



Epidemiological survey of hemoprotozoan parasites in cattle from low-country wet zone in Sri Lanka

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1 **Epidemiological survey of hemoprotozoan parasites in cattle from low-country wet zone**
2 **in Sri Lanka**

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22

23 **ABSTRACT**

24

25 The diseases caused by hemoprotozoan parasites in cattle often result in economic
26 losses. In Sri Lanka, previous studies found that the up-country wet zone, which is located in
27 central Sri Lanka, was characterized by a high rate of *T. orientalis* and a low rate of *T.*
28 *annulata* compared with the dry zone. In this study, DNA samples were prepared from the
29 blood of 121 cattle in Galle, a coastal district located in low-country wet zone in Sri Lanka,
30 and were PCR-screened for *B. bovis*, *B. bigemina*, *T. annulata*, *T. orientalis*, and *Tr. theileri*.
31 All the parasite species, except *B. bovis*, were detected among the surveyed cattle. The
32 animals had a high rate of *T. orientalis* (100%) and a low rate of *T. annulata* (1.6%), as in the
33 up-country wet zone. *Babesia bigemina* and *Tr. theileri* were detected in 19.0% and 20.6% of
34 the animals, respectively, and their infection rates were higher in the animals reared in
35 extensive management systems (32.8% and 27.9%, respectively) than in those managed in
36 intensive/semi-intensive systems (5.0% and 13.3%, respectively). Genotypic analyses found
37 that the *T. orientalis* *mmsp* type 5 was predominant similar to up-country wet zone, and that *Tr.*
38 *theileri* consisted of seven *catl* genotypes, including two new genotypes (IL and IM) and four
39 previously detected genotypes (IA, IB, II, and IK). These findings suggest that the
40 hemoprotozoan infection profiles are largely conserved within the wet zone, despite
41 differences in the geography, cattle breeds, and management practices between the up-country

42 and low-country wet zones.

43

44 *Keywords:* Cattle, Epidemiology, Hemoprotozoa, Low-country wet zone, Sri Lanka

45

46 **1. Introduction**

47

48 Hemoprotozoan parasites, including species of *Babesia*, *Theileria*, and *Trypanosoma*
49 parasites, infect cattle and cause clinical diseases, leading to economic losses in the cattle
50 industry worldwide. Although various species of *Babesia* infect cattle, severe clinical
51 babesiosis is caused by *B. bovis* and *B. bigemina* in the tropics and subtropics and by *B.*
52 *divergens* in Europe [1, 2]. On the other hand, bovine theileriosis is caused by several species
53 of *Theileria*, including *T. parva*, *T. annulata*, and *T. orientalis*, which cause east coast fever in
54 Eastern, Central, and Southern Africa, tropical theileriosis in North Africa, Southern Europe,
55 and Asia, and oriental theileriosis worldwide, respectively [3–6]. Although *T. parva* and *T.*
56 *annulata* are more virulent than *T. orientalis*, the latter sometimes causes severe disease,
57 especially when newly introduced into an area [7, 8]. The clinical signs of bovine theileriosis
58 are similar to those of bovine babesiosis, except for the lack of hemoglobinuria and the
59 presence of enlarged lymph nodes [9]. The virulence of *Trypanosoma* parasites differs among
60 the species of the genus. *Trypanosoma congolense*, *Tr. vivax*, and *Tr. brucei*, which are only
61 endemic to Africa, are more virulent than *Tr. evansi* and *Tr. theileri*, which have wide
62 distributions. However, *Tr. evansi* often induces a chronic wasting disease in several host
63 animals, including cattle [10], and *T. theileri* can also cause clinical disease, especially when
64 it coinfects animals with other hemoparasites [11, 12].

65 Once they infect their hosts, these hemoprotozoan parasites persist in the hosts'
66 bodies for a long period [13–15]. Therefore, they can be acquired by their vectors and
67 complete their life cycles. The detection of these carrier animals is vital in estimating the risks
68 they pose, because the vectors can transmit the parasites from these animals to their next hosts,
69 where the infection may result in clinical disease.

70 Sri Lanka is a tropical island in the Indian Ocean. The cattle farming systems in this
71 country differ significantly among the climatic zones [16]. Throughout the dry zone, which is
72 characterized by low annual rainfall, cattle breeds and management practice are similar, and
73 predominantly local cattle breeds are managed with an extensive management system [17].
74 However, different cattle breeds and management systems are used in the wet zone [17]. In
75 the up-country wet zone, which covers the high-altitude regions in central Sri Lanka, pure
76 European breed cattle are reared with an intensive management system [17]. In contrast, in
77 the low-country wet zone, which is situated around the low-elevation coastal areas in western
78 and southern Sri Lanka, European and zebu hybrid cattle are managed with an intensive or
79 semi-intensive system in urban areas and with an extensive system in rural areas [17].

80 In our previous studies in Sri Lanka, we surveyed cattle populations representing the
81 dry zone (Jaffna, Polonnaruwa, and Amapara districts) and the up-country wet zone (Nuwara
82 Eliya district) for infection with species of *Babesia*, *Theileria*, and *Trypanosoma* parasites
83 [15, 18, 19]. These studies indicated that the infection rates of some hemoprotozoan parasites,

84 particularly species of *Theileria*, differed between the dry and up-country wet zones [15, 18].
85 These differences were attributed to the variations in the species, densities, and activities of
86 the tick vectors in the climatic zones [15]. However, the cattle in the low-country wet zone
87 were not considered in these studies, although the geography, cattle breeds, and management
88 practices vary between the up-country and low-country wet zones [17]. Therefore, in the
89 present study, we surveyed the cattle in Galle, a coastal district located in the low-country wet
90 zone, for infection with bovine *Babesia*, *Theileria*, and *Trypanosoma* parasites.

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93

94 **2. Materials and methods**

95

96 ***2.1. Blood samples and DNA extraction***

97 A total of 121 blood samples were collected from cattle in 16 farms in Akmeemana
98 (n = 60) and 22 farms in Rathgama (n = 61) veterinary ranges in Galle in May 2017. Galle is a
99 coastal district of southern Sri Lanka, located in the low-country wet zone. The animals
100 sampled in both locations were cross-bred cattle. The cattle in Rathgama, a rural area, are
101 usually reared in an extensive system, whereas those in Akmeemana are maintained in
102 intensive or semi-intensive systems. In addition to cattle, seven buffaloes that were reared
103 with cattle (five from Akmeemana and two from Rathgama) were also sampled. From each
104 animal, approximately 2 ml of blood was collected from the jugular vein into a Vacutainer
105 tube containing EDTA (Nipro, Osaka, Japan). The DNA samples were prepared from 200 µl
106 of the whole blood from each animal with a commercial DNA extraction kit (QIAamp DNA
107 Blood Mini Kit, Qiagen, Hilden, Germany), and then stored at -30 °C until analysis. All
108 animal procedures were approved by the Committee on the Ethics of Animal Experiments,
109 Obihiro University of Agriculture and Veterinary Medicine (Approval number 29-53). In
110 addition, approval for the blood sampling was obtained from the Department of Animal
111 Production and Health, Peradeniya, Sri Lanka.

112

113 **2.2. PCR detection of Babesia, Theileria, and Trypanosoma parasites**

114 All the DNA samples prepared from the cattle and buffaloes were screened for *B.*
115 *bovis*, *B. bigemina*, *T. annulata*, *T. orientalis*, and *Tr. theileri*, with previously described PCR
116 assays based on the rhoptry-associated protein 1 (*rap-1*) [20], apical membrane antigen 1
117 (*ama-1*) [21], merozoite-piroplasm surface antigen (*tams-1*) [22], major piroplasm surface
118 protein (*mmsp*) [23], and cathepsin L-like protein (*catl*) [24] genes, respectively. The reaction
119 mixtures and cycling conditions for the PCR assays have been described previously [18, 19].

120

121 **2.3. Type-specific PCR assays for T. orientalis**

122 All the *T. orientalis*-positive DNA samples were screened with PCR assays specific
123 for *mmsp* genotypes 1, 3, 5, and 7, which are known to be endemic to cattle in Sri Lanka,
124 essentially as previously described [25].

125

126 **2.4. Cloning, sequencing, and phylogenetic analyses**

127 The PCR amplicons from selected samples of each of the parasite species were
128 gel-extracted and then cloned into the PCRTM2.1 plasmid vector (TOPO, Invitrogen, Carlsbad,
129 CA). The inserts were sequenced with an ABI Prism 3100 Genetic Analyzer (Applied
130 Biosystems, Branchburg, NJ, USA). The *B. bovis rap-1* and *Tr. theileri catl* gene sequences
131 obtained in the present study, together with those retrieved from GenBank, were used to

132 construct maximum likelihood and neighbor-joining phylogenetic trees, respectively, based
133 on the Tamura 3-parameter substitution model [26], using the MEGA version 6.0 software
134 [27].

135

136 ***2.5. Statistical analyses***

137 The confidence intervals for infection rates were calculated based on the Wilson
138 score [28] using the OpenEpi software program
139 (<http://www.openepi.com/Proportion/Proportion.htm>). The P values for the differences
140 between the infection rates were calculated using an “N-1” χ^2 test
141 (https://www.medcalc.org/calc/comparison_of_proportions.php) [29, 30]. A P value < 0.05
142 was considered to indicate a statistically significant difference between the infection rates.

143

144

145 3. Results and discussion

146

147 The findings of the present study indicated that the cattle in the Galle district were
148 infected with *B. bigemina*, *T. annulata*, *T. orientalis*, and *Tr. theileri*, whereas *B. bovis* was
149 not detected among the cattle surveyed. The commonest parasite was *T. orientalis*, which was
150 detected in all 121 animals (100%), followed by *Tr. theileri* (20.6%), *B. bigemina* (19.0%),
151 and *T. annulata* (1.6%) (Table 1). Of 121 cattle, 46 (38.0%) had co-infections with two or
152 three parasite species. Among these co-infected animals, four had co-infections with *T.*
153 *orientalis*, *B. bigemina*, and *Tr. theileri*, while 21, 19, and two were co-infected with *T.*
154 *orientalis* and *Tr. theileri*, *T. orientalis* and *B. bigemina*, and *T. orientalis* and *T. annulata*,
155 respectively. These observations are in agreement with the results of previous studies, which
156 clearly showed that the cattle in the up-country wet zone had high *T. orientalis* and low *T.*
157 *annulata* infection rates, compared with the infection rates for both these parasite species in
158 cattle in the dry zone [15, 18]. Therefore, our present findings indicate that, despite the
159 differences in the cattle-farming systems and geography of up-country and low-country wet
160 zones, the rates of hemoprotozoan parasites displayed similar patterns in both regions,
161 suggesting that the major factor influencing the hemoprotozoan parasitic epidemiology in Sri
162 Lanka is climate, not geographic location.

163 The animals in the up-country wet zone are managed exclusively with an intensive

164 management system [16]. However, the present study provided an opportunity to compare the
165 infection rates between cattle managed with intensive/semi-intensive and extensive
166 management systems in the wet zone. We found that the infection rates for *Tr. theileri* and *B.*
167 *bigemina* were higher in Rathgama (27.9% and 32.8%, respectively) than in Akmeemana
168 (13.3% and 5.0%, respectively) (Table 1). The risk of exposure to vectors is higher for cattle
169 in Rathgama than for those in Akmeemana, because the animals in these two areas are
170 maintained with extensive and intensive/semi-intensive management systems, respectively,
171 which explains why the *Tr. theileri*- and *B. bigemina*-positive rates were higher in Rathgama
172 than in Akmeemana [31]. Thus, the prevalence of bovine hemoprotozoan parasites is
173 influenced by climate, as well as management practices, in Sri Lanka. However, no
174 comparison of the *T. annulata*-positive rates in the two sampling locations was possible
175 because only two animals in Rathgama were positive for this parasite. All the animals
176 sampled at both locations were positive for *T. orientalis*. A previous study conducted in Sri
177 Lanka found that the animals had high *T. orientalis* infection rates, despite its low
178 transmission rate, because the infection displays pronounced persistence [15]. This could
179 explain the high infection rates for *T. orientalis* in both sampling locations in the present
180 study, despite the differences in the management practices there.

181 *Theileria orientalis* consists of 11 *mmsp* genotypes, including types 1–8, N1, N2, and
182 N3, and four of these (types 1, 3, 5, and 7) have been detected in Sri Lankan cattle [25].

183 Therefore, we screened all 121 cattle DNA samples, all of which were PCR-positive for *T.*
184 *orientalis*, using previously established PCR assays specific for *mpsp* genotypes 1, 3, 5, and 7.
185 The commonest genotype was type 5 (40.4%), followed by types 7 (30.5%), 1 (20.6%), and 3
186 (10.3%). These findings are also consistent with the previous observation that type 5 was
187 predominant in the up-country wet zone [25]. However, of the 121 cattle DNA samples tested,
188 only 52 were positive in at least one type-specific PCR assay, suggesting the presence of other
189 genotypes. Therefore, we cloned and sequenced the amplicons from the screening PCR assays
190 of 28 (18 from Akmeemana and 10 from Rathgama) of the 59 samples that were negative in
191 the type-specific PCR assays. The newly generated sequences represented either type 1 (n =
192 5; GenBank accession numbers LC438466–LC438470), type 5 (n = 15;
193 LC438471–LC438485), or type 7 (n = 7; LC438486–LC438492). Therefore, the low DNA
194 concentrations of individual genotypes in the DNA samples might explain the initial negative
195 results for the type-specific PCR assays of the *T. orientalis*-positive DNA samples.

196 Although buffalo farming is uncommon in the up-country wet zone, buffaloes are
197 sometimes reared together with cattle in the low-country wet zone. In the present study, DNA
198 samples from seven buffaloes that were reared together with cattle at the sampling sites were
199 also screened for hemoprotozoan parasites with PCR. *Babesia bovis*, *T. orientalis*, and *Tr.*
200 *theileri* infections were detected among these animals. In common with the cattle, all seven
201 buffaloes were positive for *T. orientalis*, and one and three animals were infected with *B.*

202 *bovis* and *Tr. theileri*, respectively. One of the *Tr. theileri*-positive buffalo was co-infected
203 with *T. orientalis* and *B. bovis*, and the remaining two were co-infected with *T. orientalis*.

204 To confirm the PCR findings, the PCR amplicons were cloned and sequenced. One
205 resultant sequence of *B. bovis rap-1* (buffalo; GenBank accession number LC438493), seven
206 sequences of *B. bigemina ama-1* (cattle; LC438494–LC438500), two sequences of *T.*
207 *annulata tams-1* (cattle; LC438501 and LC438502), and eight sequences of *Tr. theileri catl*
208 (one from buffalo and seven from cattle; LC438503–LC438510) shared high identity scores
209 with those previously reported in Sri Lanka [18, 19, 32], confirming the PCR findings in this
210 study. In a previous study, *B. bovis rap-1* variants were shown to differ between cattle and
211 buffaloes in Sri Lanka [32]. The buffalo-derived *rap-1* sequence generated in the present
212 study (LC438493) clustered together with previously reported buffalo-derived sequences
213 from Sri Lanka (AB845432–AB845437) and those from GenBank in the phylogeny, whereas
214 the previously determined cattle-derived sequences from Sri Lanka (AB690859–AB690861)
215 occurred in a separate clade (Fig. 1). This finding confirms that the *B. bovis* populations differ
216 between the cattle and buffaloes in this country. Therefore, the detection of *B. bovis* in a
217 buffalo may not necessarily indicate that the cattle in Galle are infected with this parasite
218 species. However, further studies with large number of samples are essential to rule out *B.*
219 *bovis* infection in the cattle in Galle.

220 *Trypanosoma theileri* can be divided into several genotypes, based on the *catl* gene

221 sequences [33, 34]. In Sri Lanka, 12 *catl* genotypes have been reported, including IA, IB, and
222 ID–IK within major phylogenetic clade TthI and IIE and IIF within major phylogenetic clade
223 TthII [19]. In contrast, the *catl* sequences determined in the present study were classified into
224 seven genotypes, including two new genotypes (IL and IM) and four genotypes (IA, IB, II,
225 and IK) that were previously detected in Sri Lanka (Fig. 2). Investigations using large number
226 of samples from different geographic regions are required to confirm whether the newly
227 detected *catl* genotypes are unique to the low-country wet zone.

228 In conclusion, in this study, we analyzed infections of several hemoprotozoan
229 parasite species among the cattle population in the low-country wet zone of Sri Lanka, and
230 found that the infection profiles were similar to those observed in the up-country wet zone,
231 despite the variations in cattle breeds, management practices, and geography between these
232 two regions. Therefore, the major factor that influences the epidemiology of bovine
233 hemoprotozoan parasites in Sri Lanka is not geography, but the local climatic zones.

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235

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238

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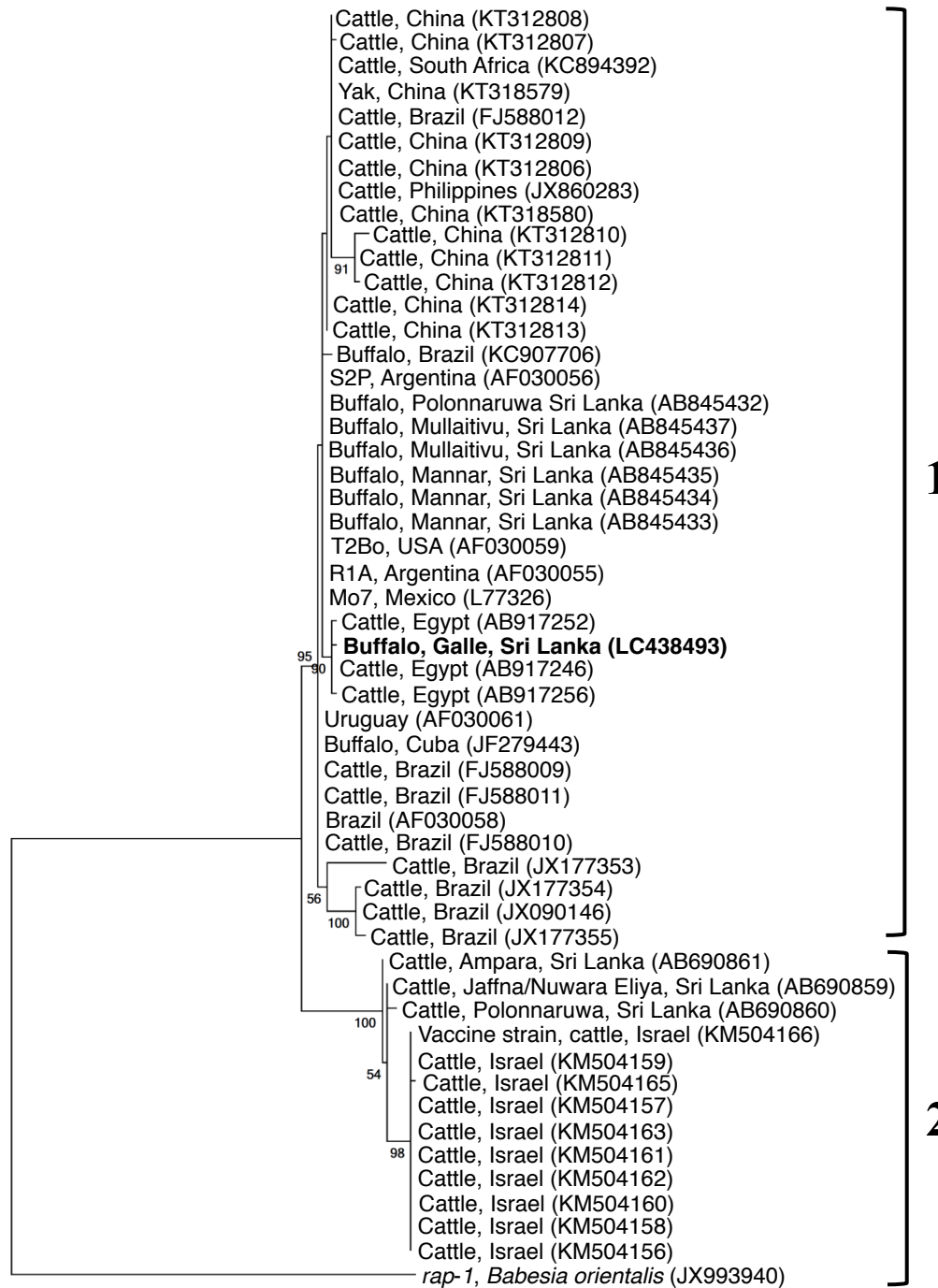
353 **Figure legends**

354

355 **Fig. 1.** Phylogeny of *Babesia bovis rap-1* gene. A buffalo-derived *B. bovis rap-1* sequence
356 (indicated in boldface type) determined in this study and those retrieved from the GenBank
357 database were used to construct a maximum likelihood phylogenetic tree. Note that the
358 buffalo-derived sequence from Galle occurs in clade 1, together with the buffalo-derived
359 sequences previously determined in Sri Lanka, and that the previously determined
360 cattle-derived sequences from Sri Lanka occur in clade 2.

361

362 **Fig. 2.** Phylogeny of *Trypanosoma theileri catl* gene. The *catl* sequences from seven cattle
363 and one buffalo from Galle (indicated in boldface type) and those previously reported in Sri
364 Lanka and other countries were used to construct a neighbor-joining phylogenetic tree. Note
365 that the newly determined sequences occur in six clades within the major TthI clade,
366 including IA, IB, II, IK, IL, and IM, and that genotypes IL and IM are reported for the first
367 time in Sri Lanka.



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0.05

Fig. 1

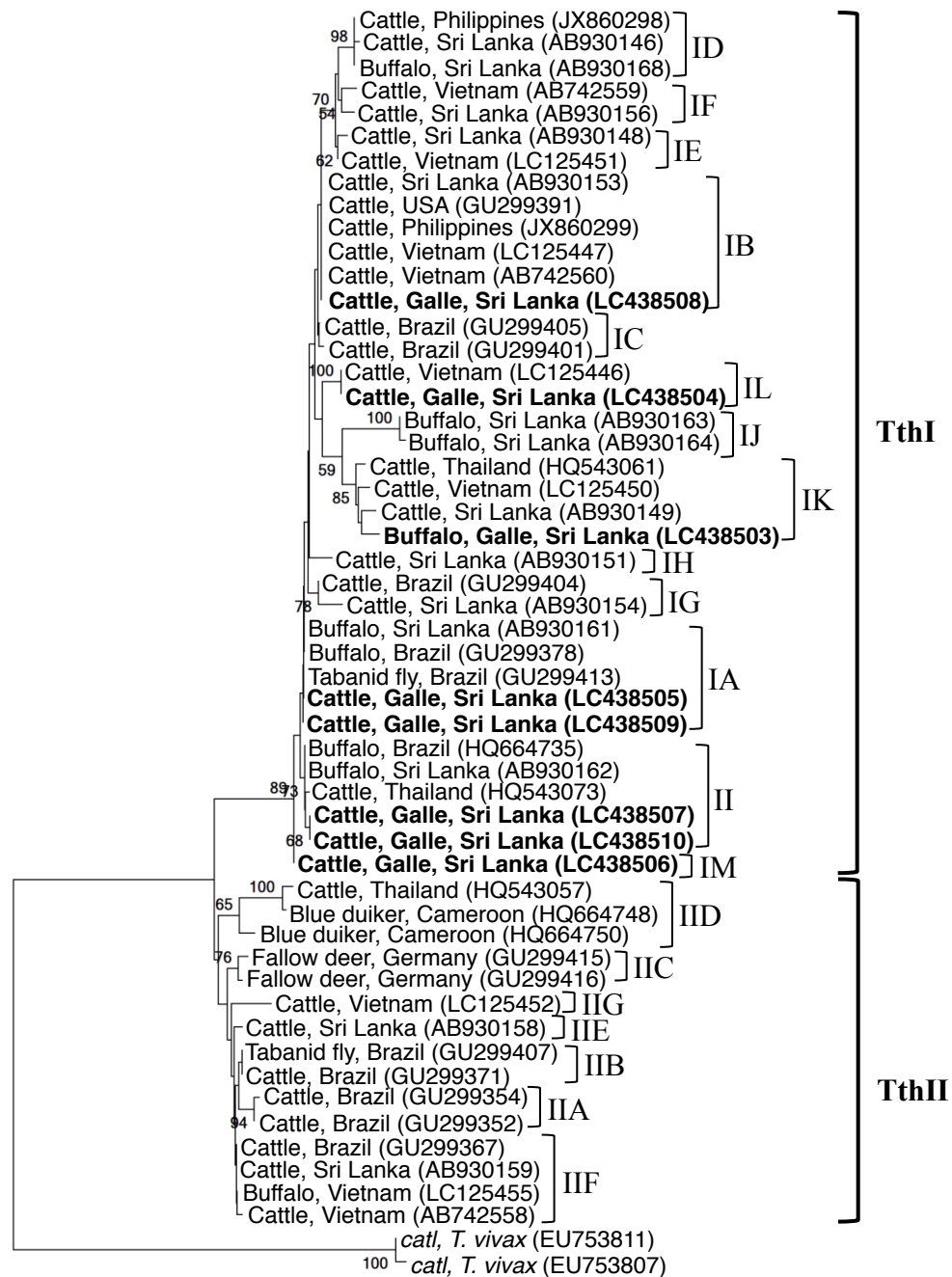


Fig. 2

Table 1. PCR detection of *Babesia*, *Theileria*, and *Trypanosoma* in 121 cattle from Galle in Sri Lanka

Parasite	Akmeemana (n=60)		Rathgama (n=61)		P value	Total	
	No. positive	% (CI)	No. positive	% (CI)		No. positive	% (CI)
<i>B. bigemina</i>	3	5.0 (1.6-12.7)	20	32.8 (22.3-45.2)	0.0001	23	19.0 (13.0-26.9)
<i>T. annulata</i>	0	0 (0.0-5.6)	2	3.3 (1.7-13.5)	0.1576	2	1.6 (0.45-5.82)
<i>T. orientalis</i>	60	100 (94.4-100)	61	100 (94.1-100)		121	100 (96.9-100)
<i>T. theileri</i>	11	13.3 (9.7-27.8)	17	27.9 (18.2-40.2)	0.0483	25	20.6 (14.4-28.7)

CI, 95% confidence interval