.

Molecular examination of ticks for haemoprotozoa: Around 2-3 engorged female ticks were grounded in liquid nitrogen in a pre-chilled mortar using pestle. Then, the powdered material was transferred in an autoclaved 1.5 ml microcentrifuge tube for genomic DNA extraction using QiaAmp DNeasy Blood & Tissue Kit. The *SSU* rRNA gene of *Babesia bigemina* and *Cytob1* gene of *Theileria annulata* was amplified using published primers (Table 1). DNA sample collected from microscopically positive *B. bigemina* and *T. annulata* solo infection in blood showing high parasitaemia was taken as positive control (Ganguly *et al.*, 2020). Whole-genomic DNA was isolated from egg samples and salivary gland using QIAamp DNA mini kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations with minor modifications.

Detection of *B. bigemina* in *H. anatolicum* ticks: The PCR reactions were set up into 25 µl volume containing 12.5 µl GoTaq® Green PCR Master Mix (Promega, USA), 1 µl of each primer (10 pmol each of BBF/BBR) and 1µl of the extracted DNA template, and the total volume was made up to 25 µl using nuclease-free water. The PCR cycling conditions were set in automated thermal cycler (Applied Biosystem, USA) with the following programme: initial denaturation at 94 °C for 2 min, 32 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 45 sec, extension at 72 °C for 1 min and the final extension at 72 °C for 7 min. All *H. anatolicum* were examined for *B. bigemina* after standardization of PCR.

Detection of *T. annulata* in *R. (B.) microplus* ticks: The PCR reactions were set up into 25 µl volume containing 12.5 µl GoTaq® Green PCR Master Mix (Promega, USA), 1 µl of each primer (10 pmol each of TAF/TAR) and 1µl of the extracted DNA template, and the total volume was made up to 25 µl using nuclease-free water. The PCR cycling conditions were set in automated thermal cycler (Applied Biosystem, USA) with the following programme: initial denaturation at 94 °C for 2 min, 32 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 45 sec, extension at 72 °C for 1 min and the final extension at 72 °C for 7 min. All *R. (B.) microplus* were examined for *T. annulata* after standardization of PCR.

RESULTS AND DISCUSSION

Identification of ticks: Out of 140 pooled tick samples, 98 pooled tick samples were identified as *R. (B.) microplus* and 42 pooled ticks as *H. anatolicum* using standard identification keys (Walker, 2003). The higher prevalence of *R. (B.) microplus* than *H. anatolicum* was also observed by Sangwan *et al.* (2000) in Haryana state. *R. microplus* was found to be the predominant tick of cattle in the state and was probably because it prefers dense hair coat present on cattle skin (Khan, 1994). Similarly, *R. (B.) microplus* had been reported as the predominant tick in cattle from various other states of India, viz., Punjab (Haque *et al.*, 2011), Karnataka (Hiregoudar and Harlapur, 1988), Uttar Pradesh (Khan and Srivastava, 1994), Uttarakhand (Vatsya *et al.*, 2008), Andhra Pradesh (Rajendran and Hafeez, 2003), Maharastra (Shahardar *et al.*, 1998) and Jammu (Khajuria *et al.*, 2015). An increase in the

Table 1

Target genes and oligonucleotides for *T. annulata* and *B. bigemina*

Name of Haemoprotozoan	Primer target gene	Primer designation (Oligonucleotide) and sequence	Region amplified	Amplicon Size	Annealing Temperature	Reference
Babesia bigemina	*SSU* rRNA	BB F: TAG TTG TAT TTC AGC CTC GCG BB R: AAC ATC CAA GCA GCT AHT TAG	Small subunit ribosomal RNA sequence of *B. bigemina*	689 bp	50ºC	Ellis *et al.*, 1992
Theileria annulata	*Cytob1*	TA F:ACT TTG GCC GTA ATG TTA AAC TA R: CTC TGG ACC AAC T GT TTG G G	Cytochrome b1 gene of *T. annulata*	312 bp	50 ºC	Bilgic *et al.*, 2010

population of *R. (B.) microplus* and decrease in the population of *H. anatolicum* may be attributed to adaptation of enhanced management system which is detrimental for the survival of multi-host ticks (Haque *et al.*, 2011).

Gaushala-wise summary of *T. annulata* infection in *R. (B.) microplus* ticks: The confirmatory host for reproduction and life cycle of *T. annulata* is *H. anatolicum* tick, but sometimes the protozoa can also be found in *R. (B.) microplus* ticks, although it doesn't complete its life cycle in the tick (Zeb *et al.*, 2019). In the present study, *cytochrome b1* gene based PCR screening of all the 98 *R. (B.) microplus* pooled tick samples revealed 16 samples positive for *T. annulata* giving the desired band of 312 bp (Fig. 1). Gaushala-wise, four *R. (B.) microplus* ticks from Shri Ladwa, Village Ladwa (Hisar), one from Shri Krishan, Tohana Road, Barwala (Hisar), three from Shri Balaji Samiti, Mangali (Hisar), three from Shri Aryavarat Gaushala Mandothi (Jhajjar), two from Gokul Dham Shri Ratiram Gauseva Samiti (Jhajjar), one from Shri 108 Bramchari Jai Ram Dass, Beri (Jhajjar) and two from Shri Krishan Parnami, Kanwarpura (Sirsa) gaushala were positive for *T. annulata* by PCR detection method (Table 2).

Gaushala-wise summary of *B. bigemina* infection in *H. anatolicum* ticks: The confirmatory host for the reproduction and life cycle of *B. bigemina* is *R. (B.) microplus* tick. Sometimes, the *B. bigemina* infected blood can also be found in *H. anatolicum* ticks, but the protozoon doesn't complete its life cycle in that tick. In the present study, molecular detection of *B. bigemina* using the primers targeting the SSU rRNA was performed in *H.*

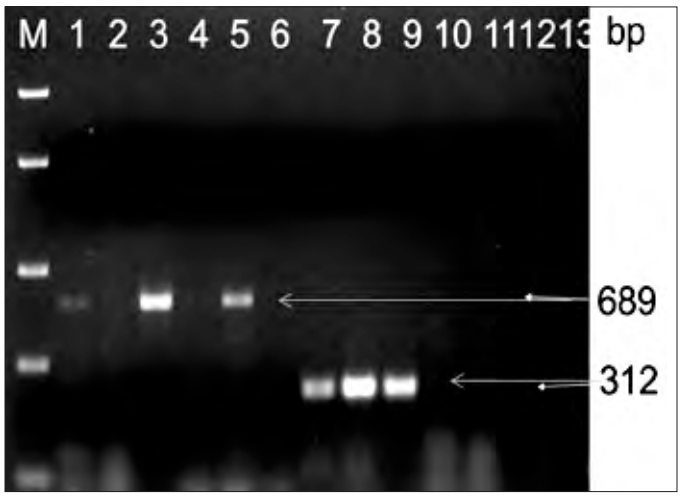

Fig. 1. AGE of amplified DNA for *T. annulata* in PCR using TAF/R primer sets; Lane M: Fast Ruler Middle Range DNA Ladder (Thermoscientific, USA); Lane 1, 3, 5: *Rhipicepalus* (B.) *microplus* ticks positive for *B. bigemina*; Lane 2, 4, 10-13: *Rhipicepalus* (*B.*) *microplus* ticks negative for both *B. bigemina* and *T. annulata*; Lane 7-9: *Rhipicepalus* (*B.*) *microplus* ticks positive for *T. annulata*; Lane 6: Negative control

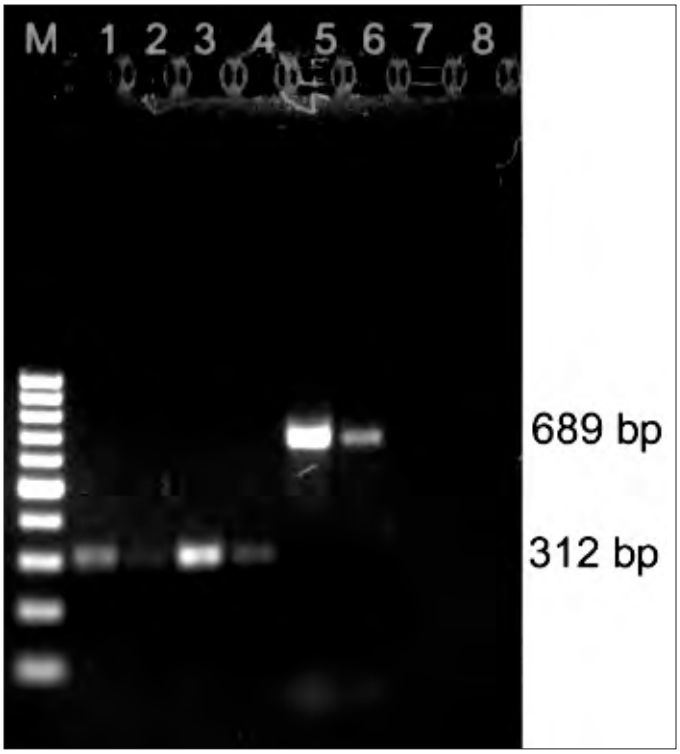

Fig. 2. AGE of amplified DNA for *B. bigemina* in PCR using BBF/R primer sets; Lane M: StepTMUp 100 bp DNA Ladder (GeNei, India); Lane 1-4: *Hyalomma* ticks positive for *T. annulata*; Lane 5-6: *Hyalomma* ticks positive for *B. bigemina*; Lane 7-8: *Hyalomma* ticks negative for both *T. annulata* and *B. bigemina*

anatolicum ticks collected from cattle. Screening of all the 42 *H. anatolicum* pooled tick samples revealed that only five samples were positive for *B. bigemina* giving the desired band of 689 bp (Fig.2). Out of five *B. bigemina* positive tick samples, two (40%) were found positive from Shri Krishan, Tohana Road, Barwala (Hisar), two (33.3%) from Shri 108 Bramchari Jai Ram Dass, Beri (Jhajjar) and one (16.6%) was found positive from Shri Krishan Parnami, Kanwarpura (Sirsa) by PCR (Table 2).

In India, *R. (B.) microplus* and *H. anatolicum* ticks are considered as main vectors for *B. bigemina* and *T. annulata*, respectively (Singh and Rath, 2013). But in the present study, examination of *R. (B.) microplus* ticks collected from three districts of Haryana revealed presence of *Theileria* infection by PCR. Briefly, 16% (8 out of 50 ticks) *Rhipicephalus* tick isolates of Hisar, 14.6% (6 out of 41 ticks) isolates of Jhajjar and 28.6% (2 out of 7 ticks) isolates of Sirsa, were found positive for *T. annulata*. Similarly, *H. anatolicum* collected from three districts of Haryana revealed *B. bigemina* infection in 10% (2 out of 20 ticks) isolates of Hisar, 12.5% (2 out of 16 ticks) isolates of Jhajjar and 16.7% (1 out of 6 ticks) isolates of Sirsa.

The reason for the present finding of cross infection of *Theileria* in *Rhipicephalus* and *Babesia* in *Hyalomma* engorged female ticks might be the feeding of high amount

Table 2

Gaushala-wise summary of hard ticks positive for haemoprotozoan infection using PCR method

Sr. No.	Gaushala / Tick Species	*R. (B.) microplus*		*H. anatolicum*	
		Examined	*Theileria* positive	Examined	*Babesia* positive
1	Shri Ladwa, Village Ladwa, Hisar	10	4	4	0
2	Shri Vaishnav Agarsen, Agroha, Hisar	10	0	4	0
3	Shri Gopal Krishan, Talwandi Rukka, Hisar	12	0	2	0
4	Shri Krishan, Tohana Road, Barwala, Hisar	9	1	5	2
5	Shri Balaji Samiti, Mangali, Hisar	9	3	5	0
6	Shri Aryavarat Gaushala, Mandothi, Jhajjar	12	3	3	0
7	Gokul Dham Shri Ratiram Gauseva Samiti, Jhajjar	11	2	3	0
8	Shri Maharishi Vidyanand, Rewari Road, Jhajjar	10	0	4	0
9	Shri 108 Bramchari Jai Ram Dass, Beri, Jhajjar	8	1	6	2
10	Shri Krishan Parnami, Kanwarpura , Sirsa	7	2	6	1
	Total	98	16 (16.3%)	42	5 (11.9%)

Figure in parenthesis show percentage

of blood (containing haemoprotozoans) by engorged female. The intestine of tick contains blood positive for haemoprotozoans and examination of these engorged ticks by PCR revealed infection equivalent to that of host. However, the adult female tick feed on the infected blood of the host containing haemoprotozoans, but the infection may not propagate further and dies in the intestine itself (Bhattacharyulu *et al.*, 1975; Zeb *et al.*, 2019).

The PCR based analysis of salivary gland of *H. anatolicum* for the *B. bigemina* infection did not revealed any positive tick. The results confirm the presence of *Babesia* protozoa only in the blood cells present in the intestine of engorged female *Hyalomma* tick and no further multiplication of the infection takes place in the salivary glands of these ticks. The absence of *Theileria annulata* DNA in the eggs of *R. (B.) microplus* ticks by PCR confirms that the infection is not transferred transovarially to the next generation of ticks. The PCR results certify the presence of *Theileria* protozoa only in the blood cells present in the intestine of engorged female *R. (B.) microplus* tick and no further propagation of the infection takes place in these ticks.

The cross-examination of *R. (B.) microplus* (n=98) and *Hyalomma anatolicum* (n=42) for *Theileria annulata* and *Babesia bigemina* revealed the positivity of 16.3% and 11.9%, respectively. The examination of salivary glands and eggs of these ticks using PCR method did not reveal any infection. The result justified the possibility of positivity of adult female engorged ticks from cross infection of haemoprotozoa, however, there is no further transmission of these infections to the next generation or stage of ticks.

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