A longitudinal study of Babesia and Theileria infections in cattle in Sri Lanka

A longitudinal study of *Babesia* and *Theileria* infections in cattle in Sri Lanka

Thillaiampalam Sivakumar [a,b], Hemal Kothalawala [b], Gayani Weerasooriya [a,b], Seekkuge Susil Priyantha Silva [b], Sumathy Puvanendiran [b], Tserendorj Munkhjargal [a], Ikuo Igarashi [a], Naoaki Yokoyama [a,*]

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

[b] *Veterinary Research Institute, P.O. Box 28, Peradeniya, Sri Lanka*

* Corresponding author: N. Yokoyama, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Tel.: +81 155 49 5649; fax: +81 155 49 5643

E-mail: yokoyama@obihiro.ac.jp

ABSTRACT

Throughout the world, infections with the *Babesia* and *Theileria* parasites often result in economically significant clinical disease in cattle. We conducted a longitudinal survey of *Babesia* and *Theileria* infections in cattle from the Polonnaruwa (n=75; dry zone) and Nuwara Eliya (n=161; wet zone) districts of Sri Lanka. DNA from blood samples collected in June, September, and December 2014 and March 2015 was screened for *Babesia bovis*, *Babesia bigemina*, *Theileria annulata* and *Theileria orientalis* using specific polymerase chain reactions (PCRs). Additionally, serum samples collected from the animals were screened using enzyme-linked immunosorbent assays (ELISAs) to detect *B. bovis*- and *B. bigemina*-specific antibodies. All of the animals surveyed in Polonnaruwa and 150 (93.2%) of the animals surveyed in Nuwara Eliya were PCR-positive for *Babesia* and/or *Theileria* at least once during the study period. A greater percentage of the cattle in Polonnaruwa were positive for *T. annulata* and *T. orientalis* than *B. bovis* or *B. bigemina* at all time points. *T. orientalis* was the most common infection in Nuwara Eliya. Additionally, more cattle were seropositive for *B. bigemina* than *B. bovis* in both districts. Although significant variations were sometimes observed in the rates of animals that were positive for *B. bigemina*, *T. annulata*, and *T. orientalis* at the different sampling time points, the rates of new infections with these parasites (by PCR or ELISA) on second, third, and fourth time points among the parasite-negative samples at the first, second, and third time points, respectively, did not

differ between the sampling in either district—suggesting that the parasite species infected cattle at a constant rate in these locations. However, in Polonnaruwa, the rates of new infection with *T. annulata* were higher than the rates of new infection with *T. orientalis*. The rates were also higher than those in Nuwara Eliya. In Nuwara Eliya, the rates of new infection with *T. orientalis* were higher than the rates of new infection with *T. annulata*. The rates were also higher than those in *T. orientalis* in Polonnaruwa. These differences might be due to variations in the density and activity of the specific tick vectors within and between the districts. Our findings suggest the need for year-round control measures against bovine *Babesia* and *Theileria* infection in Sri Lanka. Further studies to determine the densities of the vector tick species in the different geographical areas of the country are warranted.

Keywords: *Babesia*, cattle, longitudinal study, Sri Lanka, *Theileria*

1. INTRODUCTION

Piroplasmids, such as *Babesia* and *Theileria* parasites, cause economically significant disease in livestock, especially cattle (Bishop et al., 2004; Bock et al., 2004). The prevalence of *Babesia* and *Theileria* in a locality is usually related to the distribution of their transmission vectors, the ixodid ticks. The lifecycles of *Babesia* and *Theileria* parasites in their host animals begin with the injection of sporozoites by infected ticks during their blood meal (Bishop et al., 2004; Hunfeld et al., 2008). *Babesia* sporozoites directly invade the host's red blood cells (RBCs), where they transform into trophozoites and then meronts, which undergo asexual reproduction to form merozoites (Homer et al., 2000). In contrast, *Theileria* sporozoites first infect the host's leukocytes, where they develop into schizonts. The subsequent rupture of the schizonts releases merozoites, the life stage that eventually infects host RBCs (Bishop et al., 2004). Hence, parasites can be detected in the blood samples of *Babesia*- and *Theileria*-infected animals (Mans et al., 2015; Mosqueda et al., 2012).

Among the bovine *Babesia* parasites, *Babesia bovis*, *Babesia bigemina*, and *Babesia divergens* are known to be virulent species, while species with low pathogenicity—such as *Babesia ovata* and *Babesia major*—are also infective to cattle (Bock et al., 2004). Acute infections with *B. bovis* and *B. bigemina*, which are the major causative agents of clinical babesiosis in the tropical and subtropical regions of world, are characterized by anemia and anemia-related syndromes, which are associated with extensive intravascular hemolysis

(Ristic, 1981). Additionally, *B. bovis* infections can sometimes be associated with neurological and respiratory syndromes caused by the cytoadherence of parasite-infected RBCs to endothelial cells in the capillary beds (Everitt et al., 1986, Wright and Goodger, 1988). On the other hand, *Theileria* parasites, which comprise the bovine *Theileria parva* and *Theileria annulata* transforming species types, are more pathogenic than *Theileria orientalis,* which is a non-transforming species (Dobbelaere and Heussler, 1999; McKeever et al., 2009; Sivakumar et al., 2014a). Geographically, the parasite responsible for East Coast Fever (*T. parva*) has a limited distribution in Africa, while the causative agent of tropical theileriosis (*T. annulata*), is endemic in parts of Asia and Africa (Weir et al., 2010). In contrast, *T. orientalis* has a worldwide distribution and occasionally has a clinically significant impact (Eamens et al., 2013; McFadden et al., 2011; Sivakumar et al., 2014a).

Control strategies against *Babesia* and *Theileria* parasites largely depend on immunization with live vaccines and tick-control measures (Bishop et al., 2004; Bock and de Vos, 2001; Bock et al., 2004; Oura, 2007). In addition to tick surveys, longitudinal studies on *Babesia* and *Theileria* infections may provide information on the high-risk season(s) for infection—in terms of when the tick vectors are most active—thereby enabling the advanced application of systematic tick-control measures (Shimizu et al., 2000). However, while numerous cross-sectional surveys of *Babesia* and *Theileria* parasites have been carried out in a number of countries where these parasites are endemic (Altay et al., 2008; Elsify et al., 2015; García-Sanmartín et al., 2006; Ybañez et al., 2013), longitudinal studies to monitor

parasite infections have rarely been conducted in cattle populations. *Babesia* and *Theileria* parasites are endemic in the cattle and water buffalo populations of Sri Lanka (Jorgensen et al., 1992; Sivakumar et al., 2012b, 2014b; Weilgama et al., 1986, 1989). Recent polymerase chain reaction (PCR)-based investigations in Sri Lanka have confirmed the nationwide occurrence of *B*. *bovis*, *B*. *bigemina*, and *T*. *orientalis* in both cattle and water buffaloes, and *T*. *annulata* in cattle (Sivakumar et al., 2012b, 2014b). However, these studies were cross-sectional in nature. Thus, they did not provide any information on the temporal dynamics of *Babesia* and *Theileria* epidemiology in Sri Lanka. In the present study, we conducted a longitudinal survey of *Babesia* and *Theileria* infections in cattle that were reared in two distinct geographical locations in Sri Lanka: the Polonnaruwa (dry zone) and Nuwara Eliya (wet zone) districts.

2. MATERIALS AND METHODS

2.1. Study areas and animals

The study animals were cattle that had been reared in the Polonnaruwa (hereafter referred to as "P") and Nuwara Eliya (hereafter referred to as "NE") districts of Sri Lanka, which are located in dry and wet zones, respectively (Sivakumar et al., 2012b). The vegetation in the dry zone is characterized by scrub forest, interspersed with tough bushes. In contrast, the common types of vegetation in the wet zone include evergreen forest, savannah, and wet patana grassland. The mean annual rainfall in wet zone is over 2500 mm, whereas the mean annual rainfall of the dry zone is less than 1750 mm. In the P district, the mean daily maximum and minimum temperatures (in the hottest and coldest months) were 33°C and 21°C, respectively; those in the NE district were 28°C and 18°C, respectively. In the P district, local cattle (*Bos indicus*) and their crosses are maintained using extensive or semi-intensive management practices. In the NE district, European breeds (*Bos taurus*) are maintained by an intensive management system (Abeygunawardena et al., 1997). In the present study, blood samples were collected from 75 animals in six cattle farms in the P district (21, 5, 5, 6, 11, and 27 animals) and 161 animals in three cattle farms in NE district (58, 87, and 16 animals, respectively) in June 2014; resampling was performed in September 2014, December 2014, and March 2015. Two milliliters of blood were collected from each animal in vacutainer tubes with or without ethylene-diaminetetraacetic acid (EDTA; NIPRO, Osaka, Japan). All of the

119 animal protocols were approved by the Ethical Review Committee of the Veterinary Research
120 Institute, Sri Lanka.
121
122 ***2.2. DNA extraction and serum separation***
123 The blood samples that were collected with EDTA were subjected to DNA extraction
124 using a commercial kit (Qiagen, Hilden, Germany). Briefly, DNA was extracted from 200 µl
125 of whole blood, according to the manufacturer's instructions. The extracted DNA samples
126 were stored until further use at −20°C. The serum samples were prepared from the blood
127 samples that had been collected in tubes without any anticoagulants, as previously described
128 (Munkhjargal et al., 2013).
129
130 ***2.3. The PCR detection of Babesia and Theileria parasites***
131 Previously described *B. bovis*-, *B. bigemina*-, *T. annulata*-, and *T. orientalis*-specific
132 PCRs were used to screen all of the DNA samples. A nested PCR, based on the
133 rhoptry-associated protein-1 (*rap-1*) gene, was used to screen for *B. bovis* (Figueroa et al.,
134 1993). Single-step PCRs based on apical membrane antigen-1, *T. annulata* merozoite surface
135 antigen-1, and major piroplasm surface protein genes were used to specifically detect *B.*
136 *bigemina*, *T. annulata*, and *T. orientalis*, respectively (Kirvar et al., 2000; Ota et al., 2009;
137 Sivakumar et al., 2012a). The PCR primers, reaction mixtures, and cycling conditions were
138 described in a previous report (Sivakumar et al., 2012b). After separation by agarose gel

electrophoresis, the PCR products were stained with ethidium bromide, and then visualized under UV illumination. The detection of bands of similar size to those observed from the positive controls was considered evidence of parasite positivity.

2.4. The enzyme-linked immunosorbent assays (ELISAs)

All of the serum samples were analyzed by ELISAs using *B. bovis*- and *B. bigemina*-recombinant RAP-1 (rRAP-1) antigens. Briefly, 300-bp gene fragments, encoding the 100 amino acid residues located within the previously characterized *B. bovis*- and *B. bigemina*-specific N-terminal regions of the RAP-1 antigens (Boonchit et al., 2004, 2006), were amplified from PCR 2.1 plasmids containing inserts from *B. bovis* (GenBank accession number: LC157851) and *B. bigemina* (GenBank accession number: LC157860) *rap-1* genes that had been isolated in Sri Lanka (unpublished data). The *Bam*HI and *Xho*I restriction sites (underlined) were added to the forward (*B. bovis*, 5′-gcggatccAACTATCTGAAAGCCAATG-3′; *B. bigemina*, 5′-gcggatccCCTCACTACCTTTCTAAGGC-3′) and reverse (*B. bovis*, 5′-gcctcgagtcaAGCAATATTCTCGCCTAGG-3′; *B. bigemina*, 5′-gcctcgagtcaATCTTCATTTTTGGGGTCATC-3′) primers, respectively (the uppercase letters indicate the regions corresponding to the template sequences). A reverse-complement stop codon TGA (TCA) was also added to the 5′ end of the reverse primers. PCR amplification and cloning, and protein expression and purification were essentially performed

159 according to previously described methods (Tattiyapong et al., 2016). Briefly, the
160 PCR-amplified target gene fragments were digested with their respective restriction enzymes,
161 ligated to a similarly digested pGEX4T-1 (*B*. *bovis*) or pGEX6p2 (*B*. *bigemina*) plasmid
162 vector (GE Healthcare, Uppsala, Sweden), expressed as glutathione S-transferase
163 (GST)-fusion proteins, purified using a Glutathione Sepharose 4B column (GE Healthcare),
164 and finally cleaved using Thrombin or PreScission Protease (GE Healthcare) to isolate the
165 rRAP-1 antigens. The purified *B*. *bovis*–rRAP-1 and *B*. *bigemina*–rRAP-1 antigens were then
166 used in ELISAs to screen the serum samples that were collected in the present study, as
167 described previously (Terkawi et al., 2011). A serum sample was considered to be positive if
168 the OD value was greater than the cutoff value, which was the sum of the mean OD value of
169 the five negative controls (non-infected serum samples) that were used in each ELISA plate
170 and 5 × the standard deviation.
171
172 ***2.5. Statistical analyses***
173 In the analysis of the rates of parasite positivity, the OpenEpi software program
174 (http://www.openepi.com/Proportion/Proportion.htm) was used to determine the 95%
175 confidence intervals using a Wilson score interval (Wilson, 1927). In addition, the "N-1"
176 chi-squared test (Campbell, 2007; Richardson, 2011)
177 (https://www.medcalc.org/calc/comparison_of_proportions.php) was used to determine the *P*
178 values. *P* values of < 0.05 were considered to indicate statistical significance.

179 **3. RESULTS**

180

181 ***3.1. The monitoring of Babesia and Theileria parasites using parasite-specific PCRs***

182 The PCRs detected all four parasite species (*B*. *bovis*, *B*. *bigemina*, *T*. *annulata*, and
183 *T*. *orientalis*) in the DNA samples from the cattle populations of the R and NE districts.
184 During the study period, *Babesia* and/or *Theileria* infections were detected in at least one
185 sample from all of the animals surveyed in the P district and 150 (93.2%) of the 161 animals
186 surveyed in the NE district. Additionally, 63 (84.0%), 65 (86.7%), 67 (89.3%), and 68
187 (90.7%) animals in the P district, and 92 (57.1%), 124 (77.0%), 119 (79.3%), and 126
188 (78.3%) animals in the NE district were infected with at least one parasite species in the first
189 (June, 2014), second (September, 2014), third (December, 2014), and fourth (March, 2015)
190 sampling time points, respectively. Moreover, 7 (9.3%), 48 (64.0%), 74 (98.7%), and 58
191 (77.3%) of 75 animals surveyed in the P district, and 9 (5.6%), 31 (19.3%), 15 (9.3%), and
192 147 (91.3%) of 161 animals surveyed in the NE district were infected with *B*. *bovis*, *B*.
193 *bigemina*, *T*. *annulata*, and *T*. *orientalis*, respectively, in at least one sampling time point (Fig.
194 1). In the P district, the rates of *T*. *annulata* (42.7–85.3%) and *T*. *orientalis* (56.0–62.7%)
195 positivity were higher in all four sampling time points than the rates of *B*. *bovis* (0–4.0%) and
196 *B*. *bigemina* (18.7–36.0%) positivity. In contrast, the rates of *T*. *orientalis* (53.4–78.3%)
197 positivity were significantly higher than the rates of *B*. *bovis* (0–5%), *B*. *bigemina*
198 (1.2–16.1%), and *T*. *annulata* (1.2–4.3%) positivity in the NE district (Fig. 1).

199 In the P district, a significant difference in the rate of *B*. *bigemina* positivity was
200 observed between consecutive sampling time points; a significant decrease (from 36.0% to
201 18.7%) was observed at the third sampling time point. Furthermore, a significant increase was
202 observed in the rate of *T*. *annulata* positivity, which increased at the second (from 42.7% to
203 62.7%) and third (from 62.7% to 80.0%) sampling time points (Fig. 1). In the NE district,
204 significant differences were found in the rate of *B*. *bigemina* positivity, which increased at the
205 second sampling time point (from 1.9% to 16.1%) and then decreased at the third sampling
206 time point (from 16.1% to 1.2%), and in the rate of *T*. *orientalis* positivity, which increased
207 (from 57.1% to 77.0%) at the second sampling time point (Fig. 1).
208 When the new infection rates among the PCR-negative samples on the first, second,
209 and third samplings were analyzed at the second, third, and fourth samplings, respectively, the
210 rates of *T*. *annulata* positivity were higher than the rates of *B*. *bovis*, *B*. *bigemina*, and *T*.
211 *orientalis* positivity in the P district (Table 1). In contrast, in the NE district, the rates of *T*.
212 *orientalis* positivity were higher than the rates of *B*. *bovis*, *B*. *bigemina*, and *T*. *annulata*
213 positivity (Table 1). Only the rates of new infection with *B*. *bigemina* showed significant
214 variation between consecutive samples in the P district, where the positive rate decreased at
215 the third sampling and subsequently increased at the fourth sampling, which was similar to
216 the NE district, where the infection rate also decreased at the third sampling. On a per district
217 basis, the rates of new infections with *B*. *bigemina* and *T*. *annulata* in the P district were
218 higher than those in the NE district at each sampling time point, whereas the rates of new

219 infection with *T. orientalis* in the NE district were higher than those in the P district (Table 1).

220 In the P district, 0 of 7, 16 (33.3%) of 48, 62 (83.8%) of 74, and 45 (77.6%) of 58
221 animals that were infected *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis*, respectively,
222 had persistent infections for two or more consecutive sampling time points during the study
223 period (Fig. 2), demonstrating that the persistence of *Theileria* infection was more
224 pronounced than the persistence of *Babesia* infection in this district. Furthermore, in the NE
225 district, 0 of 9, 1 (3.2%) of 30, 0 of 15, and 116 (78.9%) of 147 animals infected with *B. bovis*,
226 *B. bigemina*, *T. annulata*, and *T. orientalis*, respectively, had persistent infections at two or
227 more consecutive sampling time points (Fig. 2).

228 Multiple infections were more common in the P district, where the co-infection rates
229 among the animals that were infected with at least one parasite species ranged from 49.2% to
230 77.9%, in comparison to the NE district, where the co-infection rates ranged from 2.5% to
231 12.9% (Table 2). In the P district, all of the *B. bovis*-infected cattle were co-infected with *B.*
232 *bigemina* and/or *Theileria* species. The co-infection rates among *B. bigemina*-, *T. annulata*-,
233 and *T. orientalis*-infected cattle in this district were 58.8–100%, 68.3–85.1%, and 57.4–90.9%,
234 respectively (Fig. S1). In the NE district, the PCR assay detected *B. bovis* at the first and third
235 sampling time points, and three of the eight animals that were infected with *B. bovis* at the
236 first sampling time point were co-infected with *T. orientalis*. With the exception of a single
237 animal at the second sampling time point, all of the *T. annulata*-infected cattle in the NE
238 district were co-infected with *Babesia* and/or *T. orientalis*. The co-infection rates among the *B.*

239 *bigemina*- and *T. orientalis*-infected cattle in this district were 50–100% and 2.6–14.4%,
240 respectively (Fig. S1).
241
242 ***3.2. The serological monitoring of B. bovis and B. bigemina***
243 During the study period, anti-*B. bovis* and anti-*B. bigemina* antibodies were detected
244 in 63 (84.0%) and 74 (98.7%) of the 75 cattle in the P district and 141 (87.6%) and 160 (99.4)
245 of the 161 cattle in the NE district, respectively, by ELISAs (Fig. 3). At all four sampling time
246 points, the rates *B. bigemina* positivity in the R (85.3–98.7%) and NE (84.5–96.3%) districts
247 were higher than the rates of *B. bovis* (40–57.3% and 58.4–66.5%, respectively) positivity.
248 Furthermore, the rates of *B. bigemina* and *B. bovis* positivity in the R and NE districts were
249 comparable at each sampling time point. The rate of *B. bigemina* positivity at the fourth
250 sampling time point was significantly decreased in the P district, whereas the rate of *B.*
251 *bigemina* positivity was increased at the second sampling time point and then decreased at the
252 fourth sampling time point (Fig. 3). In contrast, no statistically significant changes in the rates
253 of new infection with *B. bovis* or *B. bigemina* were observed between consecutive samplings
254 in either district (Table 3).
255

256 **4. DISCUSSION**

257 Cattle populations, especially those in the tropics and subtropics, are vulnerable to
258 *Babesia* and *Theileria* infections (Uilenberg, 1995). Investigations on the temporal dynamics
259 of parasite infections would enable veterinary authorities to implement appropriate control
260 strategies against *Babesia* and *Theileria*. We therefore conducted a longitudinal investigation
261 of *Babesia* and *Theileria* species in the cattle of two districts in Sri Lanka: Polonnaruwa (R),
262 which is located in a dry zone; and Nuwara Eliya (NE), which is located in a wet zone. The
263 two districts had different characteristics with regard to their climates, cattle breeding systems,
264 and cattle management practices (Abeygunawardena et al., 1997).

265 In agreement with a recent study (Sivakumar et al., 2012b), the PCRs—the
266 specificities of which have been confirmed in previous surveys of cattle and water buffalos in
267 Sri Lanka (Sivakumar et al., 2012b, 2014b)—detected all four of the parasites (*B. bovis*, *B.*
268 *bigemina*, *T. annulata*, and *T. orientalis*) in the cattle at both sampling locations. Within the
269 study period, all of the animals in the P district and most of the animals in the NE district
270 were infected with *Babesia* and/or *Theileria* at least once, indicating the extent of the animals'
271 exposure to these pathogens. The pronounced persistence of *T. annulata* and *T. orientalis*
272 infections in the P district and *T. orientalis* infections in the NE district may explain the large
273 percentages of animals that were positive for these parasites in the two locations. The finding,
274 that none of the *T. annulata*-infected cattle (*B. taurus*) in the NE district had persistent
275 infection, contrasts with a previous study which reported that persistent infections were more

276 prominent in *B. taurus* animals than in *B. indicus* animals (Glass et al., 2005). Host immunity
277 against *T. annulata* infection is tightly focused and strain-specific due to the
278 immunodominance of the parasite, major histocompatibility complex (MHC) class I
279 phenotype variations in cattle, and diversity in the $CD8^+$ cytotoxic T lymphocyte (CTL)
280 determinants of the parasite population (MacHugh et al., 2011; Sivakumar et al., 2014a). Thus,
281 investigations to compare the variations in the MHC class I phenotypes of cattle and the CTL
282 determinants of *T. annulata* in the NE and P districts might shed additional light on the
283 differences that we observed in the persistence of *T. annulata* infection and the percentages of
284 parasite-positive animals in these two districts.

285 Although differences were found in percentages of *T. annulata*-positive animals in
286 the P district and *T. orientalis*-positive animals in the NE district, the rates of new infection
287 with these parasites at the different sampling time points did not differ within the districts,
288 suggesting that *T. annulata* and *T. orientalis* were transmitted at constant rates within each of
289 the districts. Our findings also show that the new infection rates for *T. annulata* in the P
290 district were higher than those in the NE district, and that the rates of new infection with *T.*
291 *orientalis* were higher in the NE district. Additionally, the rates of new infection with *T.*
292 *annulata* were higher than the rates of new infection with *T. orientalis* in the P district, while
293 rates of new infection with *T. orientalis* were higher than the rates of new infection with *T.*
294 *annulata* in the NE district. These observations led us to infer that the density and activity of
295 the tick species that are capable of transmitting *T. annulata* and *T. orientalis* might differ

296 within and between the two districts. In general, these findings also suggest that the species
297 and/or density of tick vectors that transmit *T. annulata* and *T. orientalis* may differ between
298 the dry and wet zones of Sri Lanka. Several species of cattle ticks belonging to the
299 *Rhipicephalus*, *Haemaphysalis*, *Amblyomma*, and *Hyalomma* genera were reported in
300 previous studies in Sri Lanka (Liyanaarachchi et al., 2015; Weilgama et al., 1986). Many of
301 these tick species, such *R. microplus* and species of *Hyalomma* and *Haemaphysalis*, are
302 known to transmit bovine *Babesia* and *Theileria* (Bishop et al., 2004; Bock et al., 2004).
303 However, with the exception of studies investigating the experimental transmission of *T.*
304 *orientalis* by *H. bispinosa* (Weilgama et al., 1986) and the isolation of *B. bovis* from *R.*
305 *microplus* (Weilgama et al., 1989), there have been few studies to investigate the vector
306 competence of these tick species in Sri Lanka. Thus, studies to determine the vectorial
307 capacity of cattle ticks in this country have become a priority.

308 Because the persistence of *B. bovis* and *B. bigemina* infections was not pronounced
309 in the cattle of either district, the PCR results from the present investigation might not reflect
310 the actual rates of *Babesia* infection between two consecutive sampling time points. We
311 therefore used ELISAs to determine the rates of seropositivity for *B. bovis* and *B. bigemina*.
312 The findings showed that—similarly to the cases of *T. annulata* and *T. orientalis*
313 infection—the rates of new infection with *B. bovis* did not differ between the sampling time
314 points in either district. However, given that the rates of seropositivity were very high at each
315 sampling time point in both districts, it was not possible to accurately compare the rate of new

316 infection with *B*. *bigemina*. In both districts, the percentage of animals that were seropositive
317 for *B*. *bigemina* was greater than the percentage of animals that were positive for *B*. *bovis* at
318 each sampling time point, suggesting that the density and/or activity of the ticks that are
319 capable of transmitting *B*. *bigemina* are higher than those that transmit *B*. *bovis* in both
320 districts. However, no significant variations were found between the R and NE districts with
321 regard to the rates of *B*. *bovis* and *B*. *bigemina* positivity, indicating that the activities of the
322 tick species that transmit these parasites were similar in both of the districts.

323 The cattle population in the P district is composed of *B*. *indicus* and their crosses,
324 which are known to have higher resistance to piroplasmosis in comparison to the European
325 breeds (*B*. *taurus*) that are commonly kept in the NE district (Bock et al., 1999). This could
326 explain why the clinical babesiosis was more common in the NE district than in the P district;
327 however the rates of seropositivity and new infection with the *Babesia* species were
328 comparable between these districts. With the exception of the rate of *T*. *annulata* infection in
329 the NE district, the rates of exposure to the surveyed *Babesia* and *Theileria* species were high
330 in both districts during the study period, which suggests that endemic stability has been
331 reached. In this setting, there may be few (or no) clinical cases, despite the high infection
332 rates among cattle (Bock et al., 2004).

333 The new infection rates might be confounded by parasite recrudescence (Calder et al.,
334 1996; Figueroa et al., 1992). However, the confounding effect of recrudescence for *B*. *bovis*
335 and *B*. *bigemina* may be minimal in the present study, because the seropositive rates for these

parasites were considered; seropositivity among animals that were seronegative at the previous sampling time point probably indicates the presence of new infections. Furthermore, although *Theileria* parasitemia fluctuates with time, the parasites rarely become undetectable in carrier animals when a PCR is used for their detection (Mans et al., 2015). Thus, PCR positivity for *Theileria* among animals that were PCR-negative at a previous sampling time point might also be indicative of new infection. Furthermore, despite the comparable rates of *T. orientalis* positivity at both sampling locations, the new infection rates were much higher in the NE district, suggesting that sample positivity was not likely to have been caused by parasite recrudescence. Thus, the impact of parasite recrudescence on the rates of new infections is likely to have been minimal in the present investigation.

Co-infections with multiple parasite species were more common in the P district than in the NE district, as the rates of animals that were positive for *T. annulata* and *B. bigemina* at each sampling time point were higher in the P district. A previous study found that the development of anemia in *T. orientalis*-infected animals could be potentiated by co-infection with *B. ovata* (Sivakumar et al., 2012c). In the present study, among the 47, 42, 44, and 44 animals in the P district that were infected with *T. orientalis* (at the first to fourth sampling time points), 8 (17.0%), 12 (28.6%), 10 (22.7%), and 8 (18.2%) animals were co-infected with *B. bovis* and/or *B. bigemina*, respectively. It would be interesting to investigate the effects of such co-infections on the anemia status of infected cattle in Sri Lanka.

5. CONCLUSION

The present study, which found that *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis* infections were common among cattle in two Sri Lankan districts throughout a nine-month study period, suggests that year-round control strategies are essential if we are to minimize the rates of *Babesia* and *Theileria* infection in cattle in this country. The findings also suggest that the species and/or density of tick vectors involved in the transmission of *Theileria* might differ between the dry and wet zones of Sri Lanka. Epidemiological surveys to identify the specific tick vectors that are capable of transmitting different species of *Babesia* and *Theileria* and to determine their relative abundance in the dry and wet zones of Sri Lanka are now a priority.

Conflict of interest statement

The authors declare no conflicts of interest in association with the present study.

Acknowledgments

We are grateful to Drs. Kulanayagam Kuleswarakumar and Subramaniyam Sukumar (Department of Animal Production and Health, Central Province, Sri Lanka) and Dr. Asela Sanjeewa Meewewa (Department of Animal Production and Health, North-Central Province, Sri Lanka) for collecting blood samples from the cattle surveyed. We thank the staff at the Sri Lankan cattle farms and at the Veterinary Research Institute, Department of Animal Production and Health, Peradeniya, Sri Lanka, for their kind cooperation. We also thank Ms. Hiroko Yamamoto, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan, and Mr. H.A. Wijithasiri, Mr. M.M.P.S.K. Chandrasena, Ms. W.M.M.B.K. Jayathilake, Ms. D.M.M. Darshani, Ms. A.B.M.H. Priyadarshani, and Ms. D.H.S.P. Meththananda, Veterinary Research Institute, Peradeniya, Sri Lanka, for their excellent technical assistance. This study was supported by a grant from the Japan Society for Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (JSPS KAKENHI Numbers 26257417, 16H05033, and 15K14862).

REFERENCES

Abeygunawardena, H., Rathnayaka, D., Jayathilaka, W.M.A.P., 1997. Characteristics of cattle farming systems in Sri Lanka. J. Natn. Sci. Foundation Sri Lanka 25, 25–38.

Altay, K., Aydin, M.F., Dumanli, N., Aktas, M., 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. Vet. Parasitol. 158, 295–301.

Bishop, R., Musoke, A., Morzaria, S., Gardner, M., Nene, V., 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixo did ticks. Parasitology 129 (Suppl.), S271–283.

Bock, R.E., de Vos, A.J., 2001. Immunity following use of Australian tick fever vaccine: a review of the evidence. Aust. Vet. J. 79, 832–839.

Bock, R., Jackson L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129 (Suppl.), S247–S269.

Bock, R.E., Kingston, T.G., de Vos, A.J., 1999. Effect of breed of cattle on transmission rate and innate resistance to infection with *B. bovis* and *B. bigemina* transmitted by *Boophilus microplus*. Aust. Vet. J. 77, 461–464.

Boonchit, S., Alhassan, A., Chan, B., Xuan, X., Yokoyama, N., Ooshiro, M., Goff, W.L., Waghela, S.D., Wagner, G., Igarashi, I., 2006. Expression of C-terminal truncated and full-length *Babesia bigemina* rhoptry-associated protein 1 and their potential use in

412 enzyme-linked immunosorbent assay. Vet. Parasitol. 137, 28–35.
413
414 Boonchit, S., Xuan, X., Yokoyama, N., Goff, W.L., Waghela, S.D., Wagner, G., Igarashi, I.,
415 2004. Improved enzyme-linked immunosorbent assay using C-terminal truncated recombinant
416 antigens of *Babesia bovis* rhoptry-associated protein-1 for detection of specific antibodies. J.
417 Clin. Microb. 42, 1601–1604.
418
419 Calder, J.A., Reddy, G.R., Chieves, L., Courtney, C.H., Littell, R., Livengood, J.R., Norval,
420 R.A., Smith, C., Dame, J.B., 1996. Monitoring *Babesia bovis* infections in cattle by using
421 PCR-based tests. J. Clin. Microbiol. 34, 2748–2755.
422
423 Campbell, I., 2007. Chi-squared and Fisher-Irwin tests of two-by-two tables with small
424 sample recommendations. Stat. Med. 26, 3661–3675.
425
426 Dobbelaere, D., Heussler, V., 1999. Transformation of leukocytes by *Theileria parva* and *T.*
427 *annulata*. Annu. Rev. Microbiol. 53, 1–42.
428
429 Eamens, G.J., Gonsalves, J.R., Jenkins, C., Collins, D., Bailey, G., 2013.
430 *Theileria orientalis* MPSP types in Australian cattle herds associated with outbreaks of
431 clinical disease and their association with clinical pathology findings. Vet. Parasitol. 191,
432 209–217.
433
434 Elsify, A., Sivakumar, T., Nayel, M., Salama, A., Elkhtam, A., Rizk, M., Mosaab, O., Sultan,
435 K., Elsayed, S., Igarashi, I., Yokoyama, N., 2015. An epidemiological survey of bovine
436 *Babesia* and *Theileria* parasites in cattle, buffaloes, and sheep in Egypt. Parasitol. Int. 64,
437 79–85.
438

439 Everitt, J.I., Shadduck, J.A., Steinkamp, C., Clabaugh, G., 1986. Experimental *Babesia bovis*
440 infection in Holstein calves. Vet. Pathol. 23, 556–562.
441
442 Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1992. Detection of *Babesia*
443 *bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30,
444 2576–2582.
445
446 Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1993. Multiplex polymerase
447 chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and
448 *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol. 50, 69–81.
449
450 García-Sanmartín, J., Nagore, D., García-Pérez, A.L., Juste, R.A., Hurtado, A., 2006.
451 Molecular diagnosis of *Theileria* and *Babesia* species infecting cattle in Northern Spain using
452 reverse line blot macroarrays. BMC. Vet. Res. 9, 2–16.
453
454 Glass, E.J., Preston, P.M., Springbett, A., Craigmile, S., Kirvar, E., Wilkie, G., Brown, C.G.,
455 2005. *Bos taurus* and *Bos indicus* (Sahiwal) calves respond differently to infection with
456 *Theileria annulata* and produce markedly different levels of acute phase proteins. Int. J.
457 Parasitol. 35, 337–347.
458
459 Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis.
460 Clin. Microbiol. Rev. 13, 451–69.
461
462 Hunfeld, K.P., Hildebrandt, A., Gray, J.S., 2008. Babesiosis: recent insights into an ancient
463 disease. Int. J. Parasitol. 38, 1219–1237.
464
465 Jorgensen, W.K., Weilgama, D.J., Navaratne, M., Dalgliesh, R.J., 1992. Prevalence of

Babesia bovis and *Anaplasma marginale* at selected localities in Sri Lanka. Trop. Anim. Health. Prod. 24, 9-14.

Kirvar, E., Ilhan, T., Katzer, F., Hooshmand-Rad, P., Zweygarth, E., Gerstenberg, C., Phipps, P., Brown, C.G.D., 2000. Detection of *Theileria annulata* in cattle and vector ticks by PCR using the Tams1 gene sequences. Parasitology 120, 245–254.

Liyanaarachchi, D.R., Rajakaruna, R.S., Dikkumbura, A.W., Rajapakse, R.P., 2015. Ticks infesting wild and domestic animals and humans of Sri Lanka with new host records. Acta. Trop. 142, 64–70.

MacHugh, N.D., Weir, W., Burrells, A., Lizundia, R., Graham, S.P., Taracha, E.L., Shiels, B. R., Langsley, G., Morrison, W.I., 2011. Extensive polymorphism and evidence of immune selection in a highly dominant antigen recognized by bovine CD8 T cells specific for *Theileria annulata*. Infect. Immun. 79, 2059–2069.

Mans, B.J., Pienaar, R., Latif, A.A., 2015. A review of *Theileria* diagnostics and epidemiology. Int. J. Parasitol. Parasites Wildl. 4, 104–118.

McFadden, A.M., Rawdon, T.G., Meyer, J., Makin, J., Clough, R.R., Tham, K., Mullner, P., Geysen, D., 2011. An outbreak of haemolytic anaemia associated with infection of *Theileria orientalis* in naive cattle. N. Z. Vet. J. 59, 79–85.

McKeever, D.J., 2009. Bovine immunity - a driver for diversity in *Theileria* parasites? Trends Parasitol. 25, 269–276.

Mosqueda, J., Olvera-Ramirez, A., Aguilar-Tipacamu, G., Canto, G.J., 2012. Current

493 advances in detection and treatment of babesiosis. Curr. Med. Chem. 19, 1504–1518.
494
495 Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren,
496 B., Bayarsaikhan, D., Byambaa, B., Terkawi, M. A., Yokoyama, N., Igarashi, I., 2013.
497 Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. Infect.
498 Genet. Evol. 16, 178–85.
499
500 Ota, N., Mizuno, D., Kuboki, N., Igarashi, I., Nakamura, Y., Yamashina, H., Hanzaike, T.,
501 Fujii, K., Onoe, S., Hata, H., Kondo, S., Matsui, S., Koga, M., Matsumoto, K., Inokuma H.,
502 Yokoyama N., 2009. Epidemiological survey of *Theileria orientalis* infection in grazing cattle
503 in the Eastern Part of Hokkaido, Japan. J. Vet. Med. Sci. 71, 937–944.
504
505 Oura, C.A., 2007. *Theileria parva* live vaccination: parasite transmission, persistence and
506 heterologous challenge in the field. Parasitology 134, 1205–1213.
507
508 Richardson, J.T., 2011. The analysis of 2 × 2 contingency tables--yet again. Stat. Med. 30,
509 890.
510
511 Ristic, M., 1981. Babesiosis. In: Ristic, M., MacIntyre, I. (Eds.), Diseases of cattle in the
512 tropics. Martinus Nijhof Publishers, The Hague, The Netherlands, pp. 443–468.
513
514 Shimizu, S., Nojiri, K., Matsunaga, N., Yamane, I., Minami, T., 2000. Reduction in tick
515 numbers (*Haemaphysalis longicornis*), mortality and incidence of *Theileria sergenti* infection
516 in field-grazed calves treated with flumethrin pour-on. Vet. Parasitol. 92, 129–138.
517
518 Sivakumar, T., Altangerel, K., Battsetseg, B., Battur, B., AbouLaila, M., Munkhjargal, T.,
519 Yoshinari, T., Yokoyama, N., Igarashi, I., 2012a. Genetic detection of *Babesia bigemina* from

520 Mongolian cattle using *apical membrane antigen-1* gene based PCR assay. Vet. Parasitol. 187,
521 17–22.
522
523 Sivakumar, T., Hayashida, K., Sugimoto, C., Yokoyama, N., 2014a. Evolution and genetic
524 diversity of *Theileria*. Infect. Genet. Evol. 27, 250–263.
525
526 Sivakumar, T., Kothalawala, H., Abeyratne, A.S., Vimalakumar, S.C., Meewawe, A.S.,
527 Hadirampela, D.T., Puvirajan, T., Sukumar, S., Kuleswarakumar, K., Chandrasiri, A.D.N.,
528 Igarashi, I., Yokoyama, N., 2012b. A PCR-based survey of selected *Babesia* and *Theileria*
529 parasites in cattle in Sri Lanka. Vet. Parasitol. 190, 263–267.
530
531 Sivakumar, T., Tagawa, M., Yoshinari, T., Ybanez, A.P., Igarashi, I., Ikehara, Y., Hata, H.,
532 Kondo, S., Matsumoto, K., Inokuma, H., Yokoyama, N., 2012c. PCR detection of *Babesia*
533 *ovata* from cattle reared in Japan and clinical significance of coinfection with *Theileria*
534 *orientalis*. J. Clin. Microbiol. 50, 2111–2113.
535
536 Sivakumar, T., Tattiyapong, M., Fukushi, S., Hayashida, K., Kothalawala, H., Silva, S.S.P,,
537 Vimalakumar, S.C., Kanagaratnam, R., Meewawe, A.S., Suthaharan, K., Puvirajan, T., de
538 Silva, W.K., Igarashi, I., Yokoyama, N., 2014b. Genetic characterization of *Babesia* and
539 *Theileria* parasites in water buffaloes in Sri Lanka. Vet. Parasitol. 200, 24–30.
540
541 Tattiyapong, M., Sivakumar, T., Takemae, H., Simking, P., Jittapalapong, S., Igarashi, I.,
542 Yokoyama, N., 2016. Genetic diversity and antigenicity variation of *Babesia bovis* merozoite
543 surface antigen-1 (MSA-1) in Thailand. Infect. Genet. Evol. 41, 255–261.
544
545 Terkawi M.A., Huyen, N.X., Shinuo, C., Inpankaew, T., Maklon, K., Aboulaila, M., Ueno, A.,
546 Goo, Y.K., Yokoyama, N., Jittapalapong, S., Xuan, X., Igarashi, I., 2011. Molecular and

547 serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the
548 northeast region of Thailand. Vet. Parasitol. 178, 201–207.
549
550 Uilenberg, G., 1995. International collaborative research: significance of tick-borne
551 hemoparasitic diseases to world animal health. Vet. Parasitol. 57, 19–41.
552
553 Weilgama, D.J., Jorgensen, W.K., Dalgliesh, R.J., Navaratne, M., Weerasinghe, C., 1989.
554 Comparison between Sri Lankan and Australian strains of *Babesia bovis* in the vaccination of
555 imported cattle in Sri Lanka. Trop. Anim. Health. Prod. 21,141–145.
556
557 Weilgama, D.J., Perera, P.S.G., Nanayakkara, E., Nambuge, D., 1986. Observations on
558 theileriosis among cattle and buffaloes in Sri Lanka. Sri Lanka Vet. J. 34, 64–75.
559
560 Weir, W., Karagenc, T., Baird, M., Tait, A., Shiels, B.R., 2010. Evolution and diversity of
561 secretome genes in the apicomplexan parasite *Theileria annulata*. BMC. Genomics. 11, 42.
562
563 Wilson, E.B., 1927. Probable inference, the law of succession, and statistical inference. J. Am.
564 Statist. Assoc. 22, 209–212.
565
566 Wright, I.G., Goodger, B.V., 1988. Pathogenesis of babesiosis. In: Babesiosis of domestic
567 animals and man (ed. Ristic, M.), pp. 99–118. Boca Raton, Florida, CRC Press, Inc.
568
569 Ybañez, A.P., Sivakumar, T., Ybañez, R.H., Vincoy, M.R., Tingson, J.A., Perez, Z.O.,
570 Gabotero, S.R., Buchorno, L.P., Inoue, N., Matsumoto, K., Inokuma, H., Yokoyama, N., 2013.
571 Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. Vet. Parasitol. 196,

572 13–20.

573

574 **FIGURE LEGENDS**

575

576 **Fig. 1.** The PCR detection of *Babesia* and *Theileria* parasites at the four sampling time points.
577 The rates of positivity for each parasite species are expressed as the percentage of the total
578 number of animals sampled in the P district (Polonnaruwa, n=75) or the NE district (Nuwara
579 Eliya, n=161). The first, second, third, and fourth samplings are indicated by 1, 2, 3, and 4,
580 respectively. "T" indicates the percentage of animals that were infected with each parasite
581 species on at least one sampling time point during the study period. The error bars represent
582 the 95% confidence intervals. Statistically significant increases and decreases in the positive
583 rates in comparison to previous samples are indicated by single and double asterisks,
584 respectively.

585

586 **Fig. 2.** The persistence of *Babesia* and *Theileria* infections. The persistence of *B. bovis*, *B.*
587 *bigemina*, *T. annulata*, and *T. orientalis* infections is expressed as the percentage of cattle that
588 were PCR-positive for each of the parasite species at least once during the study period in the
589 R (Polonnaruwa) and NE (Nuwara Eliya) districts. Note that persistence of *T. annulata* and *T.*
590 *orientalis* infections was more pronounced than the persistence of *B. bovis* and *B. bigemina*
591 infections in the P district, and that the rate of persistent *T. orientalis* infections in the cattle
592 was higher than rates of persistent *B. bovis*, *B. bigemina*, and *T. annulata* infections in the NE
593 district.

Fig. 3. The serological detection of *B*. *bovis* and *B*. *bigemina* at the four sampling time points. The rates of positivity for each parasite species are expressed as a percentage of the total number of animals sampled in the P district (Polonnaruwa, n = 75) or the NE district (Nuwara Eliya, n = 161). The first, second, third, and fourth samplings are indicated by 1, 2, 3, and 4, respectively. "T" indicates the rate of animals that were infected with *B*. *bovis* or *B*. *bigemina* on at least one sampling time point during the study period. Statistically significant increases and decreases in the positive rates in comparison to previous samples are indicated by single and double asterisks, respectively.

Fig. S1. Co-infection with the *Babesia* and *Theileria* species. Venn diagrams are used to illustrate the numbers of animals that were co-infected with *B*. *bovis* (*Bbo*), *B*. *bigemina* (*Bbi*), *T*. *annulata* (*Ta*), and *T*. *orientalis* (*To*). The numbers in the overlapping areas indicate the numbers of animals that were co-infected with the relevant species of *Babesia* and/or *Theileria*.

Table 1. New episodes of *Babesia* and *Theileria* infections on 2nd, 3rd, and 4th sampling occasions, as determined by PCR assays

Districts	Parasite	2014/9		2014/12		2015/3	
		No. New infections[a]	%[b] (CI[c])	No. New infections	% (CI)	No. New infections	% (CI)
Polonnaruwa (P)	*B. bovis*	3	4.1 (1.4–11.4)	0	0	2	2.7 (0.7–9.2)
	B. bigemina	19	32.8 (22.1–45.6)	4	8.3[d] (3.3–19.6)	16	26.2[e] (16.8–38.4)
	T. annulata[f]	23	53.5 (38.9–67.5)	20	71.4 (52.9–84.8)	12	80.0 (54.8–93.0)
	T. orientalis	2	7.1 (2.0–22.6)	5	15.2 (6.7–30.9)	6	19.4 (9.2–36.3)
Nuwara Eliya (NE)	*B. bovis*	0	0	1	0.6 (0.1–3.4)	0	0
	B. bigemina	26	16.5 (11.5–23.0)	1	0.7[d] (0.1–4.1)	2	1.3 (0.3–4.4)
	T. annulata	3	1.9 (0.7–5.6)	2	1.3 (0.3–4.5)	7	4.4 (2.1–8.8)
	T. orientalis[g]	35	46.7 (35.8–57.8)	22	44.0 (31.2–57.7)	25	56.8 (42.2–70.3)

[a] New infections for a given parasite species at 2nd, 3rd, and 4th sampling are defined as infections among animals that were infection-negative at 1st, 2nd, and 3rd sampling occasions, respectively.

[b] New infection rate for a given parasite species is expressed as a percentage of the number of negative animals on the previous sampling occasion.

[c] 95% confidence interval.

[d] New infection rate lower than previous sampling occasion.

[e] New infection rate higher than previous sampling occasion.

[f] New infection rates for *T. annulata* were higher than those for *B. bovis, B. bigemina*, and *T. orientalis* in all four sampling occasions in district P.

[g] New infection rates for *T. orientalis* were higher than those for *B. bovis, B. bigemina*, and *T. annulata* in all four sampling occasions in district NE.

Table 2. Co-infections in cattle as determined by PCR assays

Combination	Polonnaruwa (P)				Nuwara Eliya (NE)			
	No. Positive (%[a])			No. Positive (%)				
	2014/6	2014/9	2014/12	2015/3	2014/6	2014/9	2014/12	2015/3
4 parasites								
B. bovis + *B. bigemina* + *T. annulata* + *T. orientalis*	0	1 (2.2)	0	0	0	0	0	0
3 parasites								
B. bigemina + *T. annulata* + *T. orientalis*	3 (9.7)	6 (13.3)	8 (18.6)	7 (13.2)	1 (9.1)	0	0	1 (12.5)
B. bovis + *B. bigemina* + *T. annulata*	0	1 (2.2)	0	1 (1.9)	0	0	0	0
B. bovis + *T. annulata* + *T. orientalis*	1 (3.2)	0	0	1 (1.9)	0	0	0	0
2 parasites								
T. annulata + *T. orientalis*	19 (61.3)	19 (42.2)	29 (67.4)	32 (60.4)	5 (45.5)	2 (12.5)	2 (66.7)	6 (75.0)
B. bigemina + *T. annulata*	4 (12.9)	12 (26.7)	4 (9.3)	12 (22.6)	1 (9.1)	0	0	0
B. bigemina + *T. orientalis*	3 (9.7)	5 (11.1)	2 (4.7)	0	1 (9.1)	14 (87.5)	1 (33.3)	1 (12.5)
B. bovis + *T. orientalis*	1 (3.2)	0	0	0	3 (27.3)	0	0	0
B. bovis + *T. annulata*	0	1 (2.2)	0	0	0	0	0	0
Total (%[b])	31 (49.2)	45 (69.2)	43 (64.2)	53 (77.9)	11 (12.0)	16 (12.9)	3 (2.5)	8 (6.3)

[a] Expressed as a percentage of the total number of co-infected animals.
[b] Expressed as a percentage of the number of animals positive for at least one parasite species.

Table 3. New infectious episodes with *B. bovis* and *B. bigemina* at 2nd, 3rd, and 4th sampling, as determined by ELISAs

Districts	Parasite	2014/9		2014/12		2015/3	
		No. New infections[a]	%[b] (CI[c])	No. New infections	% (CI)	No. New infections	% (CI)
Polonnaruwa (P)	*B. bovis*	17	37.8 (25.1–52.4)	7	20.6 (10.4–36.8)	9	28.1 (15.6–45.4)
	B. bigemina	5	83.3 (43.7–97.0)	0	0	0	
Nuwara Eliya (NE)	*B. bovis*	20	29.9 (20.2–41.7)	22	36.7 (25.6–49.3)	13	20.1 (14.6–37.0)
	B. bigemina	12	63.2 (41.0–80.9)	5	62.5 (30.6–86.3)	2	33.3 (9.7–70.0)

[a] New infections for a given parasite species at 2nd, 3rd, and 4th sampling is defined as infections among animals that were sero-negative at 1st, 2nd, and 3rd sampling, respectively.
[b] New infection rate for a given parasite species is expressed as a percentage of the number of sero-negative animals on the previous sampling occasion.
[c] 95% confidence interval.

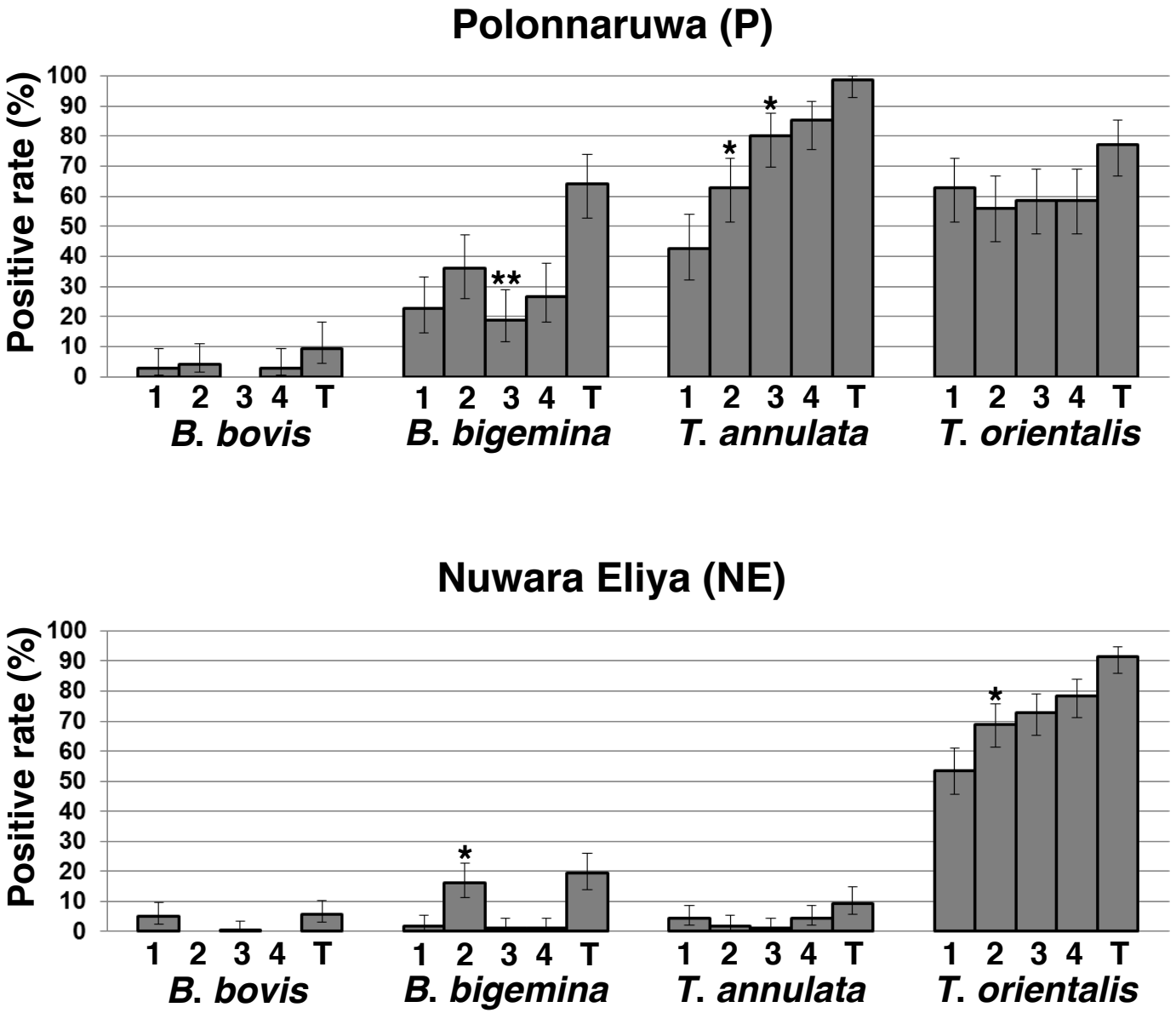
Polonnaruwa (P)
Positive rate (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
*
1 2 3 4 T
B. bovis
B. bigemina
T. annulata
T. orientalis
Nuwara Eliya (NE)
Positive rate (%)
*
*
1 2 3 4 T
B. bovis
B. bigemina
T. annulata
T. orientalis

Fig. 1

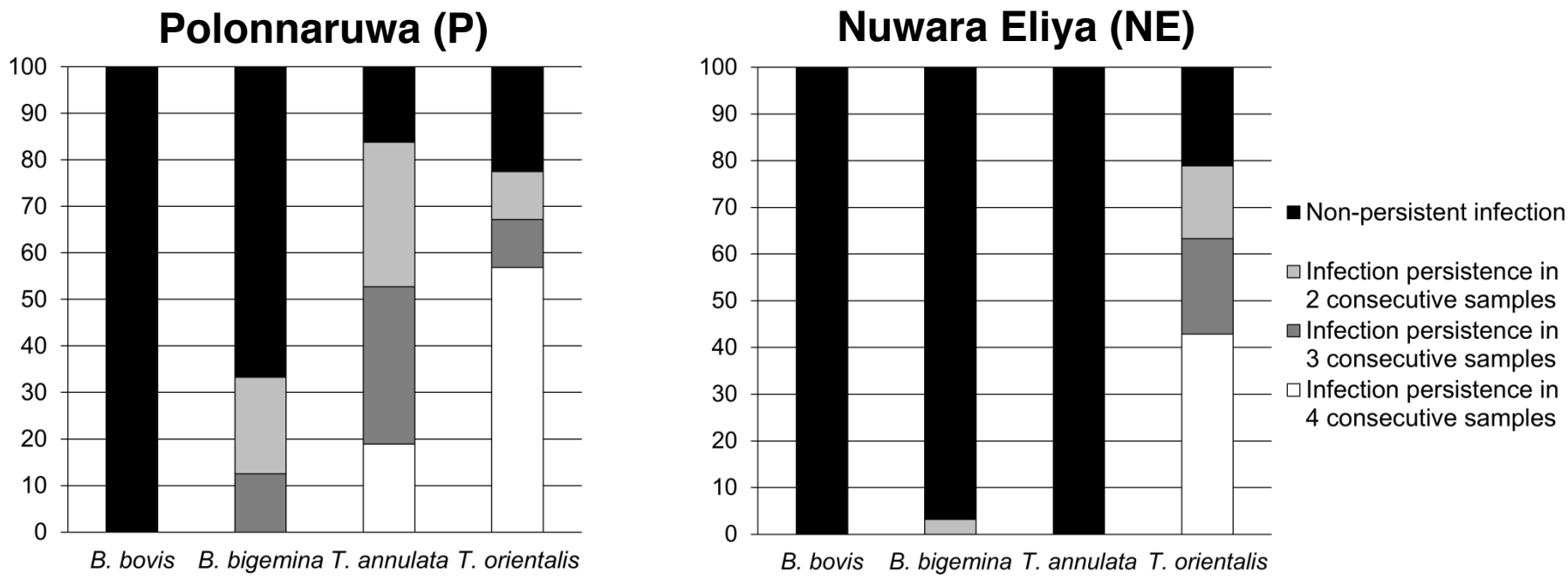
Polonnaruwa (P)
Nuwara Eliya (NE)
100
90
80
70
60
50
40
30
20
10
0
B. bovis
B. bigemina
T. annulata
T. orientalis
Non-persistent infection
Infection persistence in 2 consecutive samples
Infection persistence in 3 consecutive samples
Infection persistence in 4 consecutive samples

Fig. 2

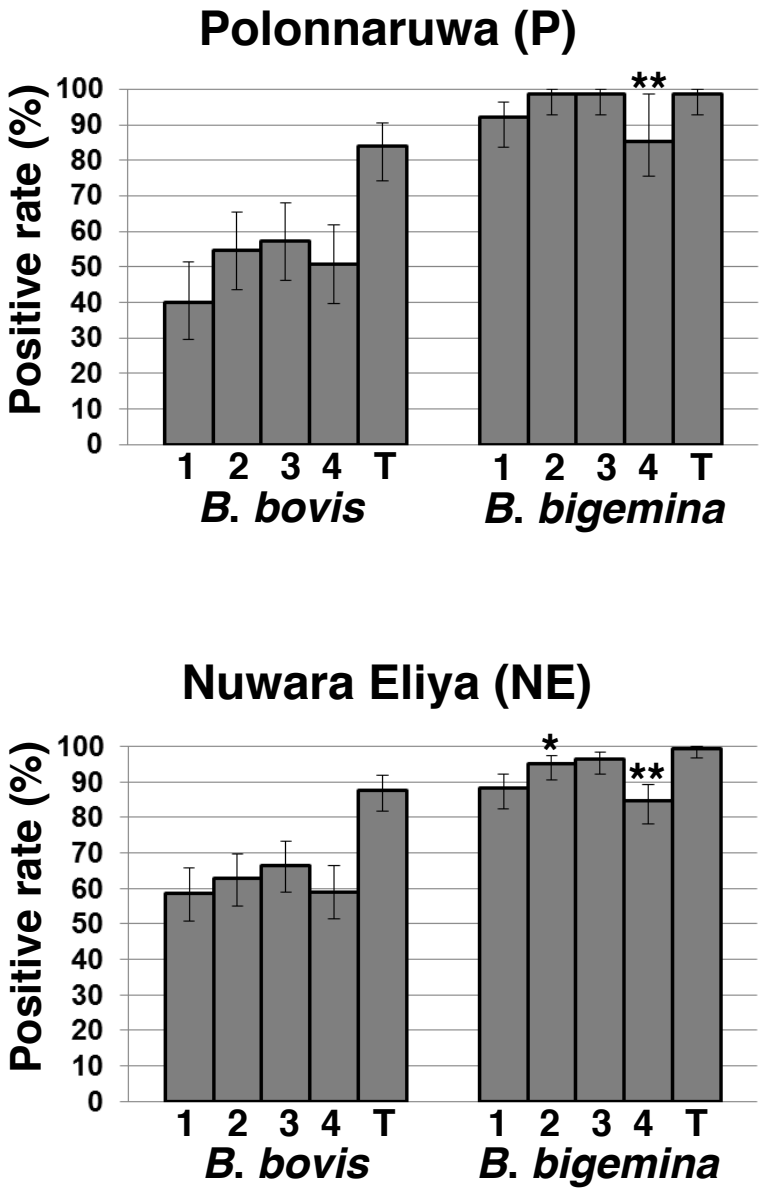
Polonnaruwa (P)
Positive rate (%)
100
90
80
70
60
50
40
30
20
10
0
**
1 2 3 4 T
B. bovis
1 2 3 4 T
B. bigemina
Nuwara Eliya (NE)
Positive rate (%)
*
**
1 2 3 4 T
B. bovis
1 2 3 4 T
B. bigemina

Fig. 3