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1           **Tick-borne haemoparasites and Anaplasmatataceae in domestic dogs in Zambia**

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42

43 **Keywords**

44 Anaplasmataceae; *Babesia*; dog; *Hepatozoon*; Multiple PCR; Zambia

45 **Abstract**

46 Tick-borne diseases (TBDs), including emerging and re-emerging infectious diseases,  
47 are important threats to human and animal health worldwide. Indeed, the number of reported  
48 human and animal infectious cases of novel TBD agents has increased in recent decades.  
49 However, TBDs tend to be neglected, especially in resource-limited countries that often have  
50 limited diagnostic capacity. The aim of this molecular survey was to detect and characterise tick-  
51 borne pathogens (*Babesia*, *Theileria*, and *Hepatozoon* parasites and Anaplasmataceae bacteria)  
52 in domestic dogs in Zambia. In total, 247 canine peripheral blood samples were collected in  
53 Shangombo, Mazabuka, Lusaka, and Monze. Conventional PCR to detect the selected pathogens  
54 was performed using DNA extracted from canine blood. One hundred eleven samples were  
55 positive for protozoa and 5 were positive for Anaplasmataceae. Sequencing of thirty-five  
56 randomly selected protozoan-positive samples revealed the presence of *Babesia rossi*, *Babesia*  
57 *vogeli*, and *Hepatozoon canis* 18S rDNA. Based on these sequences, a multiplex PCR system  
58 was developed to yield PCR products with different amplicons, the size of which depended on  
59 the parasite species; thus, each species could be identified without the need for sequence analysis.  
60 Approximately 40% of dogs were positive for *H. canis*. In particular, the positive rate (75.2%) of  
61 *H. canis* infection was significantly higher in Shangombo than in other sampling sites. Multiplex  
62 PCR assay detected *B. rossi* and *B. vogeli* infections in five and seven dogs, respectively,

63 indicating that this approach is useful for detecting parasites with low prevalence. Sequencing  
64 analysis of *gltA* and *groEL* genes revealed that two and one dogs in Lusaka were infected with *A.*  
65 *platys* and *E. canis*, respectively. The data indicated that Zambian dogs were infected with  
66 multiple tick-borne pathogens such as *H. canis*, *B. rossi*, *B. vogeli*, *A. platys*, *E. canis* and  
67 uncharacterized *Ehrlichia* sp. Since some of these parasites are zoonotic, concerted efforts are  
68 needed to raise awareness of, and control, these tick-borne pathogens.

69

## 70 **Introduction**

71 Ticks are the second most common blood sucking arthropods next to mosquitoes. They  
72 not only cause anaemia in their animal hosts, but also carry and transmit a wide variety of viruses,  
73 bacteria, and protozoa, some of which cause tick-borne diseases (TBDs) (de la Fuente et al.,  
74 2008; Otranto et al., 2014). These TBDs not only include multiple existing infectious diseases,  
75 but also comprise emerging and re-emerging infectious diseases. An example of one such  
76 emerging TBD is severe fever with thrombocytopenia syndrome, which was reported to be  
77 endemic to China in 2011 and which poses serious threats to human and animal health (Parola et  
78 al., 2005; Yu et al., 2011). Moreover, in the past two decades, the number of reported cases of  
79 infection with novel TBDs in humans and animals has increased (Kernif et al., 2016).

80           Given that some TBDs in humans are zoonoses, it is important to identify the tick-borne  
81 pathogens in pets, livestock, and wild animals and elucidate the factors that determine their  
82 prevalence. The most common tick-borne protozoan pathogens of dogs are *Babesia* and  
83 *Hepatozoon* (Homer et al., 2000; Baneth et al., 2003). These haemoparasites live in mammalian  
84 blood cells and cause severe diseases and sometimes death in infected animals (Schnittger et al.,  
85 2012). Specifically, *Babesia gibsoni*, *Babesia canis*, *Babesia rossi*, and *Babesia vogeli* are  
86 causative agents of canine babesiosis (biliary fever). *B. gibsoni* is distributed in Asia, North  
87 America, Europe, and northern and eastern Africa (Farwell et al., 1982; Jefferies et al., 2003;  
88 Lobetti, 1998). *B. canis* is transmitted by *Dermacentor reticulatus*, which is prevalent in Europe  
89 (Solano-Gallego and Baneth, 2011). The other two species, *B. rossi* and *B. vogeli*, are mainly  
90 transmitted by *Haemaphysalis leachi* and *Rhipicephalus sanguineus* sensu lato (s.l.), respectively  
91 (Apanaskevich et al., 2007; Criado-Fornelio et al., 2003). The distribution of *B. rossi* is restricted  
92 to sub-Saharan Africa, while that of *B. vogeli* is worldwide (Europe, Africa, Asia, South and  
93 North America) (Oyamada et al., 2005; Matjila et al., 2008). *Hepatozoon canis* and *Hepatozoon*  
94 *americanum* are the agents of canine hepatozoonoses that range from being asymptomatic with  
95 low levels of parasitaemia to a severe life-threatening illness characterised by high levels of  
96 parasitaemia, fever, anaemia, and lethargy (Baneth et al., 2000; Baneth et al., 2003). These two  
97 *Hepatozoon* species are genetically and geographically distinct (Baneth et al., 2000). *H. canis* is

98 distributed in Africa, southern Europe, the Middle East, and Asia (Baneth, 2006) and is mainly  
99 transmitted by *R. sanguineus* s.l. and *Haemaphysalis longicornis* (Dantas-Torres et al., 2012;  
100 Murata et al., 1995). *H. americanum* is found in the Americas and is transmitted by *Amblyomma*  
101 *maculatum* (Mathew et al., 1998). Recently, new *Hepatozoon* spp. were reported in dogs and  
102 wildlife in Turkey and Brazil (Aydin et al., 2015; Soares et al., 2017).

103         *Anaplasma* and *Ehrlichia* species are obligate intracellular bacteria that belong to the  
104 family of Anaplasmataceae. These tick-borne pathogens infect humans and animals all over the  
105 world. *Anaplasma platys* is primarily found in dogs with cyclic thrombocytopaenia (Harvey et al.,  
106 1978). In addition, new *Anaplasma* species that are closely related to *A. phagocytophilum*, which  
107 causes human granulocytic anaplasmosis, have been detected in canine blood (Inokuma et al.,  
108 2005). *Ehrlichia canis* is the causative agent of canine ehrlichiosis, which is transmitted by *R.*  
109 *sanguineus* s.l. (Groves et al., 1975; Aguiar et al., 2007). While *E. canis* was initially thought to  
110 be pathogenic in canines only, it was eventually also detected in human patients with the typical  
111 clinical findings of ehrlichiosis (Perez et al., 2006).

112         Only a few studies have examined haemoparasites and Anaplasmataceae in domestic  
113 and wild dogs in Zambia. Baba et al. (2012) described the case of a dog that was exported from  
114 Zambia to Japan and was infected with *E. canis*, while Nalubamba et al. (2011) showed that of



115 1,196 samples from domestic dogs in Lusaka, only 2.4% were positive for *Babesia* parasites.  
116 This is supported by Williams et al. (2014), whose molecular survey on 11 wild and eight  
117 domestic dogs in the Eastern and Western Provinces of Zambia showed that all were negative for  
118 *Babesia* infection. However, they did find that 65% of wild dogs and 13% of domestic dogs were  
119 infected with *Hepatozoon*. Recently, Vlahakis et al. (2017) described the first molecular  
120 evidence of *A. platys* in domestic dogs in Lusaka.

121 To better understand the infection status and distribution of tick-borne pathogens in  
122 Zambian domestic dogs, we subjected blood samples from 247 domestic dogs living in four  
123 districts of Zambia to our newly developed multiplex PCR assay, which differentiates between  
124 the main tick-borne canine haemoparasites. This molecular survey showed that some Zambian  
125 dogs are infected with *Anaplasma*, *Ehrlichia*, *Babesia*, and especially *Hepatozoon*.

126

## 127 **Materials and methods**

### 128 **Ethics**

129 All procedures were performed in accordance with the guidelines established by the  
130 Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido  
131 University (Sapporo, Japan).

## 132 **Dogs**

133 From January to May 2016, 247 peripheral blood samples were collected from privately  
134 owned dogs (149 male and 98 female) in four different locations in Zambia (Figure 1): Lusaka  
135 (15.23°S, 28.19°E) (n = 50), Mazabuka (15.51°S, 27.44°E) (n = 50), Monze (16.16°S, 27.28°E)  
136 (n = 50), and Shangombo (16.19°S, 22.06°E) (n = 97). Lusaka, Mazabuka and Monza are  
137 relatively urbanized cities/towns, while Shangombo is located in a rural area close to the border  
138 with Angola. The sampling was conducted on the randomly selected dogs, which participated in  
139 the rabies vaccination campaign. The age of the dogs ranged from three months to fifteen years  
140 with averages of thirty-nine, thirty-nine, thirty, and twenty-seven months in Lusaka, Mazabuka,  
141 Monze, and Shangombo, respectively. DNA was extracted from 200 µl of EDTA-anticoagulated  
142 whole blood using DNAzol BD Reagent (Invitrogen, Massachusetts, USA) or a QIAamp DNA  
143 Blood Mini kit (Qiagen, Tokyo, Japan) and stored at -20°C until used.

## 144 **PCR**

145           PCRs were performed by using the primers listed in Table 1. *Babesia*, *Theileria*, and/or  
146 *Hepatozoon* parasites were detected by nested PCR that amplifies a 1.4–1.6 kb fragment of the  
147 parasite’s 18S rDNA: BTH 18S 1st F and BTH 18S 1st R were used for primary amplification  
148 while BTH 18S 2nd F and BTH 18S 2nd R were used for secondary amplification, as described  
149 previously (Masatani et al., 2017). Members of the Anaplasmataceae family were firstly detected  
150 using EHR16SD and EHR16SR, which amplify a 345-bp fragment of 16S ribosomal DNA  
151 (rDNA) from these bacteria (Parola et al., 2000). The positive samples were further characterized  
152 by additional PCRs targeting citrate synthase (*gltA*) and heat-shock protein (*groEL*) genes of  
153 Anaplasmataceae. All PCR reactions were conducted in a 25  $\mu$ l-reaction mixture containing 12.5  
154  $\mu$ l of 2  $\times$  Gflex PCR Buffer ( $Mg^{2+}$ , dNTP plus) (TaKaRa Bio Inc., Shiga, Japan), 0.5  $\mu$ l of Tks  
155 Gflex DNA Polymerase (1.25 units/ $\mu$ l) (TaKaRa Bio Inc.), 200 nM of each primer, and 1.0  $\mu$ l of  
156 template DNA or 5-fold diluted first PCR product. The reaction conditions were 95°C for 3 min  
157 and 40 cycles of 95°C for 30 s, annealing temperature for 30 s (Table 1), and 68°C for 30 s  
158 (PCRs for Anaplasmataceae) or 90 s (PCR for *Babesia*, *Theileria*, and/or *Hepatozoon*), followed  
159 by a final extension at 68°C for 5 min. The PCR products were subjected to electrophoresis in a  
160 1.2% agarose gel stained with Gel-Red<sup>TM</sup> (Biotium, Hayward, CA).

## 161 **Multiplex PCR**

162           The conventional nested PCR yielded many positive samples. To reduce the need for  
163 cost- and time-intensive sequencing, we first sequenced 35 randomly selected nested PCR-  
164 positive blood samples. Since we detected *B. rossi*, *B. vogeli*, and *H. canis*, a multiplex PCR that  
165 differentiated between these species was developed. Thus, the 18S rDNA sequences of the 35  
166 samples were aligned and three forward primers that were specific for each parasite (Br\_18S\_F,  
167 Bv\_18S\_F, and Hc\_18S\_F) and a reverse primer that recognized the same sequence in all three  
168 parasites (BTH\_multi\_R) were designed (Table 1). The amplified products for *B. rossi*, *B. vogeli*,  
169 and *H. canis* comprise 522 bp, 1024 bp, and 360 bp, respectively. The multiplex PCR was  
170 conducted by using the Multiplex PCR Assay Kit (TaKaRa Bio Inc.). A 25 µl-reaction mixture  
171 that consisted of 12.5 µl of Multiplex PCR Mix 2, 0.125 µl of Multiplex PCR Mix 1, 200 nM of  
172 the four primers (Br\_18S\_F, Bv\_18S\_F, Hc\_18S\_F, and BTH\_multi\_R), and 1.0 µl of 5-fold  
173 diluted product of the PCR using the BTH 18S 1st F and BTH 18S 1st R primers was generated.  
174 The reaction conditions were 94°C for 1 min and 40 cycles of 94°C for 30 s, 57°C for 30 s, and  
175 72°C for 60 s, followed by a final extension at 72°C for 10 min. The parasite species were  
176 identified according to the size of the PCR amplicon in an agarose gel electrophoresis gel.

## 177 **Cloning**

178 To analyse parasite sequences present in samples infected with multiple parasites,  
179 parasite 18S rDNA was amplified using a high-fidelity PCR enzyme, KOD-Plus-Ver.-2 DNA  
180 polymerase (Toyobo, Osaka, Japan), in 25 µl of reaction mixture containing 2.5 µl of 10× Buffer  
181 for KOD-Plus-Neo, 300 nM of each primer, 200 µM dNTPs, 1 mM MgSO<sub>4</sub>, 0.5 unit of KOD-  
182 Plus-Neo DNA polymerase, and 1.0 µl of template DNA or diluted (5×) first-round PCR product.  
183 The reaction conditions were 94°C for 2 min, followed by 40 cycles of 98°C for 10 s, 55°C for  
184 30 s, and 68°C for 30 s, followed by a final extension at 68°C for 2 min. The second-round PCR  
185 product was A-tailed using 10× A-attachment Mix (Toyobo) and then cloned into a T-vector  
186 pMD20 (TaKaRa Bio Inc.).

## 187 **Sequencing**

188 The PCR products were purified by using a NucleoSpin Gel and PCR Clean Up Kit  
189 (Takara Bio Inc.) and sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit  
190 (Applied Biosystems, Foster City, CA, USA) utilising an ABI Prism 3130x genetic analyser  
191 according to the manufacturers' instructions. Only the sequences that were recovered from more  
192 than three clones were considered to be genuine. The sequences that were obtained were  
193 submitted to the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>) under accession numbers  
194 LC331056–LC331057 (18S rDNA of *B. rossi*), LC331058 (18S rDNA of *B. vogeli*), LC331059–

195 LC331061 (18S rDNA of *H. canis*), LC331059–LC331061 (16S rDNA of Anaplasmataceae),  
196 LC373037 and LC373038 (*gltA* of Anaplasmataceae), and LC373039–LC373041 (*groEL* of  
197 Anaplasmataceae).

## 198 **Sequence data analysis**

199 The sequences were analysed by using GENETYX version 9.1 (GENETYX  
200 Corporation, Tokyo, Japan) and were trimmed on both the 5' and 3' ends. The obtained  
201 sequences were compared with those available in public databases using nucleotide BLASTn at  
202 the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was conducted  
203 by using MEGA version 6.05 (Tamura et al., 2013). ClustalW was used to align the sequences to  
204 closely related organism sequences that were deposited in the database. A neighbour-joining  
205 method was used to perform the phylogenetic analysis. Bootstrap values were obtained with  
206 1,000 replicates.

## 207 **Statistical analysis**

208 Dogs from different regions were compared in terms of frequency of infection with  
209 specific parasites by using Fisher's exact test. For this, the Fmsb package in R 3.4.0 (R Core  
210 Team 2017) was employed. *P* values of <0.05 were considered to indicate statistical significance.

211

## 212 **Results**

### 213 **Detection of protozoan parasites**

214           Nested PCR targeting *Babesia*, *Theileria*, and *Hepatozoon* parasites was positive in 111  
215 dogs: fourteen (28.0%) in Lusaka, eight (16.0%) in Mazurka, sixteen (32.0%) in Monze, and  
216 seventy-three (75.2%) in Shangombo (Table 2). Initially, thirty-five samples were randomly  
217 selected and the second-round PCR products from these samples were subjected to sequence  
218 analysis. The resulting data indicated the presence of *B. rossi*, *B. vogeli*, and *H. canis*. To  
219 distinguish between these three parasites, we developed a multiplex PCR assay based on species-  
220 specific primers (Table 1). Since the PCR band of each parasite differs in size, the species can be  
221 identified without having to perform sequence analysis. A representative electrophoresis of the  
222 multiplex PCR products is shown in Figure 2.

223           Multiplex PCR assay of all 111 BTH-PCR-positive samples showed that five dogs were  
224 infected with *B. rossi*. Four dogs were from Mazabuka and one from Shangombo. None of the  
225 samples from Lusaka and Monze was positive for *B. rossi*. Thus, the *B. rossi* infection rates in  
226 Lusaka, Mazabuka, Monze, and Shangombo were 0%, 8.0%, 0%, and 1.0%, respectively.

227           The multiplex PCR also showed that seven samples were positive for *B. vogeli*. One, four,  
228 and two *B. vogeli* infections were found in dogs from Lusaka, Mazabuka, and Monze,  
229 respectively. None of the samples from Shangombo were positive for *B. vogeli*. Thus, the *B.*  
230 *vogeli* infection rates in Lusaka, Mazabuka, Monze, and Shangombo were 2.0%, 8.0%, 4.0%,  
231 and 0%, respectively.

232           The multiplex PCR also showed that 100 dogs were infected with *H. canis* (Table 2).  
233 None of the samples from Mazabuka were positive for *H. canis*. The positive rate of *H. canis* in  
234 Shangombo (75.2%) was significantly higher than that in Lusaka (26.0%) or Monze (28.0%)  
235 (both  $p < 0.05$ ). One dog from Shangombo was infected with both *H. canis* and *B. rossi*. The  
236 presence of the two parasites was confirmed by cloning and sequencing of the 18S rDNA PCR  
237 products (data not shown).

238           Sequencing analysis of the 18S rDNA PCR products of the five *B. rossi* infections  
239 yielded two sequence types. One (BR1) showed 99.9% (1,428/1,430 bp) nucleotide identity with  
240 *B. rossi* detected in black-backed jackals (*Canis mesomelas*) in South Africa (GenBank  
241 accession no.: KY463434). The other (BR2) showed 100% (1430/1430 bp) nucleotide identity  
242 with *B. rossi* from domestic dogs in Nigeria (GenBank accession no.: AB935165).



243 Sequencing analysis of the 18S rDNA PCR products of the seven *B. vogeli* infections  
244 yielded a single sequence type (BV1) that bore 100% (1429/1429 bp) nucleotide identity with *B.*  
245 *vogeli* detected in China, Japan, and Brazil (GenBank accession nos.: HM590440, AY077719,  
246 and AY371196, respectively).

247 Sequence analysis of the 18S rDNA PCR products from *H. canis* infections revealed four  
248 different sequence types. The most common type (HC1, 77.3% of all sequenced *H. canis*-  
249 positive samples) harboured two base pair mismatches (1,528/1,530 bp identity) with respect to  
250 *H. canis* sequences reported in dogs in Sudan (GenBank accession no.: DQ111754). Two other  
251 sequences (HC2 and HC3) exhibited 99.7% (1,526/1,530 bp) and 99.9% (1,529/1,530 bp)  
252 nucleotide identity with *H. canis* from a golden jackal (*Canis aureus*) in Eurasia (GenBank  
253 accession no.: KX712126 and KX712127).

254 The sequence types detected in this study are summarized in Table 3. Phylogenetic  
255 analysis showed that sequence types HC1–HC4 were located in the same cluster with *H. canis*,  
256 that sequence types BR1 and BR2 clustered together with *B. rossi*, and that the BV1 sequence  
257 type was located in a cluster of *B. vogeli* (Figure 3).

## 258 **Detection of Anaplasmatataceae**

259           Of the 247 dogs, five (three from Lusaka and two from Shangombo) tested positive for  
260 Anaplasmatataceae by 16S rDNA PCR (Table 2). Sequencing analysis of the amplified products  
261 from these five dogs identified three different sequences (EHR1-3). EHR1 was detected in a dog  
262 from Lusaka (LuF11) and exhibited 98.4% (300/305 bp) nucleotide identity with *E. canis* and  
263 several uncharacterized *Ehrlichia* spp. EHR2 was detected in the two infected dogs from  
264 Shangombo (ZD#55 and ZD#56) and showed 99.7% (304/305 bp) nucleotide identity with  
265 several uncharacterized *Ehrlichia* spp. EHR3 was detected in two dogs from Lusaka (LuF9 and  
266 LuF24) and showed 100% (305/305 bp) nucleotide identity with the *A. platys* sequence found in  
267 *R. sanguineus* s.l. ticks that had been collected from dogs in the Democratic Republic of the  
268 Congo (DRC) (GenBank accession no.: AF478131).

269           In order to further characterize Anaplasmatataceae, PCRs amplifying *gltA* and *groEL* were  
270 carried out using several primer sets shown in Table 1. Three samples (LuF9, LuF24, and  
271 LuF11) were successfully amplified, while two samples (ZD#55 and ZD#56) having identical  
272 16S rDNA sequence were not amplified.

273           Sequencing analysis of the amplified products identified two and three different  
274 sequences of *gltA* and *groEL*, respectively. One *gltA* sequence (GLTA1) detected from LuF11  
275 showed 100% (1,033/1,033 bp) identity with *E. canis* strain YZ-1 reported from Yangzhou,

276 China (GenBank accession no.: CP025749). The other *gltA* sequence (GLTA2) detected from  
277 LuF9 and LuF24 showed 99.7% (1,048/1,051 bp) identity with *A. platys* found in *R. sanguineus*  
278 s.l. collected from a dog in the DRC (GenBank accession no.: AF478130). Out of three *groEL*  
279 sequences (GROEL1-3), GROEL1 detected from LuF11 showed 100% (1,060/1,060 bp) identity  
280 with *E. canis* strain YZ-1. GROEL2 detected from LuF9 and GROEL3 detected from LuF24  
281 showed 99.7% (1,072/1,075 bp) and 99.8% (1,073/1,075 bp) identities with *A. platys* found in *R.*  
282 *sanguineus* s.l. ticks collected from a dog in the DRC (GenBank accession no.: AF478129),  
283 respectively.

284 Phylogenetic trees based on *gltA* and *groEL* genes are shown in Figures 4A and 4B,  
285 respectively. The sequences obtained from LuF11 were clustered with *E. canis* in both trees. The  
286 sequences obtained from LuF9 and LuF24 were located in the same cluster with *A. platys* in both  
287 trees.

288

## 289 **Discussion**

290 The aim of this study was to detect and characterise haemoparasites and  
291 Anaplasmatataceae in domestic dogs in Zambia, where TBDs tend to be overlooked due to limited  
292 diagnostic capacity. Here, we provide molecular evidence showing that Zambian domestic dogs

293 are infected with multiple tick-borne pathogens, namely, *H. canis*, *B. rossi*, and *B. vogeli*, *A.*  
294 *platys*, *E. canis* and uncharacterized *Ehrlichia* sp.

295 We detected *H. canis* infections in three of the four sampled locations (Mazabuka was  
296 the exception) (Table 2). The dogs from Monze and Lusaka have similar frequencies of *H. canis*  
297 infection (28.0% and 26.0%, respectively). By contrast, three-quarters of the dogs from  
298 Shangombo were infected with *H. canis*. This is remarkable given that other studies of dogs in  
299 Zambia, Angola, and Sudan showed 12.6%, 17.5% and 42.3% *H. canis*-positivity rates,  
300 respectively (Williams et al., 2014; Cardoso et al., 2016; Oyamada et al., 2005). It may be that  
301 there are more opportunities for dogs to be exposed to ticks in Shangombo for example due to  
302 the differences in dog management styles between urban and rural areas in Zambia. Alternatively,  
303 or in addition, the ticks in Shangombo may be more susceptible to infection with *H. canis* than  
304 the ticks in other districts. It should be mentioned that there was no significant difference in age  
305 between *H. canis*-infected and non-infected dogs in Shangombo (the average age were 26.7 and  
306 29.5 months, respectively) (Mann-Whitney U test,  $P = 0.77$ ). Future analyses of the involved tick  
307 vectors in Shangombo will help to understand the high endemicity of *H. canis* in this area.

308 Given that we found a large number of canine blood samples were positive for the  
309 nested PCR that detects *Babesia*, *Theileria*, and/or *Hepatozoon*, and sequencing of thirty-five

310 randomly selected positive samples indicated the presence of *B. vogeli*, *B. rossi*, and *H. canis*, we  
311 developed a multiplex PCR assay that easily distinguishes these three species from each other on  
312 the basis of their PCR band sizes. This approach is useful when there are so many nested PCR-  
313 positive samples that it becomes impractical in terms of cost and time to sequence them all. This  
314 is particularly true when the positive samples are dominated by a single parasite, such as *H. canis*  
315 in the present case. If we had followed the usual approach of sequencing a handful of randomly  
316 selected nested PCR samples, we may have missed the *Babesia* infections, which were only  
317 present in 10% of the nested PCR-positive samples. Indeed, despite the high frequency of *H.*  
318 *canis*, our multiplex PCR assay detected five *B. rossi*-infected and seven *B. vogeli*-infected  
319 samples. It is not clear why the dogs had a low rate of *Babesia* infections and a high rate of *H.*  
320 *canis* infections. This is primarily because very little is known about the life cycles, transmission  
321 routes, and pathogenic potential of these different parasites.

322         One dog in Shangombo was co-infected with *H. canis* and *B. rossi*. Several other  
323 studies report co-infection of *H. canis* and *Babesia* spp. in dogs (Cardoso et al., 2010; Rojas et al.,  
324 2014). Such co-infections are partly responsible for the variable pathogenicity of these parasites  
325 in dogs (Munson et al., 2008; Sasanelli et al., 2009). Veterinarians should be aware of the  
326 possibility of such parasite co-infections when a dog presents with a parasite-related illness that  
327 is unusually severe or is accompanied by uncharacteristic symptoms.

328           The present study detected *A. platys* infections in two female dogs in Lusaka. The  
329 presence of *A. platys* in dogs has been reported elsewhere, including in Africa (Carvalho et al.,  
330 2017; Inokuma et al., 2002; Beugnet and Marié, 2009; Oya,mada et al., 2005). *A. platys* infects  
331 the platelets of dogs and causes infectious canine cyclic thrombocytopaenia. It is usually a mild  
332 or asymptomatic disease. In general, *A. platys* is transmitted by the *R. sanguineus* s.l. tick. A  
333 recent study suggested that *A. platys* can also be transmitted vertically from infected bitches to  
334 their puppies, either transplacentally or during the perinatal period (Matei et al., 2017). Human  
335 cases of *A. platys* have been reported: two women in Venezuela (Arraga-Alvarado et al., 2014)  
336 and one veterinary professional in South Africa (Maggi et al., 2013) were found to be infected  
337 with *A. platys*. Thus, dog owners and veterinarians in these regions should be aware of the risk of  
338 *A. platys* infection in humans.

339           *Ehrlichia* infections were suspected in three dogs by sequencing analysis of  
340 Anaplasmataceae-specific 16S rDNA PCR. Additional PCRs targeting *gltA* and *groEL* of  
341 Anaplasmataceae were only successful in one dog from Lusaka, which was shown to be infected  
342 with *E. canis*. However, the PCR assays did not yield any amplicons from two *Ehrlichia*-positive  
343 dogs in Shangombo. Although several other sets of primers were employed to amplify these  
344 genes in the two dogs, none of the PCRs were successful (data not shown). These results may  
345 indicate that the dogs in Shangombo were infected with uncharacterized *Ehrlichia* sp. which are

346 genetically distinct form those previously reported. To better understand the genetic diversity of  
347 *Ehrlichia* sp., further studies on the *Ehrlichia* spp. in Zambian dogs are warranted.

## 348 **5. Conclusions**

349 This study shows that Zambian dogs are infected with several pathogens, namely, *H.*  
350 *canis*, *B. rossi*, *B. vogeli*, *A. platys*, *E. canis* and uncharacterized *Ehrlichia* sp. Our species-  
351 discriminating multiplex PCR is useful for screening for canine haemoparasites, even when one  
352 parasite is vastly predominant.

353

## 354 **Competing interests**

355 The authors declare no competing interests.

356

## 357 **Contributions**

358 R.N. and Y.Q. conceived and designed the experiments; Y.Q., C.K., R.N., M.K., S,  
359 E.S., W.M., M.B.H., and M.S. collected samples; Y.Q., M.J.T., and R.N. performed the  
360 experiments; Y.Q., C.K., and R.N. analysed the data; Y.Q. and R.N. wrote the paper; K.K., A.T.,  
361 H.S., and R.N. edited and approved the manuscript.

362

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371

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522

523

524 **Figure annotations**

525

526 **Figure 1. Dog sample collection sites in Zambia.**

527

528 **Figure 2. A representative electrophoresis gel of the multiplex PCR products.** M: marker;

529 HC: *Hepatozoon canis*; BR: *Babesia rossi*; BV: *Babesia vogeli*; NC: negative control.

530

531 **Figure 3. Phylogenetic analysis of the *Babesia* spp. and *Hepatozoon* spp. detected in canine**

532 **blood.** The analysis based on the almost full-length sequences of 18S rDNA was conducted

533 by using a Neighbour joining method. The tree is rooted with *Plasmodium falciparum*. All

534 bootstrap values from 1,000 replications are shown on the interior branch nodes. The

535 sequences obtained in this study are indicated in red.

536 **Figure 4. Phylogenetic analyses of the Anaplasmataceae detected in canine blood.** The

537 analyses based on partial sequences of (A) *gltA* and (B) *groEL* were conducted by using a

538 Neighbour-joining method. The trees are rooted with *Rickettsia rickettsii*. All bootstrap

539 values from 1,000 replications are shown on the interior branch nodes. The sequences  
540 obtained in this study are indicated in red.

**Table 1. PCR primers amplifying haemoparasites and Anaplasmataceae.**

Primer name	Sequence (5' to 3')	Target (PCR type)	Annealing temperature (°C)	Reference
EHR16SD	GGTACCYACAGAAAGTCC	16S rDNA of Anaplasmataceae (single PCR)	55	Parola et al., 2000
EHR16SR	TAGCACTCATCGTTTACAGC			
CS7F2	ATGRTAGAAAAWGCTGTTTT	<i>gltA</i> of <i>A. platys</i> (single PCR)	55	Ybañez et al., 2012
CS1033R	GCAAAGAATGCRGTAKACAT			
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	<i>gltA</i> of Anaplasmataceae (1st PCR)	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG			
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	<i>gltA</i> of Anaplasmataceae (2nd PCR)	50	Loftis et al., 2015
EHRCS-879R	TIGCKCCACCATGAGCTG			
EHRCS-754F	ATGCTGATCATGARCAAAATG	<i>gltA</i> of Anaplasmataceae (2nd PCR)	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG			
HS1-F	CGYCAGTGGGCTGGTAATGAA	<i>groEL</i> of Anaplasmataceae (1st PCR)	54	Sumner et al., 1997
HS6-R	CCWCCWGGTACWACACCTC			
HS3-F	ATAGTYATGAAGGAGAGTGAT	<i>groEL</i> of <i>Anaplasma</i> (2nd PCR)	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG			
groEL-fwd3	TGGCAAATGTAGTTGTAACAGG	<i>groEL</i> of <i>Ehrlichia</i> (2nd PCR)	50	Gofton et al., 2016
groEL-rev2	GCCGACTTTTAGTACAGCAA			
BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA of <i>Babesia</i> , <i>Theileria</i> and <i>Hepatozoon</i> parasites (1st PCR)	55	Masatani et al., 2017
BTH 18S 1st R	AAGTGATAAGGTTACAAAACTTCCC			
BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	18S rDNA of <i>Babesia</i> , <i>Theileria</i> and <i>Hepatozoon</i> parasites (2nd PCR)	55	Masatani et al., 2017
BTH 18S 2nd R	CGGTCCGAATAATTCACCGGAT			
Br_18S_F	GTATTTTGGCTTGCGGTTT	18S rDNA of <i>B. rossi</i> (Multiplex PCR)	55	This study
Bv_18S_F	TTAGTTTGAAACCCGCTTG	18S rDNA of <i>B. vogeli</i> (Multiplex PCR)		This study
Hc_18S_F	TAAGAGCTAAATTAATGATTGATAGGG	18S rDNA of <i>H. canis</i> (Multiplex PCR)		This study
BTH_multi_R	CCCGTGTGAGTCAAATTAAGC	18S rDNA of <i>B. vogeli</i> , <i>B. vogeli</i> , and <i>H. canis</i> (Multiplex PCR)		This study

**Table 2. Detection of Anaplasmataceae, *Babesia rossi*, *Babesia vogeli*, and *Hepatozoon canis* by PCR and species-discriminating multiplex PCR.**

Location	Sex	Total no.	Anaplasmataceae PCR positive	BTH PCR positive	Identified species <sup>a</sup>		
					<i>Babesia rossi</i>	<i>Babesia vogeli</i>	<i>Hepatozoon canis</i>
Lusaka	Male	25	0	8	0	1 (1)	7 (2)
	Female	25	3	6	0	0	6 (1)
Mazabuka	Male	25	0	1	1 (1)	0	0
	Female	25	0	7	3 (3)	4 (4)	0
Monze	Male	25	0	11	0	1 (1)	10 (2)
	Female	25	0	5	0	1	4 (2)
Shangombo	Male	74	2	56	1 <sup>b</sup>	0	56 (10) <sup>b</sup>
	Female	23	0	17	0	0	17 (5)

<sup>a</sup>Number in brackets indicates the number of samples confirmed by sequencing analysis of BTH PCR products.

<sup>b</sup>One dog was positive for both *Babesia rossi* and *Hepatozoon canis*.

**Table 3. Summary of sequence types detected for *Babesia rossi*, *Babesia vogeli*, and *Hepatozoon canis* from each sampling site.**

Parasite	Sequence type	Location				Total
		Lusaka	Mazabuka	Monze	Shangombo	
<i>Babesia rossi</i>	BR1	0	2	0	0	2
	BR2	0	2	0	0	2
<i>Babesia vogeli</i>	BV1	1	1	4	0	6
<i>Hepatozoon canis</i>	HC1	0	0	2	15	17
	HC2	3	0	0	0	3
	HC3	0	0	1	0	1
	HC4	0	0	1	0	1

Figure 1





Figure 2

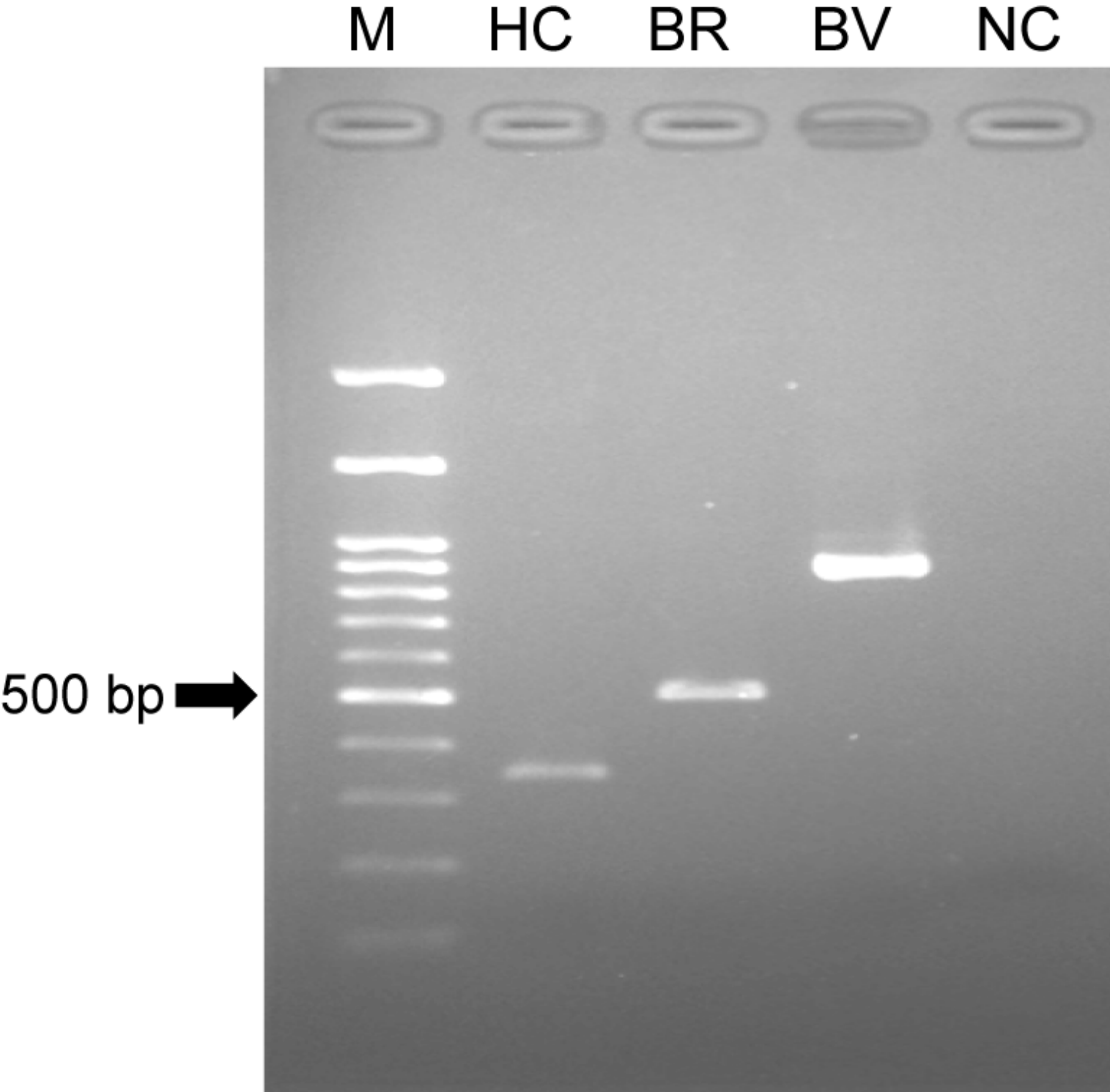
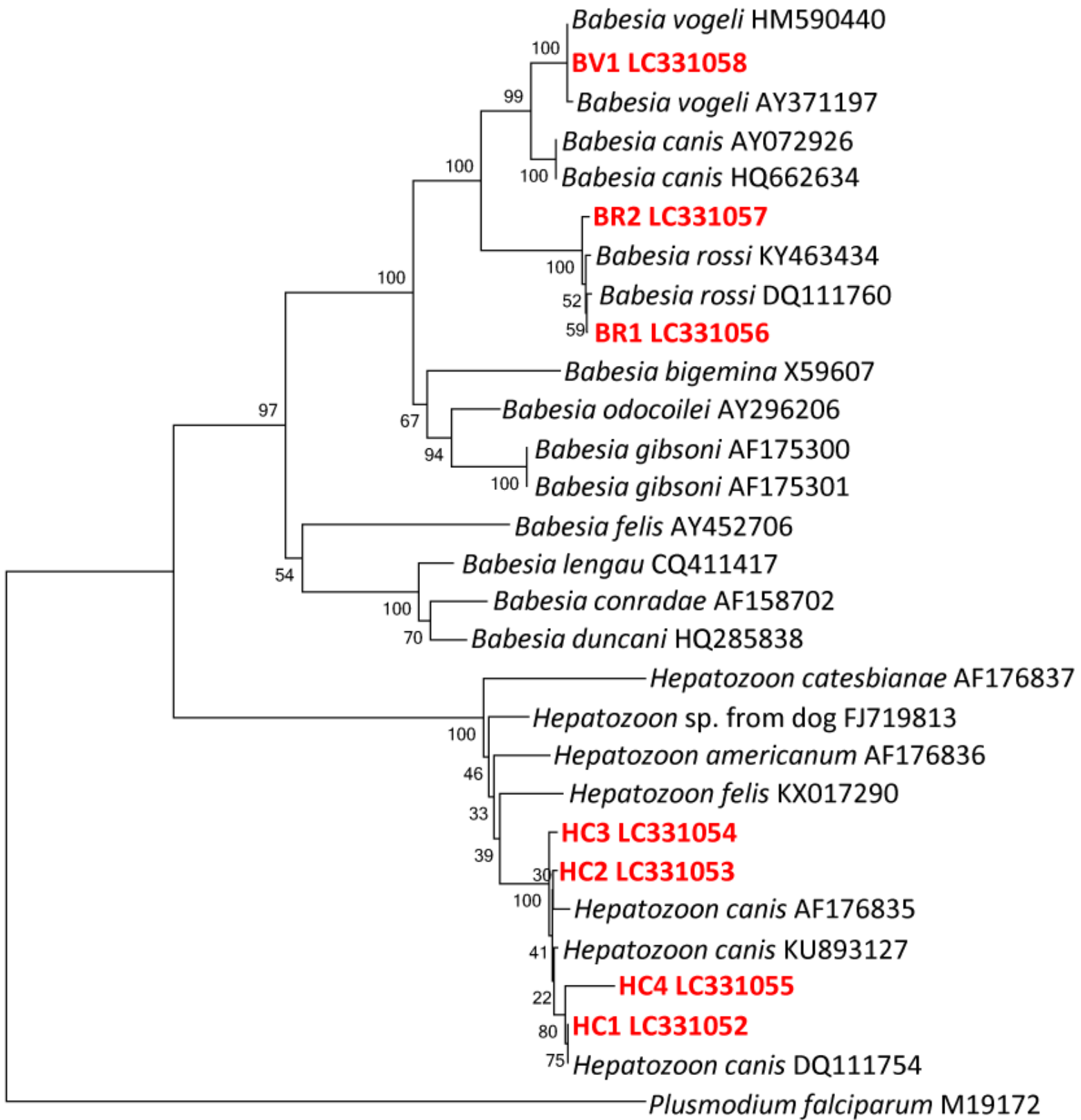


Figure 3



0.02

Figure 4

