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PCR Based Approach for Detection of Bovine Babesiosis in Suspected Carrier Cattle and Vector Ticks in Sri Lanka

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ABSTRACT

Purpose : *Cattle recovered from clinical babesiosis become carriers for a certain period posing a threat of transmitting the disease to the entire herd. Diagnosis of carrier cattle is important for preventing outbreaks of babesiosis. The objective of the present study therefore, was to establish a sensitive rapid detection method for bovine babesiosis in suspected carrier cattle using nested PCR (nPCR).*

Research Method : *Accordingly, 30 blood samples and ticks were collected from suspected carrier cattle representing two bio-climatic zones of Sri Lanka. Blood samples were analysed by both microscopically and nested nPCR methods for detection of bovine babesiosis. Further ticks were analysed for morphological idenfication using micrscopy and for babesiosis with nPCR.*

Findings : *47% (14/30) among the investigated samples became positive for the babesia infection with light microscopy, while nPCR analysis diagnosed 90% (27/30) as positive. This indicates that, 43% (13/30) of the animals which appeared to be healthy through routine light microscopical diagnosis were in fact carriers posing a major threat to the healthy herd. Further, according to the results of nPCR, 22% (6/27) of the blood samples were positive only for Babesia bovis infection, 11% (3/27) only for Babesia bigemina infection and 67% (18/27) for mixed infection with both parasites. The dominant tick vector isolated from both zones was Rhiphicephalus microplus. Out of the examined ticks, 21% (5/24) were positive for Babesia bigemina infection.*

Research limitations : *Since the sample size from all sites of both climatic zones were uneven and small, data could not be analysed for statistical significance.*

Originality/Value : *However, the results from this study indicate that nPCR provides a sensitive screening method to detect bovine babesiosis compared to the conventional microscopic analysis.*

Keywords: *Babesiosis, nested PCR, Rhiphicephalus microplus, Sri Lanka*

INTRODUCTION

Cattle and Buffalo population of Sri Lanka in 2014 has been recorded as 1.2 million and 0.41 million respectively (DAPH, 2014). In Sri Lanka, many farmers depend on animal husbandry for their livelihood. Being a developing country, dairy industry of Sri Lanka is still struggling to meet self-sufficiency. Among the major

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issues that contributed towards lowering animal production are health issues and reproductive inefficiency. Bovine babesiosis is considered as one of the major tick born diseases in the country with an endemic vector population throughout the island. *Babesia bovis* and *Babesia bigemina* are the major babesia parasites of bovines that are disseminated by a single tick vector, *Rhiphicephalus microplus* in most of the tropical and subtropical regions of the world. *Babesia bovis* infections are characterized by high fever, ataxia, anorexia, general circulatory shock, and sometimes with nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries (Maeda *et al.*, 2016; Mahoney and Mirre, 1977; Musinguzi *et al.*, 2017; Sunil *et al*, 2017). Anaemia and haemoglobinuria may appear later in the course of the disease. In *B. bigemina* infections, the major signs include fever, haemoglobinuria and anaemia. Intravascular sequestration of infected erythrocytes does not occur with *B. bigemina* infections (Bock *et al.*, 2008). In acute cases of *B.bovis* infections the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to *B. bigemina* infections, where the parasitaemia often exceeds 10% and may be as high as 30% (Bock *et al.*, 2008; Benjamin 1986; Ondej *et al.*, 2013).

In acute infection, death occurs within a few days of the onset of fever and severely affected animals would succumb within 24 hrs. Mortality is extremely variable and may reach 50 percent or higher, but in the absence of too much stress, most animals will survive. In those that survive, the febrile stage often lasts for about a week and there is usually severe weight loss, drop in milk production, risk of abortion, and a protracted recovery. Surviving bulls may have reduced fertility for several weeks (Musinguzi *et al*, 2017). Generally, calves are reasonably resistant to the severe clinical disease whereas in older animals, clinical signs can be very severe. A subacute syndrome and mild infections also occur, in which, case signs are less obvious and sometimes even difficult to detect. The fever is mild and hemoglobinuria is often absent in such cases (Benjamin, 1986). Clinical signs are not apparent during this carrier state. Nevertheless, diagnosis of asymptomatic infections are important in disease epidemiology; i.e. carrier cattle act as a reservoir for the infection during the low transmission periods that maintain the infectious agent within the herd (Moses and Phillip, 2013). Diagnosing this carrier cattle population with babesiosis is a challenge to the conventional diagnostic procedures due to the low number of parasites in peripheral blood.

The traditional method of identifying the *Babesia* spp. in acute cases is by microscopic examination of thick and thin blood films stained with Giemsa, a Romanowsky type stain (10% Giemsa in phosphate buffered saline or Sorenson's buffer at pH 7.4). The sensitivity of thick films is such that it can detect parasitaemia as low as one parasite in 10^6 red blood cells (Bose *et al.*, 1995). This technique is usually adequate for the detection of acute infections, but not for the detection of carriers where the parasitaemia are maintained at a very low level. For instance, *in vitro,* culture methods have been used to demonstrate the presence of carrier infections of *Babesia spp.* among those animals who were apparently normal through microscopic examination (Holman *et al.*, 1993). On the other hand, animal inoculation for confirmation of infection in a suspected carrier animal is also possible (Maeda *et al.*, 2016). However, these methods are cumbersome, expensive, and obviously not suitable for routine diagnostic use because of its complexity. Although serological tests have been described that detect the reactive antibodies with *Babesia spp*., they do not consistently detect the infections in carrier animals, and their specificity is limited by the cross-reactivity of the antigens of other *Babesia spp.* (Moses and Phillip, 2013).

Nucleic acid-based diagnostic assays are very sensitive particularly in detecting *B. bovis* and *B. bigemina* in carrier cattle (Buling *et al*., 2007; Venu *et al*., 2015; Criado-Fornelio, 2007). Polymerase chain reaction(PCR) based techniques are reported to be as much as 1000 times more sensitive than microscopy

for detection of *Babesia spp*., with detection of parasitaemia levels ranging from 0.001% to 0.0000001% (1 parasite in 10^9 RBCs) (Criado-Fornelio, 2007). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (Buling *et al*., 2007; Venu *et al*., 2015; Criado-Fornelio, 2007). However, these methods have not been tested in Sri Lankan context to check the feasibilty of using them to develop a more efficient screening procedure. The aim of this study therefore, was to examine the suspected carrier cattle which have recovered from clinical babesiosis and the tick vectors using a nested PCR (nPCR) assay specific for *Babesia bovis* and *Babesia bigemina* in the commercially important milk production areas in Sri Lanka. A comparative analysis was also performed with conventional microscopic methods to gauge the usefulness of the molecular method.

MATERIALS AND METHODS

Statement of Animal Ethics

This study was approved by the Institutional Animal Care and Use Committee of Animal and Research Ethics and carried out according to the Animal Experimentation Regulations of Sabaragamuwa University of Sri Lanka.

Collection of Blood Samples

The study was carried out from the first week of June up to end of July 2015, in four selected dairy farms located in North Western and Central Province and which were under National Livestock Developing Board, Sri Lanka. The study area was determined by the 2014 statistics of cow milk production by district per year in Sri Lanka; around 33.5% and 16.9% of total milk production came from Central and North Western Provinces respectively (DAPH, 2014). Andigama dairy farm and Nikaweratiya dairy farm were selected to represent the North Western Province and Menikpalama dairy farm and Rosita farm, Kotagala were selected to represent Central Province. The study area provided excellent sites that represented, two significantly different bio climatic zones, in the intermediate (Andigama and Nikawaratiya of North Western Province) and Wet zone (Menikpalama and Rosita of Central Province) zone of the island. Both *Bos taurus* origin, *Bos indicus* origin and cross bred dairy cattle were reared in the study area. The blood samples were collected from 30 suspected carrier cattle which have recovered from tick fever within 6 months to 3 years from the time of sample collection. 1 ml of blood was collected from each animal into EDTA-coated tubes by jugular or coccygeal venipuncture and transported to the laboratory on ice and stored at -20°C until further analysis. Clinical history was recorded for all suspected carrier animals recruited for the study.

Tick Collection

The animals which were identified as suspected carrier cattle for Babesia were examined for tick infestation at the ear, eye, flank, abdomen, tail and perineal regions at the time of the collection of the blood sample. With the use of a thin-tipped tweezers or forceps with a steady even pressure, ticks were removed straight upward from different body parts of the cattle. Ticks were collected and preserved in 70% ethanol for morphological identification and DNA extraction.

Identification of Tick Species

The ticks were identified up to the species level under the stereo microscope (Stemi 2000, 150X,Carl Zeiss Microscopy, Germany). The species and sex of the ticks were identified according to standard taxonomic keys for adult ticks (Walker *et al*., 2003).

Microscopic Detection of Blood Smears

The dried thin and thick blood smears were fixed in absolute methyl alcohol for one min. Staining was performed using Giemsa method as described by Benjamin (1986). Thick and thin blood smears were stained with one fourth dilution of commercially available Giemsa stain for four mins; and the smear was observed with the help of oil immersion lens (100X) to detect

the presence of *Babesia spp.* Identification was conducted using standard keys (Soulsby, 1982).

DNA Isolation from Bovine Blood and Rhiphicephalus Microplus Female Tick

The molecular detection was conducted at Genetech Molecular Diagnostics, Colombo, Sri Lanka. Genomic DNA was extracted from 300µl of cattle blood and *Rhiphicephalus microplus* fed female ticks using Wizard Genomic Extraction kit (Promega, WI, USA) according to the manufacturer's guidelines. Extracted DNA was dissolved in 100µl of rehydration buffer and stored at −20°C until further analysis.

PCR Amplification and Data Analysis

The method described by Figueroa J V *et al.,* (1993), was slightly modified to establish the PCR assay. The PCR reaction mixture contained 2.5µl of template DNA, 1xTaq PCR green buffer 1.5 mM $MgCl_2$, 1.25U of Taq DNA polymerase (PromegaGo Taq; Promega, WI, USA), 0.35mMdNTP each (Geneshun Biotech, China) and 0.6µM of eachprimer (Macrogen, Korea) in a final volume of 25µl. The amplification profile consisted of 3 mins; of initial denaturation at 95°C followed by 35 cycles of 30S at 95°C, 45S at 55°C, 1 min at 72°C, and final extension at 72°C for 10 mins; in a thermal cycler (Biorad; Mycycler). 2µl of first round PCR products were used for the second round (nested) PCR mixtures comprising of similar composition of reagents as the first round PCR, except that the external primers were replaced with the nested PCR primers. PCR mixtures were cycled using the same PCR profile. The PCR products obtained were visualized with UV transillumination after electrophoresis in 2.0 % agarose ethidium bromide gels (Figure 1). The exact fisher test was used to compare the data from blood smears and nPCR at significance level of 0.05.

Table 01: Primer sequences and amplicon sizes used to detect *Babesia bovis* and *Babesia bigemina*

Target organism	Assay	Nucleotide sequence (5' to 3')	Annealing temperature	Product length
B.bigemina	PCR	F: CATCTAATTTCTCTCCATACCCCTCC R: CCTCGGCTTCAACTCTGATGCCAAAG	55°C	278bp
B.bigemina	nPCR	F: CGCAAGCCCAGCACGCCCCGGTGC R: CCGACCTGGATAGGCTGTGTGATG	55°C	170bp
B.bovis	PCR	F: CACGAGGAAGGAACTACCGATGTTGA R: CCAAGGAGCTTCAACGTACGAGGTCA	55°C	360bp
B.bovis	nPCR	F: TCAACAAGGTACTCTATATGGCTACC R: CTACCGAGCAGAACCTTCTTCACCAT	55°C	298bp

F: Foward Primer, R: Reverse Primer

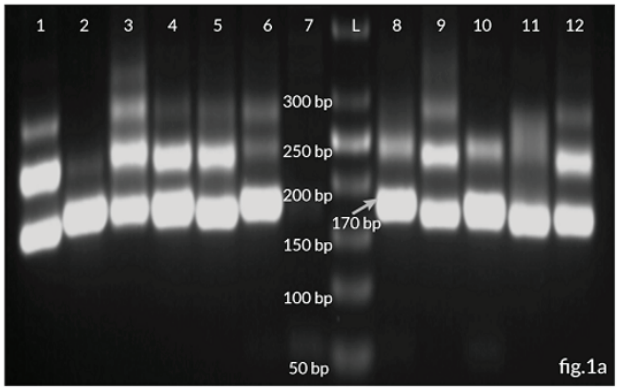

Figure 01a: Agarose gel electrophoresis photograph showing nested PCR results for the detection of *Babesia bigemina* in suspected carrier cattle.

Lane 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, and 12; Positive samples sample for *B. bigemina*, Lane 7; Negative sample for *B. bigemina* at 170bp, Lane L; 50bp DNA ladder marker.

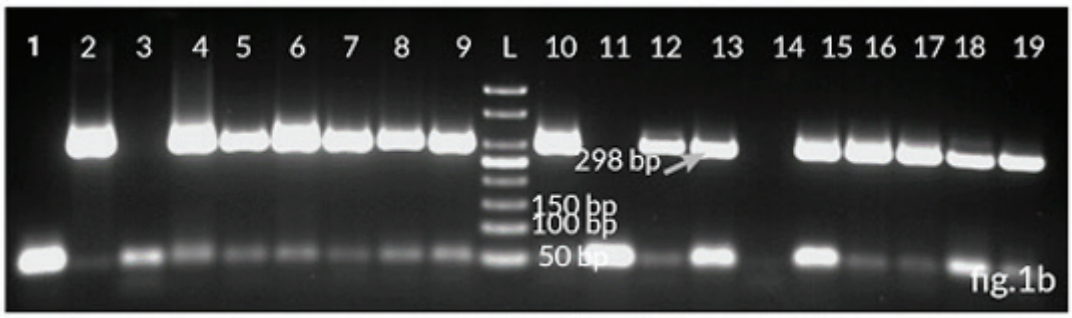

Figure 01b: Agarose gel electrophoresis photograph showing nested PCR results for the detection of *Babesia bovis* in Suspected Carrier cattle.

Lane 1,3,11 and 14; Negative samples for *B.bovis*, Lane 2,4,5,6,7,8,9,10,12,13,15,16,17,18 and 19; Positive samples for *B. bovis* at 298bp, Lane L; 50bp DNA ladder marker.

RESULTS AND DISCUSSION

Sensitivity of the Nested PCR Detection

Among the study samples (n=30), 46.66% of suspected carrier cattle were positive for either of the *Babesia spp.* when conventional thin film microscopy results were analysed. According to the nPCR results, 90% of the carrier cattle were positive for either of the two infections. However, the difference was not significant (p>0.05). Among nPCR positives, 88.88% of cattle were positive for *Babesia bovis*, 77.77% for *Babesia bigemina* and 66.66% for both *Babesia bovis* and *Babesia bigemina* infections regardless of the bio climatic zones. However, previous PCR based study by Sivakumar *et al.,* 2012 demonstrated that the dry zones (Polonnaruwa, Ampara, and Jaffna) had more *Babesia*-positive animals than the hill country wet zone (Nuwara Eliya) regardless their carrier stage.

This study shows that the nPCR assay for the detection of *Babesia spp*. is more sensitive compared to the conventional microscopic method in detecting carrier animals. However, statistical comparison of the two mthods did not show a significant diffence with respect to the sensitivity. Similarly, the occurance of *B.bovis* and *B.bigemina* did not show a significant difference (P>0.05). It is likely that the uneven and small sample size from the collected sites from both climatic zones have masked the possible significant effects. Increasing the sample size would give more accurate results and would assist in confiming the observed high senisitinity of nested PCR over microscopy. Prevalence of carrier cattle in a herd as identified by the nPCR, creates the biggest threat by providing reservoirs for the *Babesia spp.* which leads to rapid transmission of the disease to other animals in the herd.

Table 02: Detection of *Babesia bovis* and *Babesia bigemina* in whole blood from cattle in Central and NorthWestern Province of Sri Lanka

Site		Total positives for microscopy		Total positives for nPCR		Presence of diffrent infections in positive samples as detected by nPCR					
						B.bovis		*B.bigemina*		*B.bovis* and *B. bigemina*	
		+	%	+	%	+	%	+	%	+	%
Central Province	site 1(n=4)	0	0	2	50	2	100	0	0	0	0
	site 2(n=11)	2	18.2	10	90.9	8	80	8	80	6	60
Northwestern Province	site 1(n=2)	1	50	2	100	1	50	2	100	1	50
	site 2(n=13)	11	84.6	13	100	13	100	11	84.6	11	84.6
Total	n=30	14	46.6	27	90	24	80	21	70	18	60

Central Province; Site 1:Menikpalama dairy farm, Site 2: Rosita farm, Kotagala
North Western Province; Site 1: Nikaweratiya dairy farm , Site 2: Andigama dairy farm
+ :Positive, %: Percentage, No: Number
Statistical significance of differences between microscopic detection and nPCR were tested at of 0.05 level

Prevalence of Babesia bovis and Babesia bigemina in Suspected Carrier Cattle

More than 50% of the herd carry at least a single *Babesia spp.* within the first six months from the recovery of the clinical disease (Table 3). One third of the herd would still carry them even after one year from the recovery. Long-term persistence of Babesia infection may develop premune immunity; resistance to re-infection resulted by continuous presence of the parasite in blood (Chauvin *et al.,* 2009).

Heifers examined in this study were aged between 5 to 10 months and cows in lactation were between the first to eighth lactation. However, tick infestation was higher with uncountable numbers in heifers compared to lactating cows as observed. Probably, this massive tick infestation itself may have boosted the immunity among heifers towards *Babesia* spp. making them more resistant to the infection, assuming that majority of the ticks they harbour were infected. As shown in Table 4, out of the two pathogenes, *Babesia bovis* was predominant compared to *Babesia bigemina* in both heifers and cows in lactation. Mixed infections were also present. However, calves rarely showed clinical signs of the disease after contracting the infection regardless of the *Babesia spp*. involved or the immune status of the dams.

Young calves exhibit a strong innate immunity compared to adult cattle (Ondrej *et al*., 2013; Goff *et al*., 2001). Within the study sites, all heifers or calves (n=10) of Jersey sahiwal cross breed, aged from 5 to 10 months were subclinical for babesiosis at parasitaemia levels detectable by PCR indicating high disease transmission rates. It might further indicate the tolarence for clinical babesiosis for reinfections with the same strain of the parasite. However, calves rarely showed clinical signs of the disease after contracting the infection regardless of the *Babesia spp*. involved or the immune status of the dams. This is in agreement with our result which indicates the low level of infections in calves/young heifers than lactating cows (Bock *et al.*, 2008; Monica *et al*., 2013).

Other than the passive immunity developed from maternal antibodies, immunization of cattle with *Babesia* live vaccine provides a durable, long-lasting immunity against *B. bovis* and *B. bigemina.* However, according to our results, more than 80% of pre-immunized cattle with locally manifactured vaccines were positive for both *B. bovis* and *B.bigemina* infections. A single vaccination usually provides lifelong immunity. Protective immunity develops in 3–4 weeks. But live *Babesia* vaccines are not entirely safe (Bock *et al.*, 2008). A practical recommendation is to limit their use to calves, preferably less than 1 year old, when nonspecific, immunity will minimize the risk of vaccine reactions. However, the cattle examined to check the pre-immunized status are aged between 5 to 10 months and they continue to carry *Babesia* infection even after pre-immunization in test herds. It is likely that antibodies produced by the attenuated vaccine of babesia may not work against the certain isotypes of *Babesia spp.*

Tick Infestations

The cattle identified as suspected carriers of babesiosis in this study were noticed for clinical symptoms of tick infestation as damaged hides, lethargy, lower weight gain, reduced appetite and other secondary infections irrespective of them being subclinical for babesiosis. The entire herd examined were carrying severe tick infestation even after recent application of acaricide. It is possible that ticks which have infested the examined cattle population may have acquired resistance to certain acaricide used in the farms over the years. According to the samples collected from the two different localities, the major tick species observed in cattle was *Rhiphicephalus microplus* (Figure 2). The tick infestations in cattle were high in certain sites of both Central and Northwestern provinces (Table 6).

Table 03: Prevalence of *Babesia bovis* and *Babesia bigemina* in suspected carrier cattle as detected by nPCR according to the time elapsed after the recovery from the clinical *Babesia* infection

Period between sample collection and the recovery from the clinical disease	Presence of *Babesia spp.*						Total no.of infected cattle	
	Babaesia bovis		*Babesia bigemina*		*Babesia bovia* and *Babesia bigemina*			
	+	%	+	%	+	%	No.	%
0 to 6 months (n=11)	6	54.5	8	72.7	5	45.4	9	81.8
6 to 12 months (n=6)	5	83.3	3	50	3	50	5	83.3
1 year to 3 years (n=3)	3	100	1	33.3	1	33.3	3	100
Total (n=20)	14	70	12	60	9	45	17	85

+ :Positive, %: Percentage, No: Number

Table 04: Prevalence of *Babesia spp.* in suspected carrier cattleas detected by nPCR in relation to their physiological status

Physiological status of cattle	*Presence of Babesia spp.*						Total no.of infected cattle	
	Babesia bovis		*Babesia bigemina*		*B.bovis* and *B.Bigemina* mixed *infection*			
	+	%	+	%	+	%	No.	%
Heifers (n=12)	7	53.3	5	41.6	3	25	9	75
Cows in lactation (n=18)	17	94.4	16	88.8	15	83.3	18	100
Total(n=30)	24	80	21	70	18	60	27	90

+ :Positive, %: Percentage,No: Number

Table 05: The presence of *Babesia bovis* and *Babesia bigemina* infections in pre-immunized suspected carrier cattle as detected by nPCR

Period between pre-immunization and sample collection	Presence of *Babesia spp.*						Total no.of infected cattle	
	Babesia bovis		*Babesia bigemina*		*B.bovis* and *B.Bigemina mixed infection*			
	+	%	+	%	+	%	No.	%
2 months (n=5)	5	100	4	80	4	80	5	100
6 months (n=6)	6	100	6	100	6	100	6	100
Total (n=11)	11	100	10	90.9	10	90.9	11	100

+ :Positive, %: Percentage,No: Number

Table 06: Number of *Rhiphicephalus microplus* females infected with *Babesis bovis* and/or *Babesia bigemina* as detected by nPCR; Ticks (n=24) were collectd from Heifers (n=10) in Northwestern Province of Sri Lanka

Infection dected in Heifers	Infection detected in female ticks			
	B.bovis	*B.bigemina*	*B.bovis* and *B.bigemina*	Negative
B.bovis	0	0	0	2
B.bigemina	0	0	0	0
Mixed infection *with B.bovis* and *B.bigemina*	0	5	0	17
Total	0	5	0	19

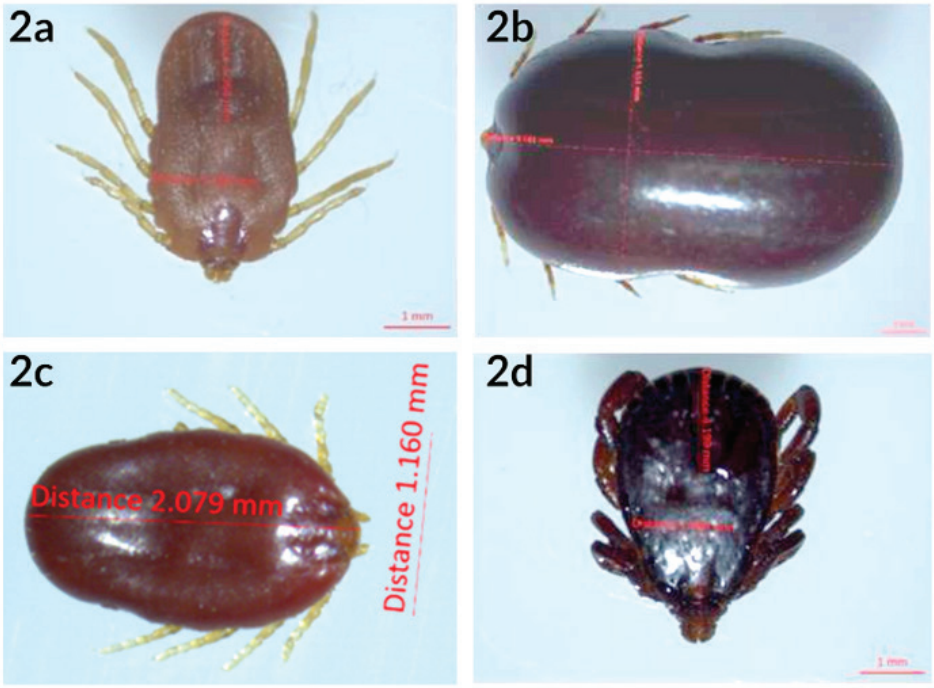

Figure 02: Morphological features of *Rhiphicephalus microplus,* the major tiks associated with bovine babesiosis in Sri Lanka.

Figure 02a: *Rhiphicephalus microplus* female tick.

Figure 02b: *Rhipicephalus microplus* - Fed female.

Figure 02c: *Rhiphicephalus microplus* nymph.

Figure 02d: *Rhiphicephalus microplus* male tick.

Low detection levels of *Babesia spp*. in ticks is comparable to the result reported in previous reseach found in the liteature (Sunil *et al*., 2017). High prevelance of *Babesia bigemina* compared to *Babesia bovis*is is also supported by previous studies (Sandra *et al*., 2014). Under field conditions, the rate of tick transmission is generally higher for *B. bigemina* than for *B. bovis* (Bock *et al*., 2008) because *B. bovis* infection does not persist in *Rhiphicephalus microplus* beyond the larval stage while *B. bigemina* can be passed from one generation to the next even when the adult ticks feed on non-susceptible hosts (De Vos *et al.,* 2004).

Further, the result obtained indicating lower tick transmission may be due to lack of enzootic stability. Enzootic stability fails to develop due to low tick transmission rates caused by ecological factors such as drought or use of acaricides (Neelu *et al*., 2015). But the establishment of low and long-lasting parasitemia is favourable for babesia. Importantly, it will increases the duration of the transmission period and consequently the chance of being acquired by the tick (Chauvin *et al*., 2009). Furthermore, tick infestations predispose cattle to bacterial and fungal infections, as well as screw-worm attack, in the wounds left by tick bites (Bose *et al*., 1995; Venu *et al*., 2015). By direct observation of cattle rearing practices and analyzing clinical history of individual cows, it was clear that there were deficiencies in optimal management practices along with well formulated disease diagnostics, treatment and follow-up programs which must have caused a high prevalence of babesiosis among the herds investigated. Routine disease diagnosis, increase sanitation to minimize the tick infestation, strict quarantine and management practices (such as isolation of carrier animals from the herd) and early detection will be the possible options to reduce the babesiosis occurrence in these farms.

CONCLUSION

In conclusion, early detection of bovine babesiosis by nPCR screening will help in identifying the carrier animals and will facilitate preventive measures through isolating carriers from the entire herd.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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