



## NOTE

Bacteriology

# Epidemiological survey of *Anaplasma marginale* in cattle and buffalo in Sri Lanka

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**ABSTRACT.** Bovine anaplasmosis caused by *Anaplasma marginale* represents a serious threat to cattle farming worldwide, especially in the tropics and subtropics. In the present study, archived DNA samples from the blood of cattle (n=437) in the Nuwara Eliya, Galle, Ampara, Polonnaruwa, and Jaffna districts and buffalo (n=327) in the Galle, Polonnaruwa, Mannar, and Mullaitivu districts in Sri Lanka, were screened for *A. marginale* using a major surface protein 5 (*msp5*) gene-based PCR assay. The findings showed that 32.7 and 57.5% of cattle and buffalo, respectively, were *A. marginale*-positive. The rate of positivity differed significantly among geographical regions. In conclusion, the high rates of *A. marginale* infection in cattle and buffalo highlight the importance of effective control measures in Sri Lanka.

**KEY WORDS:** *Anaplasma marginale*, buffalo, cattle, PCR, Sri Lanka

*Anaplasma marginale* is an intracellular Gram-negative bacterium that belongs to the family *Anaplasmataceae*, in the order *Rickettsiales* [17]. *A. marginale* infects a variety of ruminants, including cattle and buffalo [1, 18, 20]. However, clinical disease is common only in cattle, whereas the infection is usually asymptomatic in other host animals [1]. *A. marginale* is transmitted by ticks, and several tick species, including species of *Rhipicephalus* (*Boophilus*), *Dermocenter*, *Ixodes*, and *Hyalomma*, were reported to be involved in its transmission [12]. In addition, *A. marginale* can also be transmitted mechanically by biting flies or contaminated fomites, such as needles, and transplacentally from cow to offspring [1]. *A. marginale* reproduces in host erythrocytes, and the infected erythrocytes are removed by the reticuloendothelial system, leading to mild to severe hemolytic anemia [18]. Fever, abortion, and sometimes death are some of the other clinical signs observed in *A. marginale*-infected cattle. Infected animals remain as carriers for a long time, sometimes for life [12]. The major control methods include treatment of clinically-infected animals with antibiotics, vaccination, and tick control [1].

Epidemiological surveys have often been conducted to estimate the risk of *A. marginale* infection in cattle in several endemic countries [4, 8, 15, 28, 29, 31, 32]. Industry in Sri Lanka is based heavily on agriculture. The livestock sector in this country, however, suffers huge economical losses due to various infectious diseases. Among them, bovine babesiosis and theileriosis caused by *Babesia bovis* and *Babesia bigemina*, and *Theileria annulata* and *Theileria orientalis*, respectively, are widespread in this country. In the recent past, a series of epidemiological surveys were conducted in various geographical locations in Sri Lanka to detect and genetically characterize bovine *Babesia* and *Theileria* parasites in cattle and buffalo [7, 14, 21–25, 33]. In addition to babesiosis and theileriosis, anaplasmosis causes substantial economic losses in cattle industry in Sri Lanka due to treatment and control costs and production losses [5]. However, epidemiological surveys were not carried out, except a serological study that had been conducted more than 25 years ago [9], to determine the prevalence of *A. marginale* in this country. The aim of the present study was to detect *A. marginale* in cattle and buffalo reared in various locations within Sri Lanka, using a PCR assay.

Archived DNA samples extracted from blood collected from 437 cattle reared in the Galle (n=121), Polonnaruwa (n=84), Jaffna (n=61), Nuwara Eliya (n=83), and Ampara (n=88) districts and from buffalo (n=327) reared in the Galle (n=7), Polonnaruwa (n=118), Mannar (n=146), and Mullaitivu (n=56) districts in Sri Lanka, were used in the present study. The blood samples were collected from European cattle breeds (*Bos taurus*) reared by an intensive management system in Nuwara Eliya district. In the remaining districts, local (*Bos indicus*) or their crosses reared by extensive or semi-intensive management system were sampled.

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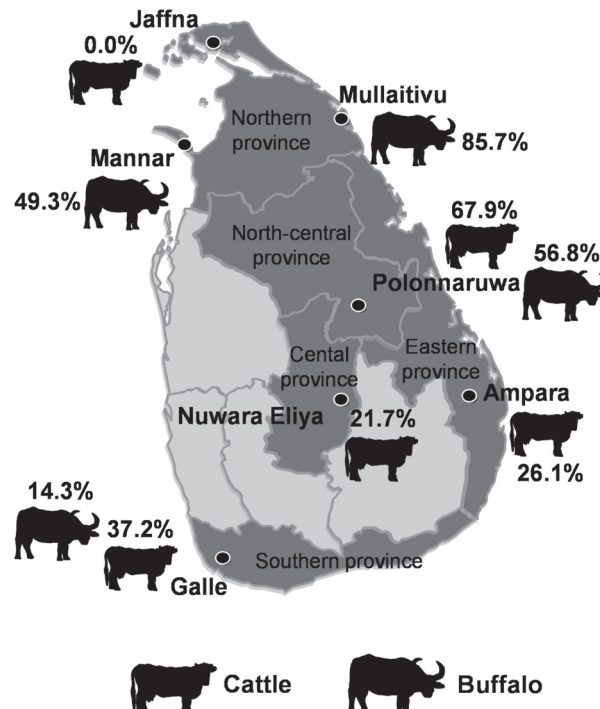
For buffalo, information on breeds was not known due to extensive cross breeding. Buffalo from all of the surveyed districts were managed extensively. The buffalo in the sampled locations usually share common grazing lands with cattle. All animals were apparently healthy during sampling. Among the sampling districts, Nuwara Eliya and Galle districts are located in the wet zone, while the others (Ampara, Polonnaruwa, Mannar, Mullaitivu, and Jaffna districts) are located in the dry zone. The DNA samples were extracted using a commercial DNA extraction kit (Qiagen, Hilden, Germany) from 200  $\mu$ l of whole blood collected from the jugular vein of each animal. These DNA samples had been used to analyze the epidemiology of *Babesia*, *Theileria*, and *Trypanosoma* parasites in previous studies [6, 21, 24, 33].

A previously described PCR assay based on the major surface protein 5 (*msp5*) gene was employed to screen the DNA samples for *A. marginale* infection [31]. Briefly, a 10- $\mu$ l PCR reaction mixture was prepared including 1  $\mu$ l DNA sample, 1  $\mu$ l of 10 $\times$  PCR buffer (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ, U.S.A.), 1  $\mu$ l of 2 mM dNTPs, 1  $\mu$ l of 10  $\mu$ M forward (AM-49F1, 5'-GTGTTCTGGGGTACTCCTATGTGAACAAG-3') and reverse (AM-595R1, 5'-AAGCATGTGACCGCTGACAACTTAAACAG-3') primers, 0.1  $\mu$ l of 5 units/ $\mu$ l AmpliTaq Gold polymerase (Applied Biosystems), and 4.9  $\mu$ l distilled water. After an initial enzyme activation step at 95°C for 5 min, the reaction mixture was subjected to 35 cycles each containing a denaturation step at 95°C for 30 sec, an annealing step at 68°C for 30 sec, and an extension step at 72°C for 1.5 min. After a final elongation step at 72°C for 5 min, PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and then observed under UV light. Detection of a band with an approximate size of 547 bp was considered a positive result. The OpenEpi online program (<http://www.openepi.com/v37/Proportion/Proportion.htm>) was used to calculate confidence intervals for the positive rates, based on the Wilson score [30].

On a per district basis, *A. marginale* positivity in cattle and buffalo ranged from 0 to 67.9% and 14.3 to 85.7%, respectively (Table 1). The cattle in Polonnaruwa had a higher positive rate of detection (67.9%) than those in Galle (37.2%), Nuwara Eliya (21.7%), and Ampara (26.1%) (Table 1). By contrast, none of the surveyed cattle were positive for *A. marginale* in Jaffna, possibly due to good management practices that result in a low prevalence of tick-borne pathogens in this district [21]. For buffalo, *A. marginale* positivity was higher in Polonnaruwa (56.8%), Mannar (49.3%), and Mullaitivu (85.7%) than in Galle (14.3%) (Table 1). The overall positive rates of *A. marginale* were significantly higher in buffalo (57.5%) than in cattle (32.7%) (Table 1). However, regardless of what the host species is, the positive rates were generally higher in the districts located in North-Central (Polonnaruwa district) and Northern provinces (Mannar and Mullaitivu) than those from the districts in Southern (Galle), Central (Nuwara Eliya), and Eastern (Ampara) provinces (Table 1 and Fig. 1). Notably, of 327 buffalo surveyed in the present study, 320 were from North-Central and Northern provinces. Therefore, it seems that the differential overall positivity in cattle and buffalo might be due to regional

**Table 1.** PCR detection of *Anaplasma marginale* in cattle and buffalo in Sri Lanka

Animal type	District	No. samples	No. positive	%	Confidence interval
Cattle	Nuwara Eliya	83	18	21.7	14.2–31.7
	Galle	121	45	37.2	29.1–46.07
	Ampara	88	23	26.1	18.1–36.2
	Polonnaruwa	84	57	67.9	57.3–76.9
	Jaffna	61	0	0	
Total		437	143	32.7	28.5–37.3
Buffalo	Galle	7	1	14.3	2.6–51.3
	Polonnaruwa	118	67	56.8	47.8–65.4
	Mannar	146	72	49.3	41.3–57.3
	Mullaitivu	56	48	85.7	74.3–92.6
Total		327	188	57.5	52.1–62.7



**Fig. 1.** Geographical distribution of *Anaplasma marginale* in Sri Lanka. *Anaplasma marginale*-positive rates in cattle and buffalo in the surveyed districts, which are located in different provinces, are shown. Note that the *A. marginale*-positivity is higher in North-Central and Northern provinces, with the exception of Jaffna district in Northern province, compared with Central, Eastern and Southern provinces.

variations. In Polonnaruwa, the positive rate was comparable between cattle and buffalo (Table 1), supporting our assumption that differences in the overall positivity between cattle and buffalo were not influenced by host type.

The samples used in the present study were used to survey *B. bovis*, *B. bigemina*, *T. annulata*, *T. orientalis*, and *Trypanosoma theileri* in previous investigations [6, 21, 24, 33]. In the current study, we analyzed the rates of co-infections with the above parasite species among *A. marginale*-positive and *A. marginale*-negative DNA samples. An N-1 chi-squared test ([https://www.medcalc.org/calc/comparison\\_of\\_proportions.php](https://www.medcalc.org/calc/comparison_of_proportions.php)) was used to calculate *P* values to determine the significance of variations between the rates [3, 16]. *P* value <0.05 was considered to indicate a statistically significant difference. We found that the rates of *B. bovis*, *B. bigemina*, and *T. annulata* infections were significantly higher among *A. marginale*-positive cattle (21.0, 48.3, and 15.4% respectively) compared with those among *A. marginale*-negative cattle (3.9, 18.5, and 3.9% respectively) (Table 2). These findings suggest that *A. marginale* might be transmitted by ticks capable of transmitting *B. bovis*, *B. bigemina*, and *T. annulata* in Sri Lanka. *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus sanguineus*, which are known vectors of *B. bovis* and *B. bigemina*, as well as *A. marginale*, infest cattle in Sri Lanka [13]. This could explain why the *B. bovis*- and *B. bigemina*-positivity was high among *A. marginale*-positive cattle. However, the differential positivity among geographical regions cannot be explained by the distribution of tick vectors transmitting *B. bovis* and *B. bigemina*, as a previous study suggested that the tick vectors involved in transmission of these parasite species may not differ between geographical locations in Sri Lanka [22]. A recent study suggested that ticks transmitting *T. annulata* might have a different distribution among geographical locations in Sri Lanka, as the density and/or the activity of ticks transmitting *T. annulata* were suggested to be higher in Polonnaruwa than in Nuwara Eliya [22]. Consistent with this, in the present investigation, we found that the *A. marginale*-positive rate was higher in Polonnaruwa than in Nuwara Eliya. This could explain the differential *A. marginale*-positivity in different geographical regions. However, further studies to identify the transmission vectors of *B. bovis*, *B. bigemina*, *T. annulata*, and *A. marginale* and to determine their relative densities and activities in different geographical areas are important to confirm our assumptions.

The co-infection data also demonstrated the lower *T. orientalis* infection rate among *A. marginale*-positive cattle and buffalo compared with *A. marginale*-negative cattle and buffalo (Table 2). Although the reasons for this negative correlation are not clear, such a relationship is not uncommon among bovine blood pathogens. For example, recent studies found negative correlations between *Babesia ovis* and *Theileria ovis*, as well as between *T. orientalis* and hemoplasmas [19, 26]. Experimental infections in cattle and buffalo with *A. marginale* and *T. orientalis* might shed additional light on the negative correlation found between *A. marginale* and *T. orientalis* in the present study.

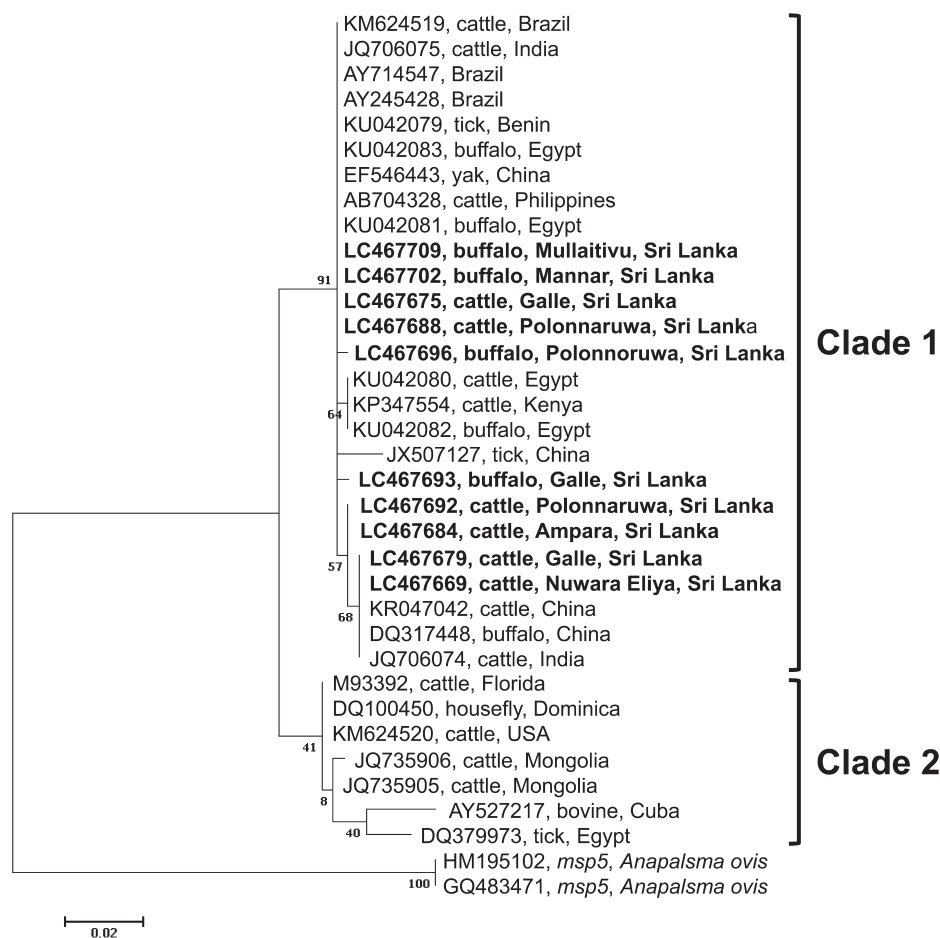
By contrast, the positive rates of *B. bovis* and *B. bigemina* were comparable between *A. marginale*-positive and *A. marginale*-negative buffalo, possibly due to low positive rates of these parasite species in the surveyed buffalo [6, 24]. In addition, the *T. theileri*-positive rates were comparable between *A. marginale*-positive and *A. marginale*-negative cattle and buffalo, as *T. theileri* is transmitted mainly by tabanid flies [2].

To confirm the PCR results, PCR amplicons from 24 cattle (six from each district) and 19 buffalo (six from Polonnaruwa, Mannar, and Mullaitivu, and one from Galle) were cloned and sequenced. Briefly, PCR amplicons were ligated into a pCR 2.1 plasmid vector (Invitrogen, Carlsbad, CA, U.S.A.), and subsequently transformed into One Shot Top10 chemically competent *Escherichia coli*, using the TA Cloning kit (Invitrogen). The gene inserts were then sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The nucleotide identity scores shared among the *msp5* sequences (GenBank accession no. LC467669-LC467711) from both cattle and buffalo ranged from 99.1 to 100%. Next, the newly generated *msp5* gene sequences, together with those retrieved from GenBank, were aligned using MAFFT online software program (<https://mafft.cbrc.jp/alignment/server/>) [10]. The alignment was then analyzed by the MEGA version 7.0 software program [27] to predict best-fitting substitution model based on the lowest Akaike Information Criterion (AIC) value. Finally, a maximum likelihood phylogeny based on the Kimura 2-parameter substitution model [11] was constructed with 1,000 bootstrap replicates, using the MEGA software. In the resultant phylogeny, the Sri Lankan *msp5* sequences from both cattle and buffalo occurred in a single clade (Fig. 2). The sequencing data and phylogenetic analysis suggested that the *A. marginale msp5* gene is conserved in Sri Lanka. Based on genetic

**Table 2.** Co-infections between *Anaplasma marginale* and hemoprotozoan parasites

Parasite <sup>a)</sup>	Cattle <sup>b)</sup>			Buffalo		
	<i>A. marginale</i> -positive (n=143)	<i>A. marginale</i> -negative (n=233)	<i>P</i> value <sup>e)</sup>	<i>A. marginale</i> -positive (n=188)	<i>A. marginale</i> -negative (n=139)	<i>P</i> value
<i>B. bovis</i>	30 (21.0% <sup>e)</sup> )	9 (3.9%)	<0.0001	3 (1.6%)	3 (2.2%)	0.6914
<i>B. bigemina</i>	69 (48.3%)	43 (18.5%)	<0.0001	3 (1.6%)	2 (1.4%)	0.8838
<i>T. annulata</i>	22 (15.4%)	9 (3.9%)	0.0001	ND <sup>d)</sup>	ND	
<i>T. orientalis</i>	94 (65.7%)	180 (77.3%)	0.0142	149 (79.3%)	122 (87.8%)	0.0438
<i>Tr. theileri</i>	18 (12.6%)	31 (13.3%)	0.8450	32 (17.0%)	19 (13.7%)	0.4169

a) PCR detection of *Babesia* and *Theileria* in cattle and buffalo was described by Sivakumar *et al.* [21, 24], while that of *Tr. theileri* in cattle and buffalo was described by Yokoyama *et al.* [33] in all sampling locations except Galle district. The data on PCR screening of *Babesia*, *Theileria*, and *Tr. theileri* in cattle and buffalo in Galle was described by Gunasekara *et al.* [6]. b) cattle from Jaffna district were not considered, as none of them were *A. marginale*-positive. c) The rates of *Babesia*, *Theileria*, and *Trypanosoma* infection were calculated among 143 *A. marginale*-positive and 233 *A. marginale*-negative cattle and among 188 *A. marginale*-positive and 139 *A. marginale*-negative buffalo. d) ND, *T. annulata* was not detected in buffalo in Sri Lanka. e) *P* value <0.05 indicates a statistically significant difference.



**Fig. 2.** Phylogenetic analysis of the *Anaplasma marginale msp5* gene. The newly determined *A. marginale msp5* sequences (highlighted in boldface type) from cattle and buffalo in Sri Lanka, together with those from other countries, were used to construct a maximum likelihood phylogeny. Note that the Sri Lankan *msp5* gene sequences from cattle and buffalo are located in clade 1.

analyses, previous investigations in Sri Lanka suggested that buffalo in this country may not be a reservoir for *T. orientalis* and *B. bovis* that infect cattle [21, 24]. By contrast, the present findings highlight that buffalo might be a reservoir host for *A. marginale* infecting cattle. However, further studies using different marker genes are essential to confirm our assumption.

In conclusion, the present study, which analyzed *A. marginale* infections in Sri Lanka, found that a substantial proportion of cattle and buffalo were infected with this pathogen. The positive detection rates of *A. marginale* differed among geographical regions. Control strategies to reduce the *A. marginale* burden in both cattle and buffalo are now a priority in Sri Lanka.

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