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PARASITOLOGY

Genetic Analysis of *Babesia* Isolates from Cattle with Clinical Babesiosis in Sri Lanka

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ABSTRACT Bovine babesiosis is a serious threat to the cattle industry. We prepared blood DNA samples from 13 cattle with clinical babesiosis from the Badulla ($n = 8$), Jaffna ($n = 3$), and Kilinochchi ($n = 2$) districts in Sri Lanka. These DNA samples tested positive in PCR assays specific for *Babesia bovis* ($n = 9$), *Babesia bigemina* ($n = 9$), and *Babesia ovata* ($n = 1$). Twelve cattle were positive for *B. bovis* and/or *B. bigemina*. One cow was negative for the tested *Babesia* species but was positive for *Babesia* on microscopic examination; the phylogenetic positions of 18S rRNA and cytochrome oxidase subunit III gene sequences suggested that the cow was infected with *Babesia* sp. Mymensingh, which was recently reported from a healthy cow in Bangladesh. We then developed a novel *Babesia* sp. Mymensingh-specific PCR assay and obtained positive results for one other sample. Analysis of gene sequences from the cow with positive *B. ovata*-specific PCR results demonstrated that the animal was infected not with *B. ovata* but with *Babesia* sp. Hue-1, which was recently reported from asymptomatic cattle in Vietnam. The virulence of *Babesia* sp. Hue-1 is unclear, as the cow was coinfected with *B. bovis* and *B. bigemina*. However, *Babesia* sp. Mymensingh probably causes severe clinical babesiosis, as it was the sole *Babesia* species detected in a clinical case. The present study revealed the presence of two bovine *Babesia* species not previously reported in Sri Lanka, plus the first case of severe bovine babesiosis caused by a *Babesia* species other than *B. bovis*, *B. bigemina*, and *Babesia divergens*.

KEYWORDS *Babesia*, cattle, clinical babesiosis, Sri Lanka

Clinical bovine babesiosis is mainly caused by *Babesia bovis* and *Babesia bigemina* in the tropics and subtropics (1), while *Babesia divergens* causes clinical bovine babesiosis in Europe (2). *Babesia* sporozoites injected by infected tick vectors directly invade the host's red blood cells (RBCs) and develop into merozoites (3, 4). Merozoites egress from the infected RBCs, causing massive intravascular hemolysis that leads to clinical signs such as fever, anemia, hemoglobinuria, and jaundice (1). Additionally, bovine babesiosis caused by *B. bovis* is characterized by neurological and respiratory syndromes because of the cytoadherence of infected RBCs in capillary beds of vital

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organs such as the brain and lungs (1, 5). Early treatment with antibabesial agents is essential for recovery, and a lack of or delay in treatment may result in severe babesiosis that causes death (6, 7). Thus, bovine babesiosis results in huge economic losses to the cattle industry because of treatment and tick control costs, production losses, and animal mortality (8). Moreover, babesiosis disrupts international cattle trade, as World Organisation for Animal Health (OIE) regulations stipulate rules for exporting cattle from countries in which bovine babesiosis is endemic (9).

Disease development in infected cattle is affected by multiple factors, including age, management practices, immunity, and breed (1). Animals less than 9 months old are usually resistant to clinical babesiosis, while adults are not (10, 11). The immunity acquired by young animals protects them from developing clinical babesiosis when they become infected as adults. In addition, *Bos indicus* is relatively resistant to clinical babesiosis compared with *Bos taurus* (12).

Cattle in several countries of endemicity are immunized against *B. bovis* and *B. bigemina* via live attenuated vaccines (13). The attenuation of *B. bovis* and *B. bigemina* is performed by serially passaging the organisms in splenectomized calves (1). However, the global use of live vaccines is limited due to the expense and time required to produce the vaccines, vaccine breakthrough due to strain variations, and risk of contamination with other bovine blood pathogens (14, 15).

In addition to *B. bovis*, *B. bigemina*, and *B. divergens*, several other species of *Babesia* are known to infect cattle, such as *B. ovata* (16), *B. occultans* (17), *B. major* (18), and several unclassified species, including *Babesia* sp. Oshima (19), *Babesia* sp. Kashi (20), *Babesia* sp. Hue-1 (21), *Babesia* sp. Mymensingh (22), and *Babesia* species isolated in South Africa (23, 24). Most of these *Babesia* species have low pathogenicity; however, the virulence of the remaining species is unknown, as they have been reported only from apparently healthy animals (21, 22). To determine the virulence of the *Babesia* species with unknown pathogenicity, there is a need for experimental infections or investigations to identify the *Babesia* species present in cattle with clinical babesiosis.

Clinical babesiosis is common among cattle in the tropical country of Sri Lanka. A few decades ago, clinical cases of bovine babesiosis were concentrated mainly in the wet zone of Sri Lanka, whereas the disease was uncommon in the dry zone (25). This discrepancy was due to the variation in cattle breeds and management practices between the wet and dry zones (12, 26); most cattle in the wet zone were temperate breeds (*Bos taurus*) that were managed intensively, while the dry zone contained mostly extensively managed local cattle (*Bos indicus*) (27). However, farmers in the dry zone have recently started to maintain temperate breeds and their crosses and to adopt an intensive management system, leading to an increased incidence of clinical babesiosis. Hence, clinical babesiosis in cattle is now common in both the wet and dry zones (28). Although live vaccines against both *B. bovis* and *B. bigemina* are available in Sri Lanka, only a few hundred calves are vaccinated annually (29, 30).

A recent series of molecular epidemiological surveys was conducted in Sri Lanka to detect and genetically characterize hemoprotozoan parasites in apparently healthy cattle (31–35). In contrast, genetic techniques have never been used to investigate clinical cases of bovine babesiosis in Sri Lanka. Therefore, in the present study, we employed molecular tools to identify the *Babesia* species present in cattle with clinical babesiosis in Sri Lanka.

MATERIALS AND METHODS

Animals and blood samples. Between June and December 2017, approximately 2-ml samples of whole blood were obtained from the jugular veins of 13 cattle with high fever and hemoglobinuria in the Badulla ($n = 8$), Jaffna ($n = 3$), and Kilinochchi ($n = 2$) districts of Sri Lanka (see Fig. S1 in the supplemental material) using Vacutainer tubes containing EDTA (NIPRO, Osaka, Japan) (Table 1). The Jaffna and Kilinochchi districts are located in the dry zone, while the sampling locations within the Badulla district were located in the wet zone. The affected animals were either Friesians, Jerseys, or Jersey and Sahiwal crosses, and their ages ranged from 3 to 6 years. None of the affected animals had been vaccinated against *B. bovis* and/or *B. bigemina*. Immediately after sampling, the animals were treated with diminazene aceturate and long-acting oxytetracycline. Blood samples were analyzed in a commercial laboratory to determine the hemoglobin concentration (HGB), hematocrit (HCT), and RBC count. DNA

TABLE 1 Clinical presentation of bovine babesiosis in Sri Lanka

Cow no.	District	Animal ID[a]	Breed	Age (yrs)	Clinical signs				RBC indices[b]		
					Temp (°C)	Anemia	Hemoglobinuria	Nervous signs	HGB	HCT	RBC
1	Badulla	I	Jersey	4	40.6	Y	Y	Y	NT	NT	NT
2	Badulla	L1	Friesian	3	41.4	N	Y	N	8.4	22.6	5.65
3	Badulla	L2	Jersey	4	41.1	N	Y	N	9.6	27.6	5.94
4	Badulla	R	Friesian	4	41.1	Y	Y	N	6.8	19.6	3.94
5	Badulla	S[c]	Jersey	5	39.4	Y	Y	Y	2.8	7.9	1.38
6	Badulla	T1	Jersey	4	41.1	Y	Y	Y	8.7	24	5.59
7	Badulla	T2	Jersey	4	41.1	N	Y	N	9.4	25.7	5.51
8	Badulla	V	Jersey	5	42.2	Y	Y	N	NT	NT	NT
9	Jaffna	C2	Jersey	4	40.6	Y	Y	N	6.8	21.9	3.88
10	Jaffna	C5	Jersey	5	40	Y	Y	Y	6.7	21.5	3.5
11	Kilinochchi	C6[c]	Jersey × Sahiwal	6	39.4	Y	Y	N	2.83	10.6	2.22
12	Kilinochchi	C7[c]	Jersey	3	41.1	Y	Y	Y	4.8	13.3	3.03
13	Jaffna	C8	Jersey × Sahiwal	6	40	Y	Y	N	NT	NT	NT

[a]All animals were female. ID, identification; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; F, female; Y, yes (observed); N, no (not observed); NT, not tested.
[b]RBC indices, including the HGB concentration (grams per deciliter), HCT (percent), and RBC count ($\times 10^6$ per microliter), were measured for 10 of the 13 animals.
[c]This animal died despite treatment with diminazene aceturate.

samples were extracted from blood samples using a commercial kit (QIAamp DNA Blood minikit; Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and stored at −30°C until use. All animal procedures were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number 29-53).

Diagnostic PCR assays. A previously described PCR assay based on the rhoptry-associated protein 1 gene (36) was used to detect *B. bovis*, while apical membrane antigen 1 (*ama-1*) gene-based PCR assays were employed to detect *B. bigemina* (37) and *B. ovata* (38). All parasite species were detected by single-step PCR, although *B. bovis*- and *B. bigemina*-specific PCR assays were originally described as nested PCR assays. The list of primer sequences used in the present study is provided in Table S1.

Cloning and sequencing. An amplicon from the *ama-1* PCR assay targeting *B. ovata* was cloned and sequenced as previously described (21), as this *Babesia* species has not been reported in Sri Lanka. In addition, the 18S rRNA and cytochrome oxidase subunit III (*cox3*) gene sequences of babesial origin from a DNA sample that was PCR positive for *B. ovata* and from a sample that was PCR negative for all three *Babesia* species tested in the present study were amplified by PCR using sets of common primers. Briefly, a 25-μl reaction mixture containing 1 μl of DNA, 1× PCR buffer (Toyobo, Osaka, Japan), 400 μM each deoxynucleoside triphosphate (dNTP; Toyobo), 0.4 μM each forward primer (18S rRNA, 5′-CATTACAAC AGTTATAGTTTCTTTGG-3′ [21]; *cox3*, 5′-TCAACAAAATGCCAATATGTTCCAA-3′) and reverse primer (18S rRNA, 5′-CTAGGCATTCCTCGTTCATGATTTAG-3′; *cox3*, 5′-TACAAAGTGCATCTTTGGGAGAAG-3′), 0.5 μl of 1-U/μl KOD FX Neo DNA polymerase (Toyobo), and 4 μl of distilled water was subjected to an initial predenaturation step at 94°C for 2 min and then to 35 cycles of denaturation at 94°C for 10 s, annealing at 58°C (18S rRNA) or 64°C (*cox3*) for 30 s, and extension at 68°C for 90 s. The PCR amplicons were cloned into a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA) and then sequenced as previously described (37).

Development of a PCR assay specific to *Babesia* sp. Mymensingh. One animal that produced negative results in the PCR assays for *B. bovis*, *B. bigemina*, and *B. ovata* was determined to be infected with *Babesia* sp. Mymensingh (22) based on an 18S rRNA sequence. A pair of common forward (5′-TGGACCAGGTACATGATCAAGT-3′) and reverse (5′-AATCATCGTGCTGACGACCCTTC-3′) PCR primers (37) was used to amplify the 1,372-bp *ama-1* gene fragment from *Babesia* sp. Mymensingh, as described for 18S rRNA or *cox3* amplification, except that the annealing temperature was changed to 62°C. The amplicon was cloned and sequenced. The newly generated *ama-1* gene sequence and those already available in the NCBI GenBank database were subjected to multiple alignment, and a set of forward (5′-TGGCGCCGACTTCCTGGAGCCCATCTCCAA-3′) and reverse (5′-AGCTGGGGCCCTCCTTCGATGAACCGTC GG-3′) primers specific to *Babesia* sp. Mymensingh was designed. A 10-μl PCR mixture containing 1 μl of DNA, 1× PCR buffer (Applied Biosystems, Branchburg, NJ), 200 μM each dNTP (Applied Biosystems), 0.5 μM each forward and reverse primers, 0.1 μl of 5-U/μl AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5.9 μl of distilled water was subjected to initial enzyme activation at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. After a final elongation step at 72°C for 7 min, the PCR products were resolved by gel electrophoresis and then visualized under UV illumination. Detection of an approximately 371-bp band was considered positive. The specificity of the PCR assay was evaluated using a panel of DNA samples derived from several bovine blood pathogens and uninfected cattle (38, 39). The *Babesia* sp. Mymensingh-specific PCR assay was then used to screen all 13 cattle DNA samples analyzed in the present study.

Sequencing and phylogenetic analyses. The identity scores among gene sequences were determined by the EMBOSS NEEDLE online program (http://www.bioinformatics.nl/cgi-bin/emboss/needle). The 18S rRNA, *cox3*, and *ama-1* gene sequences determined in the present study and those retrieved from GenBank were aligned using the MAFFT software program (40). MEGA software (41) was then used

TABLE 2 PCR detection of *B. bovis*, *B. bigemina*, and *B. ovata* in clinical cases of bovine babesiosis in Sri Lanka

Cow no.	Animal ID	*Babesia bovis*	*Babesia bigemina*	*Babesia ovata*
1	I	+	−	−
2	L1	−	+	−
3	L2	−	+	−
4	R[a]	−	−	−
5	S	+	+	−
6	T1	+	+	−
7	T2	+	+	−
8	V	+	+	−
9	C2	−	+	−
10	C5	+	−	−
11	C6	+	−	−
12	C7	+	+	−
13	C8	+	+	+
Total		9	9	1

[a]Animal R was negative for all three *Babesia* species tested in the present study.

to construct three separate maximum likelihood phylogenetic trees based on Tamura-Nei (18S rRNA and *cox3*) (42) or General Time Reversible (*ama-1*) (43) substitution models.

Accession number(s). The gene sequences determined in the present study were registered with GenBank under the accession numbers LC385886 to LC385894.

RESULTS

A total of 13 cattle with high fever and hemoglobinuria were sampled in three districts: Badulla, Kilinochchi, and Jaffna. Clinical examination determined that five of the eight animals sampled in the Badulla district were anemic, whereas all five of the animals sampled in the Kilinochchi and Jaffna districts were anemic (Table 1). Five of the affected animals also showed nervous system abnormalities, such as incoordination. In the anemic animals, except cow T1, all three RBC indices, including HGB concentration, HCT, and RBC count, were below the lower limits of the reference ranges for these parameters in healthy cattle (8 g/dl, 24%, and $5 \times 10^6/\mu$l, respectively) (44). Two animals in the Kilinochchi district (C6 and C7) and one animal in the Badulla district (S) with very low HGB concentrations, HCT, and RBC counts died despite treatment with diminazene aceturate and oxytetracycline, while the remaining animals recovered following treatment. PCR assays revealed that 12 of the 13 DNA samples were positive for *B. bovis* and/or *B. bigemina* (Table 2). *B. bovis* and *B. bigemina* were each detected in nine animals, while six animals were positive for both *Babesia* species. The result of the *B. ovata*-specific *ama-1* PCR assay was positive for only one animal (C8 in the Jaffna district).

Cow R from the Badulla district was PCR negative for all three *Babesia* species tested, but microscopic examination revealed *Babesia* piroplasms in a thin blood smear prepared from this animal (Fig. 1). The length and width of the paired pyriforms were 2.25 to 3.04 μm and 1.58 to 2.20 μm, respectively, whereas the ring forms were 1.52 to 1.97 μm in diameter. The paired pyriforms formed an obtuse angle that reached 180° in some cases. Single forms were often elongated or irregularly shaped. 18S rRNA is the most commonly used molecular marker for species identification of eukaryotes, including *Babesia* (18, 20, 45–47). Therefore, in an attempt to identify the species of *Babesia* detected in cow R, a 1,385-bp 18S rRNA sequence was isolated from cow R (GenBank accession number LC385886) and compared with the *Babesia* sequences in the GenBank database. The analyzed gene sequence shared 99.6% identity with the *Babesia* sp. Mymensingh sequence (GenBank accession number MF576177) that was recently reported from an asymptomatic cow in Bangladesh (22), followed by 97.8% identity with a *B. bigemina* sequence (GenBank accession number FJ426361) (48); this confirmed that the *Babesia* species detected in cow R was *Babesia* sp. Mymensingh. In the 18S rRNA phylogeny based on a 1,310-bp alignment with no gaps, the *Babesia* sp. Mymensingh sequence determined in the present study clustered together with that previously reported in Bangladesh (22) and formed a sister clade to *B. bigemina* (Fig. 2).

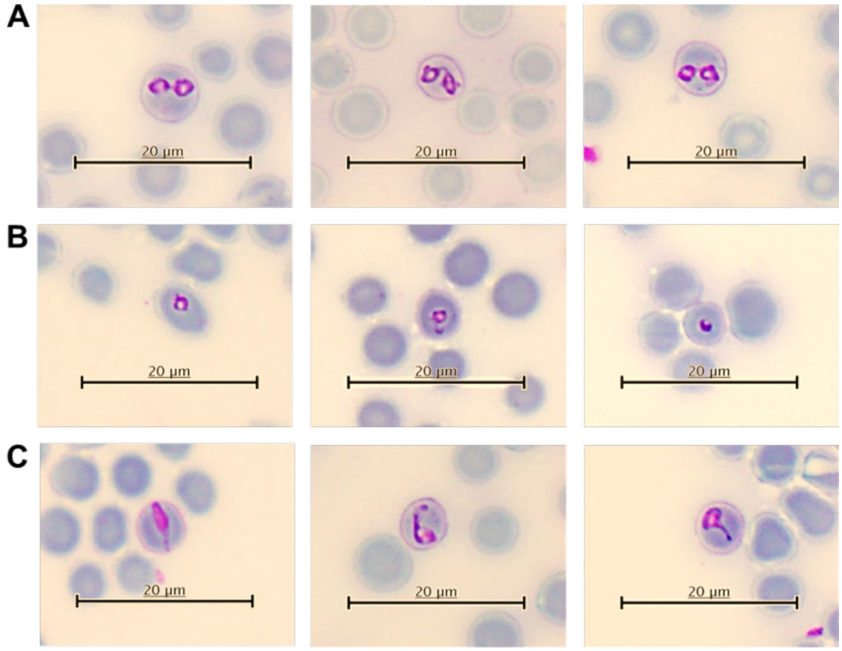

FIG 1 Morphology of the *Babesia* parasites in cow R. A Giemsa-stained thin blood smear prepared from cow R was observed under a light microscope. Paired pyriforms (A), ring forms (B), and elongated and irregularly shaped single forms (C) of *Babesia* parasites were detected in cow R.

As *B. ovata* has not been previously reported in Sri Lanka, the amplicon of the PCR assay targeting *B. ovata* from cow C8 was cloned and sequenced. The resultant *ama-1* gene fragment (GenBank accession number LC385892) shared only 93.3% identity with the *B. ovata* sequence (GenBank accession number AB634843) (37), while the sequence shared 97.4% to 98.4% identity scores with *ama-1* sequences (GenBank accession numbers LC125412 to LC125415) from recently reported *Babesia* sp. Hue-1 in Vietnam (21). The 18S rRNA amplified from cow C8 included *B. bigemina* sequences (data not shown), as well as a 1,381-bp sequence (GenBank accession number LC385887) that shared a high identity score (99.0%) with a *Babesia* sp. Hue-1 sequence (GenBank accession number LC125456) (21). These findings suggest that cow C8 was infected with *Babesia* sp. Hue-1, as the identity scores shared between the 18S rRNA and *ama-1* gene sequences from cow C8 and *Babesia* sp. Hue-1 are comparable to the identity scores shared among these sequences from different isolates of other bovine *Babesia* species (49–52). Phylogenetically, *Babesia* sp. Hue-1 from cow C8 formed a sister clade to the common ancestor of the *B. bigemina* clade and the *Babesia* sp. Mymensingh clade (Fig. 2). Moreover, in a phylogeny based on a short alignment (635 bp) of 18S rRNA, *Babesia* sp. Hue-1 sequences from cow C8 in Sri Lanka and that reported in Vietnam occurred together and formed a sister clade to the clade formed by sequences from *Babesia* species reported in China (GenBank accession number AY603403) (18) and Korea (GenBank accession number AY081192) (53) (Fig. S2).

In addition to 18S rRNA, phylogenies based on mitochondrial genes are widely used for the identification of species, including *Babesia* species (54–57). Therefore, to further investigate the phylogenetic positions of *Babesia* sp. Mymensingh and *Babesia* sp. Hue-1, *cox3* gene sequences (556 bp) were amplified from DNA samples that were positive for these *Babesia* species (from cows R and C8, respectively). The *cox3* sequence from *Babesia* sp. Mymensingh (GenBank accession number LC385889) shared 93.5% identity with *B. bigemina* (GenBank accession number LK054939) (58) and *B. ovata* (GenBank accession number LC146482) (52) sequences and formed a phylogenetic sister clade to *B. bigemina* (Fig. 3). Only the *cox3* sequence from *Babesia* sp. Hue-1 (GenBank accession number LC385890) was isolated from cow C8. The *cox3* sequence from *Babesia* sp. Hue-1 shared 94.9%, 94.1%, and 93.5% identity with sequences from

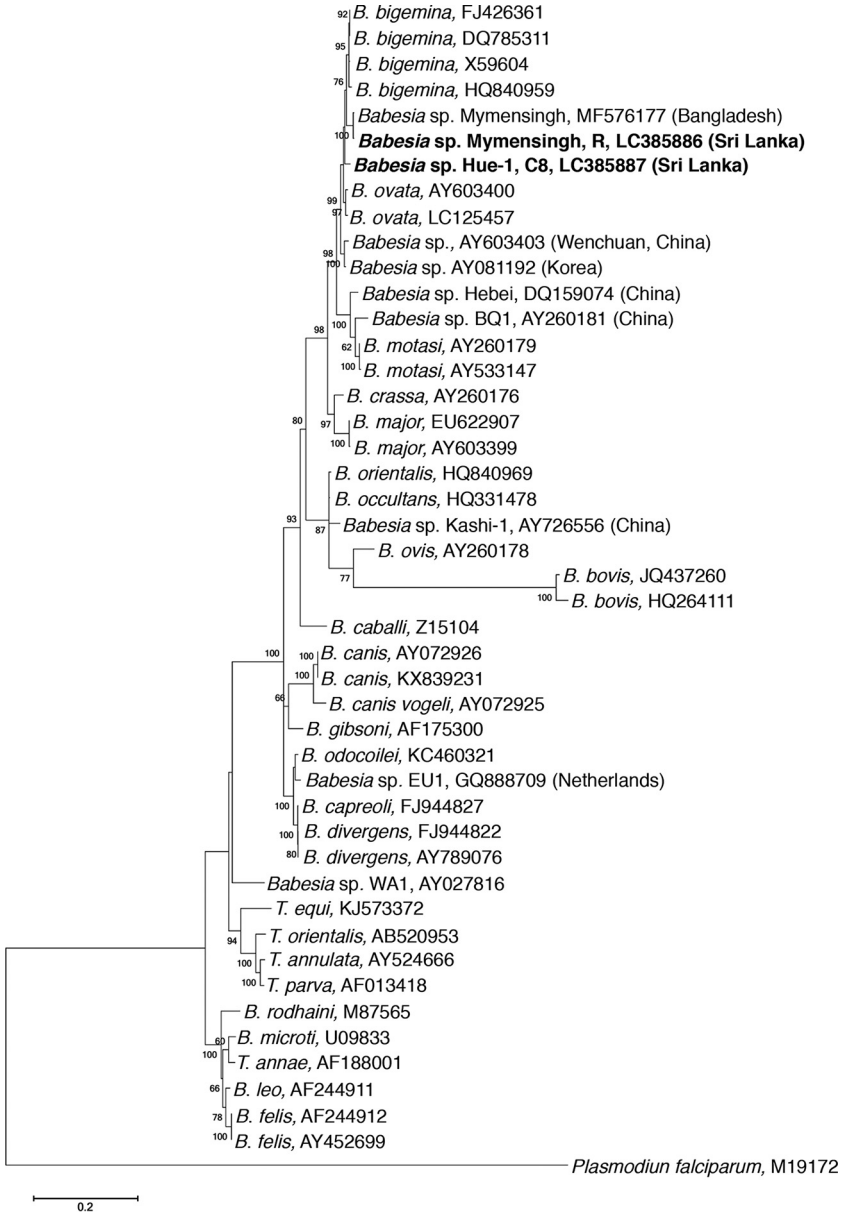

FIG 2 Phylogeny of 18S rRNA. A maximum likelihood phylogeny was constructed using 18S rRNA sequences determined in the present study and those retrieved from GenBank. The gene sequences determined in the present study are indicated by boldface type. Note that the *Babesia* sp. Mymensingh sequence determined in the present study and that previously reported from Bangladesh clustered together and formed a sister clade to *B. bigemina*. Additionally, *Babesia* sp. Hue-1 occurred separately, forming a sister clade to the common ancestor of *Babesia* sp. Mymensingh and *B. bigemina*.

an unidentified *Babesia* sp. (Wenchuan, China) (GenBank accession number JN859545) (56), *B. ovata* (GenBank accession number LC146482), and *B. bigemina* (GenBank accession number LK054939), respectively, and formed a sister clade to the clade formed by *Babesia* species reported in China (Wenchuan) and Korea (Fig. 3).

The *ama-1* gene is an attractive target for the development of species-specific PCR assays for detecting *Babesia* parasites, as the gene is conserved within a given *Babesia* species but is diverse between species (37, 59–61). Therefore, a 1,372-bp *ama-1* gene fragment (GenBank accession number LC385893) was isolated from *Babesia* sp. Mymensingh to develop a specific PCR assay to detect this *Babesia* species in the DNA samples. The gene sequence shared 83.9% and 83.3% identity with *B. bigemina* (GenBank accession number AB481200) (62) and *B. ovata* (GenBank accession number

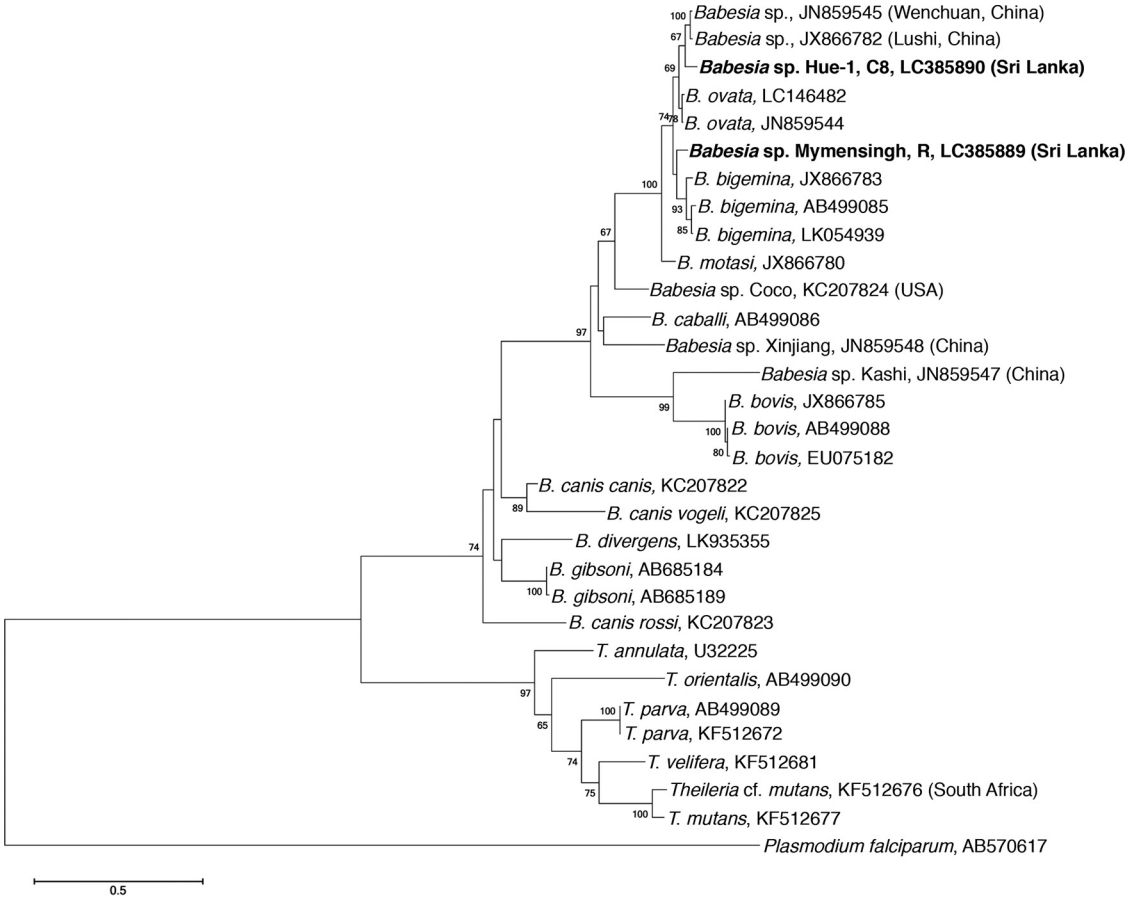

FIG 3 Phylogeny of *cox3*. A maximum likelihood phylogeny was constructed using *cox3* sequences from *Babesia* sp. Mymensingh and *Babesia* sp. Hue-1 and those available in the GenBank database. The gene sequences determined in the present study are indicated by boldface type. Note that *Babesia* sp. Mymensingh formed a sister clade to *B. bigemina*, whereas *Babesia* sp. Hue-1 formed a sister clade to *Babesia* sp. (Wenchuan, China)/*Babesia* sp. (Lushi, China).

AB634843) (37) sequences, respectively. The PCR assay developed based on the *ama-1* gene amplified only *Babesia* sp. Mymensingh, while no amplicons were observed in DNA samples from several other bovine blood pathogens and cattle DNA, confirming its specificity (Fig. 4A). When this PCR assay was employed to screen all 13 cattle DNA samples, *Babesia* sp. Mymensingh was detected not only in cow R but also in cow V (Fig. 4B). PCR amplicons from cows R and V were cloned and sequenced. The 371-bp *ama-1* sequences from cows R and V (GenBank accession number LC385894) were identical to each other and to the long *ama-1* gene fragment initially isolated from cow R (GenBank accession number LC385893). Phylogenetic trees based on the *ama-1* gene were used to investigate the evolutionary relationships of apicomplexan parasites, such as species of *Plasmodium* and *Babesia* (61, 63–65). In a phylogenetic construction used to investigate the positions of *Babesia* sp. Mymensingh and *Babesia* sp. Hue-1, the *ama-1* sequences of *Babesia* sp. Mymensingh occurred distant to the sequences of *B. bigemina* (Fig. 5). In addition, the *Babesia* sp. Hue-1 *ama-1* sequence generated in the present study clustered with those previously reported in Vietnam and formed a sister clade to *B. ovata*. To further confirm that cow V was infected with *Babesia* sp. Mymensingh, 18S rRNA and *cox3* sequences were amplified, cloned, and sequenced. The 18S rRNA sequence (GenBank accession number LC385888) from cow V shared 99.6% identity with the *Babesia* sp. Mymensingh sequence from cow R (GenBank accession number LC385886), whereas the *cox3* sequence from cow V (GenBank accession number LC385891) was identical to that from cow R (GenBank accession number LC385889), confirming that cow V was also infected with *Babesia* sp. Mymensingh.

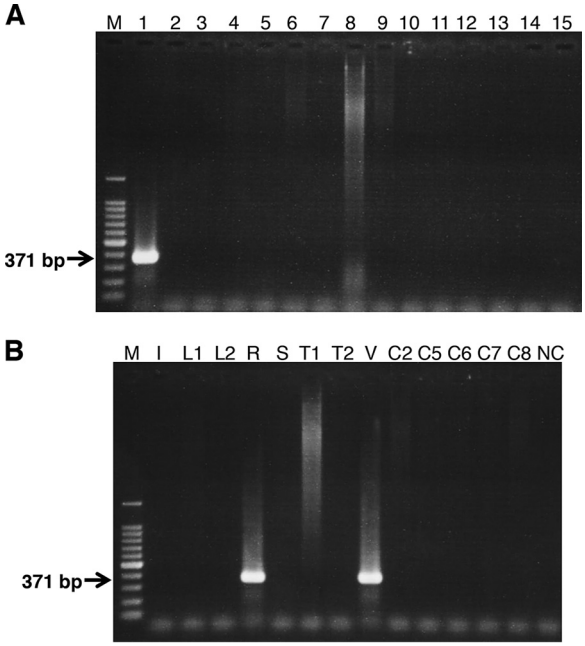

FIG 4 A PCR assay specific to *Babesia* sp. Mymensingh was developed. (A) Specificity testing. The specificity of the newly developed PCR assay was tested using DNA samples from *Babesia* sp. Mymensingh, *B. bigemina*, *B. bovis*, *B. ovata*, *B. divergens*, *Babesia* sp. Hue-1, *Theileria annulata*, *Theileria parva*, *Th. orientalis*, *Trypanosoma evansi*, *Trypanosoma theileri*, *Tr. brucei*, *Anaplasma marginale*, *Anaplamsa bovis*, and uninfected cattle (lanes 1 to 15, respectively). Lane M, 100-bp DNA marker. Note that the amplicon with the expected size was observed only with *Babesia* sp. Mymensingh. (B) Screening of 13 clinical samples for *Babesia* sp. Mymensingh. The PCR assay specific to *Babesia* sp. Mymensingh was used to screen DNA samples from 13 clinical cases. Lanes M and NC contained the 100-bp DNA marker and nontemplate control, respectively. Note that *Babesia* sp. Mymensingh was also detected in cow V.

DISCUSSION

Various species of *Babesia* infect a wide range of host species worldwide, including wild and domestic animals, humans, birds, and reptiles (66). The *Babesia* species that infect cattle are of great economic importance, as they cause severe clinical diseases leading to significant production losses (1). Among them, *B. bovis*, *B. bigemina*, and *B. divergens* are highly virulent species that cause a severe form of bovine babesiosis. Although bovine babesiosis is very common in Sri Lanka, a detailed examination of clinical cases using molecular techniques has never been carried out in this country. Therefore, in the present study, we used molecular diagnostic tools to investigate clinical babesiosis in Sri Lanka.

Anemia was detected in 10 of the 13 animals with fever and hemoglobinuria investigated in the present study, while 3 animals from the Badulla district were not anemic at the time of sampling, suggesting that these animals were sampled in the early stage of disease development. Three animals with very low RBC indices died even after treatment with an antibabesial drug (diminazene aceturate), suggesting that early veterinary intervention is of paramount importance in bovine babesiosis (7). The neurological signs observed in five animals might have been due to *B. bovis* infection, which can cause cerebral babesiosis in cattle (1, 5). However, such neurological signs can also occur in anemic animals due to hypoxia-related brain injury caused by low hemoglobin levels (67, 68). Postmortem examination could have clarified whether the actual cause of the neurological signs in cows S and C7 was cerebral babesiosis or hypoxic brain injury. Unfortunately, however, postmortem examinations were not carried out for any of the animals.

The PCR assays and sequencing analyses detected *B. bovis* and *B. bigemina*, as well as two other *Babesia* species (*Babesia* sp. Mymensingh and *Babesia* sp. Hue-1) that had

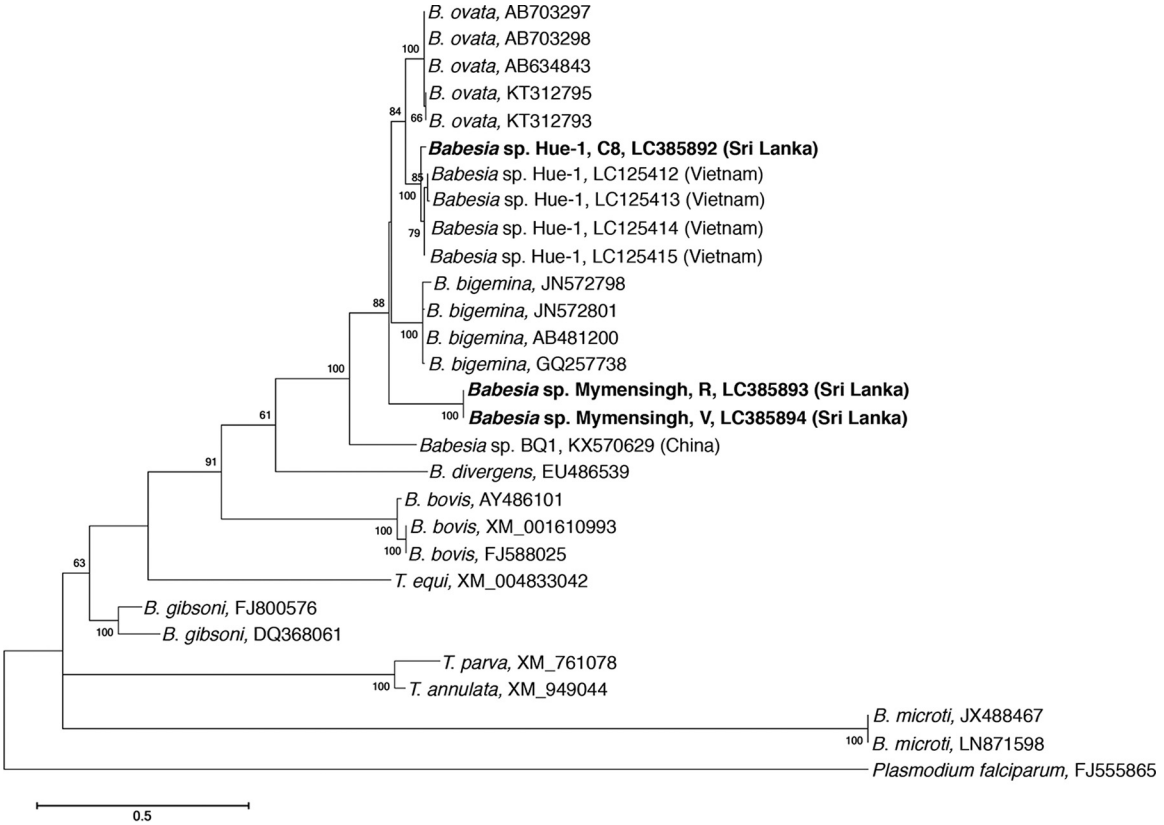

FIG 5 Phylogeny of *ama-1*. The *ama-1* sequences from *Babesia* sp. Mymensingh from cows R and V (amplified by *Babesia* sp. Mymensingh-specific PCR) and from *Babesia* sp. Hue-1 from cow C8 (amplified by *B. ovata* PCR) together with those retrieved from GenBank were used to construct a maximum likelihood phylogeny. The gene sequences determined in the present study are indicated by boldface type. Note that the *Babesia* sp. Hue-1 sequences clustered with those previously reported in Vietnam and formed a sister clade to *B. ovata*, while the *Babesia* sp. Mymensingh formed a separate clade.

not been previously reported in Sri Lanka. In addition to *Babesia* parasite species, previously described species-specific PCR assays (69–72) determined that the samples contained *Theileria annulata*, *Theileria orientalis*, *Trypanosoma theileri*, and *Anaplasma marginale* (Tables S1 and S2). However, the involvement of these parasite species in the animals' clinical disease was unclear, as none of these parasite species induce hemoglobinuria.

Based on morphological observation, *Babesia* sp. Mymensingh can be classified as a large *Babesia* organism. However, the piroplasms were morphologically different from those of *B. bigemina*, as the paired pyriforms of the latter usually form an acute angle (73). In addition, the size of the ring forms for *Babesia* sp. Mymensingh was smaller than that for *B. bigemina*. *Babesia* sp. Mymensingh was initially identified based on an 18S rRNA sequence from an apparently asymptomatic cow in Bangladesh (22). In contrast, the parasite in the present study was detected as the sole *Babesia* species in a cow (R) with typical signs of clinical babesiosis, suggesting that *Babesia* sp. Mymensingh is a virulent species.

The phylogenetic position of *Babesia* sp. Mymensingh was further analyzed in a *cox3*-based phylogeny, in which it formed a sister clade to *B. bigemina*. The sister clades formed in 18S rRNA and *cox3* phylogenies may identify *Babesia* sp. Mymensingh as a new genotype of *B. bigemina*. However, the low identity scores shared between the 18S rRNA, *cox3*, and *ama-1* gene sequences from these parasite species, the *ama-1* phylogeny in which *Babesia* sp. Mymensingh formed a separate clade, and the morphological differences confirm that *Babesia* sp. Mymensingh is a distinct *Babesia* species. The PCR detection of *Babesia* sp. Mymensingh in an additional cow suggested that *Babesia* sp. Mymensingh infection might be common among cattle in Sri Lanka. *Babesia*

sp. Mymensingh may have a wide distribution, as this parasite species was also detected in Bangladesh. Therefore, the PCR assay developed in the present study will be a useful diagnostic tool for specific detection of *Babesia* sp. Mymensingh in different geographical territories.

Cow R, which was infected with *Babesia* sp. Mymensingh, was reexamined 1 week after sampling. No hemoglobinuria was present, and the anemia was milder than previously. Furthermore, laboratory examination showed improvements in the HGB concentration (8 g/dl), HCT (23.4%), and RBC count ($4.53 \times 10^6/\mu$l) compared with the first sampling, indicating that diminazene aceturate is effective against *Babesia* sp. Mymensingh. However, *Babesia* sp. Mymensingh might have implications for the immune control and diagnostics of bovine babesiosis. For example, live attenuated vaccines are used in some countries of endemicity, including Sri Lanka, to immunize cattle against bovine babesiosis caused by *B. bovis* and *B. bigemina* (13). If *Babesia* sp. Mymensingh is not immunologically cross-reactive to these parasite species, especially to *B. bigemina*, *Babesia* sp. Mymensingh may cause clinical disease in vaccinated animals. However, if they are immunologically cross-reactive parasites, the serodiagnostic techniques commonly used in epidemiological surveys might generate unreliable data.

The PCR assay targeting *B. ovata* detected *Babesia* sp. Hue-1. Phylogenetically, 18S rRNA from *Babesia* sp. Hue-1 formed a clade that was clearly separate from the other *Babesia* species, identifying *Babesia* sp. Hue-1 as a separate *Babesia* species. The morphology of *Babesia* sp. Hue-1 was not analyzed, as the blood smear from the infected cattle was unfit for microscopic examination. Even if high-quality blood smears had been available, microscopic detection might have been impossible, as the animal was coinfected with *B. bovis* and *B. bigemina*. *Babesia* sp. Hue-1 was recently reported in Vietnam based on 18S rRNA and *ama-1* sequences from healthy cattle (21). Therefore, the clinical significance of *Babesia* sp. Hue-1 remains unknown.

In conclusion, the present study demonstrated the presence of two *Babesia* species not previously detected in Sri Lanka and also identified severe clinical bovine babesiosis caused by a *Babesia* species other than *B. bovis*, *B. bigemina*, and *B. divergens*. Priorities in *Babesia* research include isolation of the newly detected *Babesia* species, experiments to investigate their virulence in different cattle breeds and immunological cross-reactivity with *B. bovis* and *B. bigemina*, and identification of specific tick vectors.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00895-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no conflicts of interest associated with the present study.

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