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Molecular detection of *Babesia bigemina* in the natural cattle tick vector *Rhipicephalus annulatus* of Kerala by the nested PCR

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Abstract
The objective the present study was to detect the *Babesia bigemina* DNA in the *Rhipicephalus annulatus* ticks engorged on domestic cattle of Northern part of Kerala, South India. A total of 15 engorged *R. annulatus* female ticks (n=15) were collected. The DNA isolated from the ticks was used as template for the nested PCR amplification of rhoptry-associated protein-1a (*rap-1a*) gene specific for *B. bigemina.* Out of 15 ticks examined, the pathogen DNA was detected in seven ticks.

Keywords: *Babesia bigemina*, *Rhipicephalus annulatus*, tick, cattle, India

Introduction
Ticks and tick-borne diseases are one of the most important problems of the livestock industry due to the enormous economic losses. Globally, 80 per cent of the world's cattle population (1.3 billion) are at risk of TTBDs and the economic loss estimated during 1997 was between US$ 13.9 billion and US$ 18.7 billion per annum (de Castro, 1997) [3]. With the 1.47 billion cattle available globally and taking into account the inflation rates from 1996 to 2015 at 52.3%, the losses were estimated at US$ 22-30 billion per annum due to the ticks and tick-borne diseases (Lew-Tabor and Rodriguez Valle, 2016) [8]. In India, the annual control cost for TTBDs in livestock was estimated at US$ 498.7 million (Minijauw and McLeod, 2003) [10].

Babesiosis, theileriosis and anaplasmosis are the important bovine tick-borne diseases (TBD) prevalent in India (Ghosh *et al*., 2006) [5]. Among them, the bovine babesiosis is a major intraerythrocytic apicomplexan tick-borne haemoprotozoan disease caused by the parasites of the genus *Babesia*, which is found worldwide and infects wide range of domesticated and wild cattle, as well as humans (McCrosker, 1981) [9]. In India, the bovine babesiosis is mainly caused by *B. bigemina* followed by *B. bovis* (Seshadri *et al.*, 1985; Kolte *et al*., 2017) [19, 7]. The presence of *Babesia bigemina* and *B. ovata* was reported from the south Indian state, Kerala (Ravindran *et al*., 2002, 2007; Nair *et al*., 2011; Pradeep *et al*., 2019) [15, 16, 12, 14].

There are only very few studies conducted in India, discussing about the identification of the tick vectors of these babesial organisms (Ravindran *et al*., 2006; Bhat *et al*., 2017) [17, 2]. The molecular detection of *Babesia* in the prevalent tick species is an urgent need especially for developing strategic control measures. Thus, the present study focuses on the molecular detection of *B. bigemina* in the natural vector *R. annulatus* of cattle in Kerala by the nested PCR amplification of the rhoptry-associated protein-1a (*rap-1a*) gene.

Materials and Methods
A total of 15 engorged female ticks were collected from the Instructional Livestock Farm Complex, College of Veterinary and Animal Sciences, Pookode. The ticks were collected in a clean vial which was wrapped with a muslin cloth. They were stored at 4 ^{0}C until morphological identification was performed. All the ticks were morphologically identified based on the diagnostic keys (Trapido *et al*., 1964; Auffenberg and Auffenberg, 1990; Voltzit and Keirans, 2002; Geevarghese and Mishra, 2011) [20, 1, 21, 4]. After the morphological identification, the ticks were transferred to -80 ^{0}C. The frozen ticks were crushed into a powder using sterile mortar and grinding pestle. The genomic DNA was isolated from the tick samples using DNeasy® Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's protocol, the quality and quantity were analysed using a NanoDrop™ 2000

spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and then stored at -20 ^{0}C deep freezer. The genomic DNA (~20 ng) was used as the template for polymerase chain reaction (PCR). The polymerase chain reactions were conducted in an automated thermal cycler with heated lid (M/s. Eppendorf, Hamburg, Germany).

For the detection of *B. bigemina*, the nested PCR amplification (Table 1) was performed according to the protocol described by Molad *et al*. (2015) [11]. The product of first amplification (1µL) was used as a template for the nested PCR.

Table 1: Primers, cycling conditions, reaction volume and components

Target gene	Primer name	Oligonucleotide sequence (5'- 3')	Product size (bp)	cycling Conditions	Reaction volume and components	Reference
Rap-1a	RAP1A-1501-F (Primary)	TATGGCACATTGCGCATA	1501	Initial denaturation at 95 °C for 3 min which was followed by 35 cycles, each consisting of denaturation step for 10 sec at 98 °C, an annealing step of 30 sec at 54 °C, and an extension step of 1 min 30 sec at 72 °C and final extension at 72 °C for 7 min.	Total volume= 25 µL [12.5 µL of master-mix (Takara®), 1 µL (10 pmol) each of forward and reverse primer, 1 µL of template DNA and 9.5 µL of nuclease free water (Takara®)].	Molad *et al*. (2015) [11]
	RAP1A-1501-R (Primary)	TCGCTGTTAACCTCCTGAGTAGT				
	RAP1A-758-F (Nested)	TTCTTGGGTGTG TGTTTTGGA	758	Initial denaturation at 95 °C for 3 min followed by 35 cycles, each cycle consisting of denaturation at 98 °C for 10 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 50 sec. The final extension was at 72 °C for 7 min.		
	RAP1A-758-R (Nested)	ATCATGTACTC GCCGTAGCC				

Results

The ticks were identified morphologically as *R. annulatus*. Out of 15 ticks, seven amplified an amplicon specific for the rhoptry associated protein-1a (*rap-1a*) gene with ~758 bp product (Fig. 1).

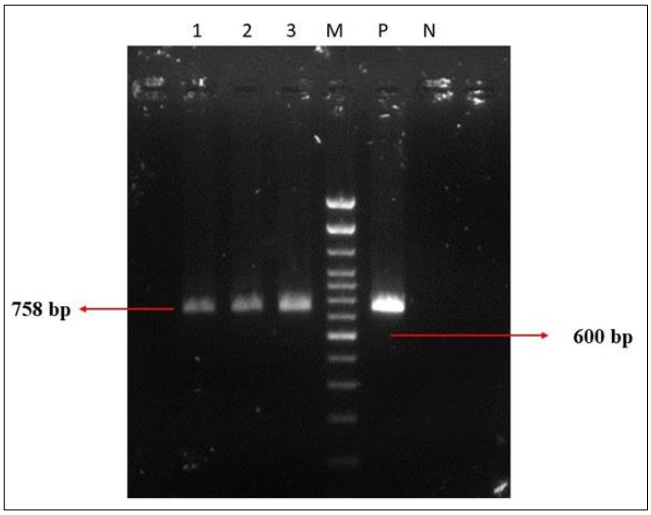

Fig 1: Nested PCR amplification of *rap-1a* gene (~758bp) of *Babesia bigemina*
Lane 1, 2, 3: Samples; Lane M: 100 bp plus ladder,
Lane P: Positive control; Lane N: Negative control

Discussion

Babesiosis among cattle in India is mainly caused by *B. bigemina*, *B. bovis* (Kolte *et al*., 2017) [7] and *B. ovata* (Pradeep *et al*., 2019) [14]. There were no reports of *B. bovis* from Kerala. *Babesia bigemina* and *B. bovis* are transmitted cyclically by *Rhipicephalus* spp. of ticks (Ozubek *et al*., 2020) [13].

In the present study, *B. bigemina* was detected in *R. annulatus* ticks collected from cattle. These findings agree with the results of a previous study involving the detection of *B. bigemina* in *R. annulatus* ticks from different parts of the world (Ica *et al*., 2007; Molad *et al*., 2015; Rajabi *et al*., 2017) [6, 11, 18]. To our knowledge this study forms the first report of the detection of *B. bigemina* in *R. annulatus* ticks from South India. In addition, the vector status of the *B. bigemina* in *R. microplus* ticks was already reported from India (Ravindran *et al*., 2006; Bhat *et al*., 2017) [17, 2]. The epidemiological analysis of the tick-borne diseases requires information on the pathogens and their potential vector ticks in a particular region. The prevalence of bovine babesiosis is assessed mainly based on the detection of the parasite in the host. The detection of the parasite in the engorged ticks is an indirect assessment of the status of parasite inside the hosts. The technique thus acts as a non-invasive methodology for finding the prevalence of the infection.

Conclusion

In the present investigation, *B. bigemina* was detected in *R. annulatus* ticks collected from cattle. To the best of our knowledge, this is the first report of the detection of *B. bigemina* DNA in *R. annulatus* ticks in India.

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