High infection rate of tick-borne protozoan and rickettsial pathogens of cattle in Malawi and the development of a multiplex PCR for *Babesia* and *Theileria* species identification

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Abstract

Malawi has an estimated cattle population of 1,884,803 heads, the indigenous Malawi zebu breed accounts for 91.2%, while the exotic and crossbred accounts for the remaining 8.8%. Although ticks and tick-borne diseases are widespread in Malawi, no molecular study has been conducted to investigate the tick-borne Anaplasmataceae and piroplasms infecting cattle. To provide an insight into the current status of tick-borne pathogens (TBPs) of cattle, a molecular survey was conducted in the central and southern regions of Malawi. A total of 191 cattle of which 132 were Malawi zebu, 44 were Holstein Friesian and 15 were Holstein-Friesian/ Malawi zebu crosses were screened for Anaplasmataceae and piroplasms using the heat shock protein *groEL* and 18S rDNA, respectively. A new 18S rDNA multiplex PCR assay was designed for *Babesia* and *Theileria* species identification without sequencing. Overall, 92.3% (*n* = 177) of the examined animals were infected with at least one TBP. Anaplasmataceae-positive rate was 57.6% (*n* = 110) while for piroplasms it was 80.1% (*n* = 153). The detected Anaplasmataceae were *Anaplasma bovis* 2.6% (*n* = 5), *Anaplasma marginale* 24.6% (*n* = 47), *Anaplasma platys*-like 13.6% (*n* = 26), uncharacterized *Anaplasma* sp. 14.1% (*n* = 27), and uncharacterized *Ehrlichia* sp. 16.2% (*n* = 31). The detected piroplasms were *Babesia bigemina* 2.6% (*n* = 5), *Theileria mutans* 73.8% (*n* = 141), *Theileria parva* 33.0% (*n* = 63), *Theileria taurotragi* 12.6% (*n* = 24), and *Theileria velifera*

53.4% (*n* = 102). Mixed infection rate was 79.6% (*n* = 152) of the samples analyzed. This study has shown a high burden of TBPs among cattle in Malawi which highlights the need to conceive new methods to control ticks and TBPs in order to improve animal health and productivity. The newly developed multiplex PCR assay would be a useful tool especially in resource limited settings where sequencing is not available and when mixed infections are expected.

Keywords: Anaplasmataceae, cattle, Malawi, molecular epidemiology, piroplasms.

1. Introduction

Tick-borne pathogens (TBPs) that belong to the genera *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* are widespread in sub-Saharan Africa where the vector ticks are present. In Malawi, 15 tick species namely: *Amblyomma variegatum*, *Hyalomma marginatum rufipes*, *Hyalomma truncatum*, *Rhipicephalus appendiculatus*, *Rhipicephalus compositus*, *Rhipicephalus decoloratus*, *Rhipicephalus kochi*, *Rhipicephalus masseyi,* *Rhipicephalus microplus*, *Rhipicephalus planus, Rhipicephalus pravus*, *Rhipicephalus punctatus*, *Rhipicephalus sanguineus* sensu lato, *Rhipicephalus simus* and *Rhipicephalus tricuspis*, have been reported to infest cattle (Walker et al., 2003; Berggren, 1978).

Bovine anaplasmosis (gall sickness) is caused by *Anaplasma marginale* infection, and the clinical outcome is characterized by anemia, icterus, fever, weight loss, abortion, and lethargy and in severe cases, it may be fatal (Kocan et al., 2003). The occurrence of clinical disease is dependent on the age of the animal, as susceptibility increases with age. Calves under 6 months of age are generally less susceptible (Kocan et al., 2003; Richey, 1991). In Malawi, cases of bovine anaplasmosis have been reported but its causative agents have not yet been characterized. *Anaplasma marginale* is transmitted by the vector ticks such as *A.*

variegatum, *R. decoloratus*, *R. evertsi evertsi* and *R. microplus* (Walker et al., 2003). Cattle may also be infected with *Anaplasma centrale,* and *Anaplasma bovis* formerly known as *Ehrlichia bovis* (Dumler et al., 2001), which cause benign anaplasmosis. *Anaplasma platys*-like infection in cattle has also been reported in Bolivia (Ogata et al., 2021), Kenya (Peters et al., 2020), and Tunisia (Ben Said et al., 2017) however, its clinical impact on cattle has not yet been fully explored.

Bovine ehrlichiosis (heartwater) is caused by *Ehrlichia ruminantium* formerly known as *Cowdria ruminantium*. The clinical disease is commonly associated with endocarditis in cattle and other non-specific signs such as fever, anorexia, aggressiveness and sudden death (Allsopp, 2015). The vector ticks of *E. ruminantium* in southern Africa are *Amblyomma hebraeum* and *A. variegatum* (Walker et al., 2003). Other uncharacterized *Ehrlichia* species closely related to *Ehrlichia minasensis* but separated from the pathogenic *E. ruminantium* have been reported to infect cattle in Kenya, in apparently healthy dairy cattle (Peters et al., 2019, 2020). In Malawi, *E. ruminantium* infection in goats has been reported using molecular techniques (Chatanga et al., 2021a).

Bovine babesiosis (red water) which is caused by *Babesia bovis* and *Babesia bigemina* has also been documented in Malawi (Lawrence et al., 1996). The newly described pathogenic *Babesia* sp. Mymegnsingh has also been documented to cause clinical disease in cattle in Argentina, Philippines, Sri Lanka, Uganda and Vietnam (Sivakumar et al., 2018, 2020). The clinical disease is characterized by high fever, hemoglobinuria, dark colored urine, anorexia, lethargy and neurological signs which usually result in fatal outcome (Uilenberg et al., 1995). The vector ticks for *B. bigemina* and *B. bovis* in southern Africa are *R. microplus*, *R. decoloratus*, and *R. evertsi evertsi* (Walker et al., 2003).

97 East Coast fever (ECF) (one of bovine theilerioses) is caused by *Theileria parva* and
98 is considered the most economically important tick-borne diseases in Africa (Lubembe et al.,
99 2020). Calves of indigenous zebu cattle below the age of 6 months are highly susceptible to
100 the infection (Moll et al., 1984, 1986). The disease is severe in exotic and crossbred cattle
101 with morbidity and mortality rates ranging from 80-100% (Oura et al., 2007).The clinical
102 disease is characterized by anemia, icterus, tachypnea, tachycardia, lethargy, froth coming out
103 of the mouth, anorexia, fever, abortion, and enlarged superficial lymph nodes (Nene et al.,
104 2015). *Theileria parva* is transmitted by the brown ear tick, *R. appendiculatus* (Walker et al.,
105 2003). Other *Theileria* species that infect cattle but cause benign theileriosis in southern
106 Africa are *Theileria mutans, Theileria taurotragi,* and *Theileria velifera*.

107 Malawi cattle population is estimated at 1,884,803 heads of which the indigenous
108 Malawi zebu accounts for 91.2% (1,719,641) while the exotic and crossbred cattle account
109 for the remaining 8.8% (165,862) as of 2020 (DAHLD, 2020). With an estimated human
110 population of 17,563,749 as of 2018 (NSO, 2018), Malawi has one of the lowest cattle
111 population per capita in Africa. Approximately 71% of the total pure and crossbred dairy
112 cattle in Malawi are found in the southern region which is considered to be non-endemic to
113 ECF (DAHLD, 2006). The endemic status of ECF has hampered the establishment of the
114 dairy industry in the central and northern regions of the country (Lawrence et al., 1996;
115 Chinombo et al., 1988).

116 This study aimed to provide molecular epidemiological data on the current prevalence
117 and characterization of TBPs infecting cattle in Malawi. Cattle blood samples were screened
118 for Anaplasmataceae and piroplasms using conventional PCR and a newly developed
119 multiplex PCR assay. This epidemiological data is a pre-requisite for the development of
120 novel control measures against tick and TBPs in Malawi to achieve the national goal of
121 Malawi being self-sufficient in safe locally produced livestock and livestock products.

2. Material and methods

2.1 Ethical consideration.

This was a parallel study of our previous study (Chatanga et al., 2020) which was approved by Ministry of Agriculture, Irrigation and Water Development (MoAIWD) in Malawi through the Department of Animal Health and Livestock Development (DAHLD) reference number 10/15/32/D and permission for sampling was obtained from the host institutions and farmers.

2.2 Study site and sample collection

Cattle blood samples (*n* = 191) were collected from five districts in the central region and southern regions of Malawi (Figure 1) from February 2018 to March 2019 during the rainy seasons which runs from November to April from apparently healthy animals. Specifically, the samples were collected from Kasungu (*n* = 26), Nkhotakota (*n* = 37), Katete (*n* = 33), Likasi (*n* = 34), Lilongwe University of Agriculture and Natural Resources (LUANAR) (*n* = 33) and Mikolongwe (*n* = 28). Overall, the sampled animals were Malawi zebu (*n* = 132), Holstein Friesian (*n* = 44) and Holstein Friesian/ Malawi zebu crosses (*n* = 15).

The animals investigated in Kasungu and Nkhotakota were Malawi Zebu cattle that were managed under extensive grazing in communal grazing lands. In this management system, animals from different smallholder farms were mixed freely and there was no ECF vaccination or dipping history to control ticks. The animals investigated at Katete farm in Lilongwe were Holstein-Friesian which were kept under a semi-intensive management with no contact with other herds. They were dipped weekly during rainy season and fortnightly during dry season to control ticks and Muguga cocktail (MC) vaccine was also used to control ECF in some animals. A total of 20 of the sampled animals at Katete farm were vaccinated with Muguga cocktail vaccine 2 years and 3 months prior to sampling period while the remainder (n = 13)

147 were unvaccinated but these animals had been co-grazing for more than 2 years. At Likasi,
148 LUANAR and Mikolongwe farms both exotic dairy breed Holstein Friesian and local Malawi
149 zebu were kept. The animals were dipped fortnightly and MC vaccine was used to immunize
150 the exotic breeds but not the local Malawi zebu. Approximately 5 ml of whole blood was
151 collected by venipuncture of the external jugular vein after disinfection of the puncture site
152 with methylated spirit cotton swab into ethylene diamine tetraacetic acid (EDTA) vacutainer
153 tube.

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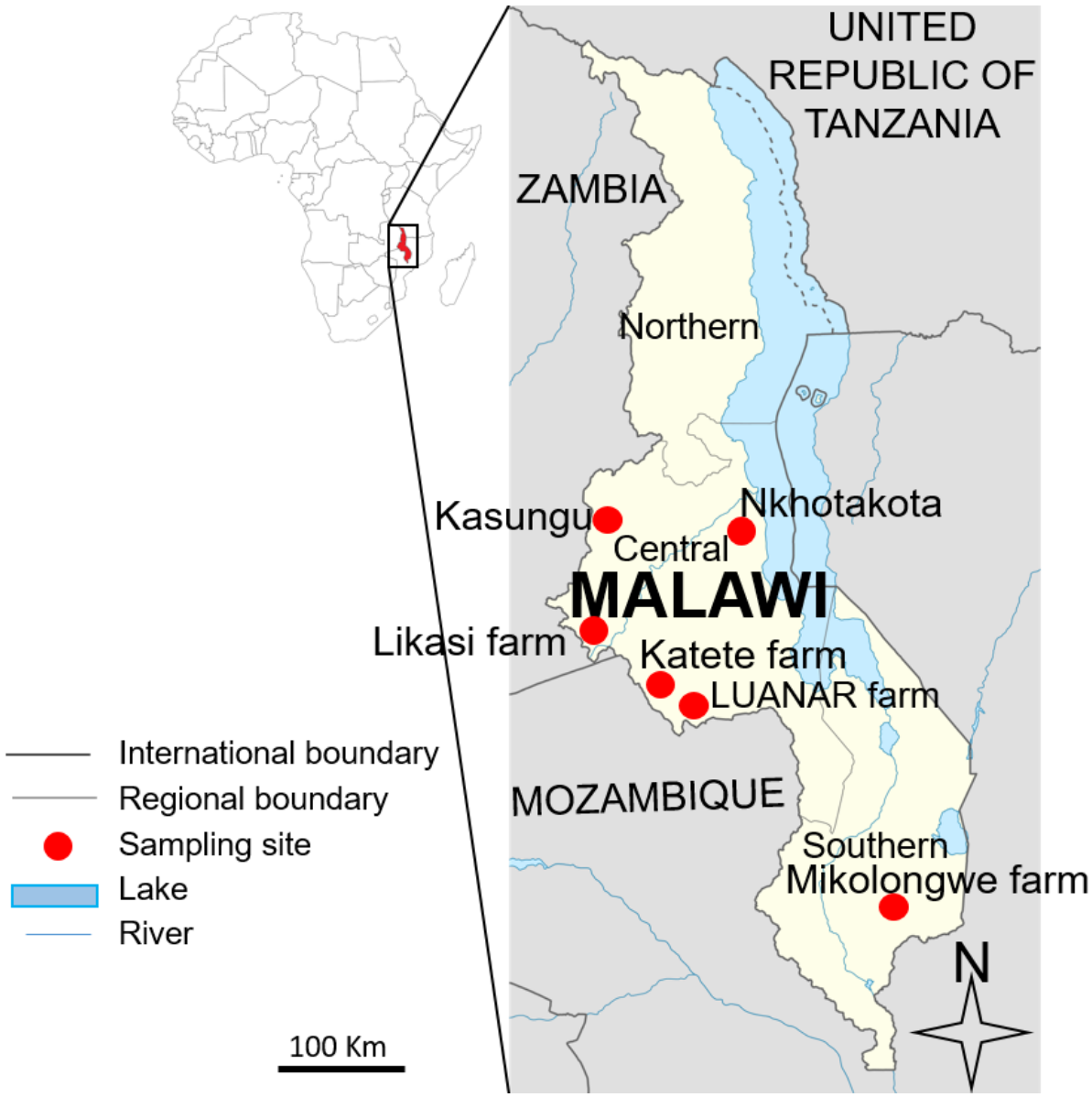

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156 Figure 1. Map of Malawi showing the sample collection sites.

157

158 **2.3 DNA extraction**

159 DNA was extracted from 200 µl of whole blood using the Quick Gene DNA whole blood
160 kit S (DB-S) (Kurabo Industries Ltd., Osaka, Japan) according to the manufacturer's
161 recommendations. The extracted DNA was stored at -20°C until required for use.

162 **2.4 Polymerase chain reaction**

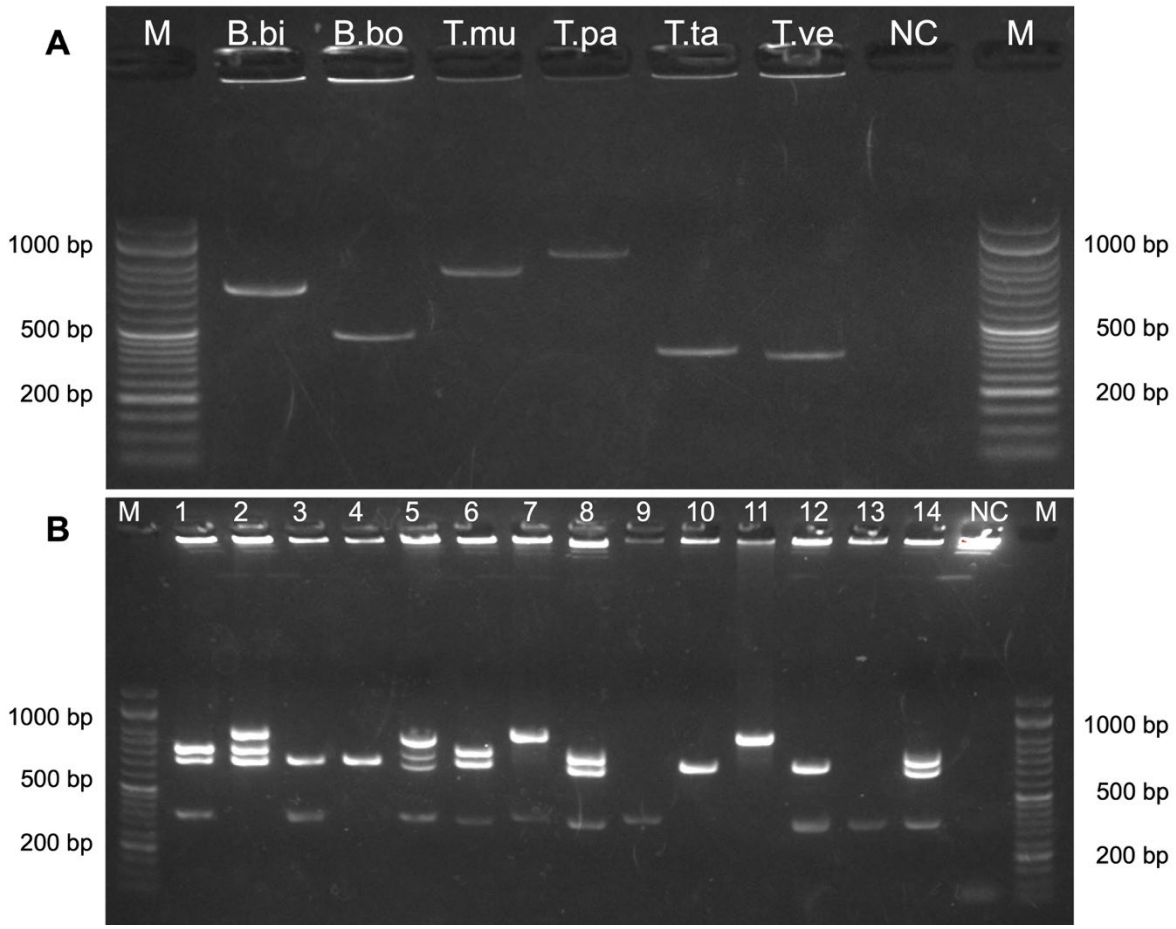
163 All the 191 samples from six sampling sites were screened for *Anaplasma*, *Ehrlichia,*
164 *Babesia* and *Theileria* species. The screening of Anaplasmataceae was done targeting the heat
165 shock protein gene (*groEL*) nested PCR assays as previously described (Rar et al., 2011; Liz
166 et al., 2000; Gofton et al., 2016). *Anaplasma ovis* DNA extracted from positive sheep blood
167 was used as positive control while molecular grade water instead of genomic DNA was used
168 as negative control for quality control.

169 The *groEL* gene secondary PCR assays make use of two primer sets: one that is specific
170 for the genus *Anaplasma* and another that is specific for the genus *Ehrlichia*. This allows
171 detection of mixed infection of *Anaplasma* and *Ehrlichia* spp. in a sample. Further,
172 characterization was done using the citrate synthase gene (*gltA*) nested PCR assays as
173 previously described (Inokuma et al., 2005). The PCR conditions for both 1st and 2nd reactions
174 were set at initial denaturation at 94°C for 1min, followed by 35 cycles of denaturation at 98°C
175 for 10 sec, annealing at 50/54/55°C for 15sec and extension at 68°C for 1 min and final
176 extension at 68°C for 5min.

177 The screening for piroplasms was done targeting almost the full length of the *18S*
178 *ribosomal RNA gene* (rDNA) nested PCR assays targeting *Babesia*, *Theileria* and *Hepatozoon*
179 (BTH) species as described by Masatani et al. (2017), and Qiu et al. (2018). All PCR reactions
180 were conducted in a 25 µl reaction mixture containing 0.5 µl of Tks Gflex DNA Polymerase

181 (1.25 units/µl) (TaKaRa Bio Inc., Shiga, Japan), 12.5 µl of 2× Gflex PCR Buffer (Mg^{2+}, dNTP
182 plus), 200 nM of each primer, 1.0 µl of template DNA and molecular grade water. *Theileria*
183 *orientalis* Ikeda strain DNA extracted from cattle blood was used as positive control while
184 molecular grade water instead of genomic DNA was used as negative control for quality control.
185 Due to mixed signals observed in selected sequenced 2nd BTH PCR products a new item
186 multiplex PCR assay for *B. bigemina, B. bovis, T. mutans, T. parva, T. taurotragi*, and *T.*
187 *velifera* was designed in this study to identify the detected pathogens to species level (Figure
188 2A).

189 To design the new multiplex PCR primers, sequences of the 18S rDNA of *Babesia*
190 species (*Babesia bovis*, AY150059, HQ264127; and *Babesia bigemina*, KM046917,
191 JQ437264), *Theileria* species (*Theileria mutans*, AF078815; *Theileria parva*, MG952923,
192 AF013418; *Theileria taurotragi*, L19082 and *Theileria velifera*, AF097993) deposited in the
193 GenBank were downloaded. The sequences were aligned using ClustalW in Molecular
194 Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al., 2016) and were visually
195 checked for regions that have homologies within each species. Primers were designed by using
196 Primer 3 plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The specificity of the
197 designed primers was confirmed by additional BLAST searches
198 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 100-fold diluted first BTH PCR product was used
199 as a template for the *Babesia*/ *Theileria* multiplex PCR assay. The multiplex PCR was used to
200 identify the detected pathogens based on the 2nd BTH PCR results to species level. The
201 multiplex PCR primers were used for sequencing of the detected pathogens to generate
202 sequences for phylogenetic tree analysis. The primers, their annealing temperature and the
203 expected amplicon size are listed in Table 1.



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205 Figure 2. Representative gel electrophoresis images of the multiplex PCR products.

206 A: M = 50 bp marker; B.bi = *Babesia bigemina*, B.bo = *Babesia bovis*, T.mu = *Theileria*

207 *mutans*, T.pa = *Theileria parva*, T.ta = *Theileria tautotragi*, T.ve = *Theileria velifera*, NC

208 = negative control.

209 B: M = 50 bp marker; lanes 1-13 samples, NC = negative control.

210 Table 1. List of primers used for the detection and characterization of tick-borne Anaplasmataceae and piroplasms of cattle in Malawi.

Primer name	Primer sequence (5′ to 3′)	Target gene/ organism	PCR type	Amplicon size (bp)	Annealing temperature (°C)	Reference
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA/ *Babesia*, *Theileria* and *Hepatozoon*	1st PCR	1400 - 1600	55	Masatani et al., 2017
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG		2nd PCR	1400 - 1600		
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
BTH_Bbig_ F	AGCCTTGGTAATGGTTAATAG	18S rDNA/ *Babesia*, and *Theileria*	Multiplex PCR	848	57	This study
BTH_Bbov_ F3	TGTCCTTTCTTGATTCTCTGGGTAG			469		
BTH_Tmut_ F	GGCCCTTGCCTTGAATACTTTAG			915		
BTH_Tpar_F	CTCTGCATGTGGCTTATTTCGG			995		
BTH_Ttau_F	TGCTAAATAGGGTACGGGAGC			330		
BTH_Tveli_F	AGGCTTTTGCCGTCCCGTGATCGC			310		
BTH_ NEW_R	TCCGAATAATTCACCGGATCAC					
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* gene/ Anaplasmataceae	1st PCR	1300	54	Rar et al., 2011
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* gene/ *Anaplasma*	2nd PCR	1256	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* gene/ *Ehrlichia*	2nd PCR	1100	50	Gofton et al., 2016
groEL_rev2	GCCGACTTTTAGTACAGCAA					
F4b	CCAGGCTTTATGTCAACTGC	*gltA* gene/ Anaplasmataceae	1st PCR	800	55	Inokuma et al., 2005
R1b	CGATGACCAAAACCCAT					
EHR-CS136F	TTYATGTCYACTGCTGCKTG	*gltA* gene/ *Anaplasma* and *Ehrlichia*	2nd PCR	650	55	Inokuma et al., 2005
EHR-CS778R	GCNCCMCCATGMGCTGG					

211 PCR = Polymerase chain reaction; F = Forward; R = Reverse

212 **2.5 Sequencing**

213 All samples that were positive on 2nd *groEL* gene PCR (*n* = 110) were purified using
214 ExoSAP-IT™ PCR Product Clean-up Reagent (Applied Biosystems, CA, USA) and
215 sequenced. Thereafter, based on the species identified from the obtained *groEL* genes
216 sequences, i.e. 4, 3, 1, 1 and 7 for *A. platys*, *A. marginale*, *A. bovis*, uncharacterised
217 *Anaplasma* sp., and uncharacterised *Ehrlichia* sp., respectively, we selected 5 samples per
218 *Anaplasma* or *Ehrlichia* species of the 2nd *gltA* gene PCR for sequencing. For *Babesia* and
219 *Theileria* species, five randomly selected samples per species from the designed new item
220 (multiplex PCR) products were cut from the gel and purified using NucleoSpin Gel and PCR
221 Clean-Up Kit (Takara Bio Inc.). Sequencing was done in both directions using BigDye™
222 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and ABI genetic
223 analyzer 3500xl (Applied Biosystems, CA, USA). Sequence data editing was conducted
224 using ATGC software (GENETYX Corporation, Tokyo, JP) by trimming the primers
225 annealing sites and the consensus sequence was extracted for phylogenetic analysis. The
226 sequences generated in this study were submitted to the DNA Data Bank of Japan (DDBJ)
227 (http://www.ddbj.nig.ac.jp/) under the accession numbers LC664051 to LC664059 for
228 piroplasms 18S rDNA, LC664060 to LC664073 and LC671361 for Anaplasmataceae *gltA*
229 gene and LC664074 to LC664088 and LC671362 for Anaplasmataceae *groEL* gene.

230 **2.6 Statistical analysis**

231 The significance of co-infections was examined by statistical modelling using
232 generalized linear model (Glm) function in R version 4.1.2. Thereafter, the odds ratio were
233 calculated based on modelling results using odds ration function in R which were eventually
234 used for calculation of the correlation of co-infection using kendall method cor function in
235 stats package in R software (http://www.rstudio.com/). The "Holm" method was used for

236 adjusting the p-value over multiple testing (https://www.jstor.org/stable/2346101). The $p <$
237 0.05 was considered significant.

238 **2.7 Phylogenetic analysis**

239 Alignments of the consensus nucleotide sequences generated from the amplified DNA
240 fragments were done using ClustalW in MEGA version 7. To understand the relationship of
241 the sequences obtained in this study and those deposited in the GenBank, maximum
242 likelihood (ML) phylogenetic trees were constructed using MEGA version 7 software using
243 the Tamura 2 parameter model.

244

245 **3. Results**

246 **3.1 PCR screening and sequence analysis**

247 The screening for Anaplasmataceae using *groEL* gene nested PCR assay showed that
248 110/191 (57.6%) were positive. To identify the detected *Anaplasma* and *Ehrlichia* species
249 sequencing of all 2nd *groEL* gene PCR positive samples (n = 110) was done. The
250 Anaplasmataceae sequence data showed that the examined cattle in Malawi were infected
251 with *Anaplasma bovis* 5/191 (2.6%), *Anaplasma marginale* 47/191 (24.6%), *Anaplasma*
252 *platys*-like 26/191 (13.6%), uncharacterized *Anaplasma* sp. 27/191 (14.1%) and
253 uncharacterized *Ehrlichia* sp. 31/191 (16.2%) as shown in Table 2.

254 The sequences of all the uncharacterised *Anaplasma* sp. (n = 27) were identical and
255 closely related to *A. bovis* in both the *groEL* and *gltA* genes. However, the sequences of the
256 uncharacterised *Ehrlichia* sp. (n = 31) produced 7 and 10 sequences for the *groEL* and *gltA*
257 genes, respectively. Since, we did not expect any wider variation between the results of
258 *groEL* gene and *gltA* gene, five (5) for each of the above detected pathogens were also
259 sequenced for *gltA* gene for further characterization.

260 The screening for *Babesia* and *Theileria* species using 18S rDNA gene BTH nested
261 PCR assay showed that 153/191 (80.1%) were positive. Further screening of the 2nd BTH
262 PCR amplicons using the new multiplex PCR assay showed that the examined animals were
263 infected with *B. bigemina* 5/191 (2.6%), *T. mutans* 141/ 191 (73.8%), *T. parva* 63/191
264 (33.0%), *T. taurotragi* 24/191 (12.6%), and *T. velifera* 102/191 (53.4%), as shown in Table 2.
265 The samples from Katete showed that among the 20 samples from vaccinated cattle 14 were
266 positive for *T. parva* representing 70% positive rate, while among the 13 unvaccinated
267 samples 3 were positive for *T. parva* representing 23% positive rate.

Table 2. Positive detection rates of tick-borne pathogens (TBPs) in the studied cattle in Malawi.

Pathogen	Study site						Overall
	Kasungu	Nkhotakota	Katete	Likasi	LUANAR	Mikolongwe	*n* = 191
	n = 26	*n* = 37	*n* = 33	*n* = 34	*n* =33	*n* = 28	
A. bovis	0	0	0	0	2 (6%)	3 (11%)	5 (2.9%)
A. marginale	5 (19%)	6 (16%)	7 (21%)	11 (32%)	6 (18%)	12 (43%)	47 (24.6%)
A. platys	2 (8%)	13 (35%)	4 (12%)	6 (18%)	0	1 (4%)	26 (13.6%)
Anaplasma sp.	7 (27%)	4 (11%)	5 (15%)	4 (11%)	3 (9%)	4 (14%)	27 (14.1%)
Ehrlichia sp.	12 (46%)	5 (14%)	2 (6%)	6 (17%)	1 (3%)	5 (18%)	31 (16.2%)
B. bigemina	1 (4%)	2 (5%)	1 (3%)	2 (6%)	0	0	6 (3.1%)
B. bovis	0	0	0	0	0	0	0
T. mutans	19 (73%)	28 (77%)	22 (67%)	26 (76%)	28 (85%)	18 (64%)	141 (73.8%)
T. parva	9 (35%)	10 (27%)	17 (52%)	9 (26%)	11 (33%)	7 (25%)	63 (33.0%)
T. taurotragi	4 (15%)	2 (5%)	6 (18%)	3 (9%)	3 (9%)	6 (21%)	24 (12.6%)
T. velifera	14 (54%)	19 (51%)	13 (39%)	19 (56%)	25 (76%)	12 (43%)	102 (53.4%)

n = number of samples, number of samples positive and percentage is provided in parenthesis.

272 **3.2 Infection status of TBPs in cattle**

273 Overall, 177 cattle were infected with at least one TBP, representing 92.7% positive
274 rate. Co-infections were observed in 152 cattle (79.6%), and the number of pathogens co-
275 infecting a sample ranged from two to six (Table 3). Triple co-infections had the highest
276 frequency at 39.3% (*n* = 75), followed by double co-infections at 28.3% (*n* = 54), as shown in
277 Table 3. Sextuple co-infection had the least frequency at 0.5% (*n* = 1) (Table 3). LUANAR
278 farm had the highest co-infection rate at 91%, followed by Likasi, Mikolongwe, Nkhotakota,
279 Kasungu and Katete, at 85%, 79%, 78%, 77% and 67%, respectively (Table 3). The double
280 infections combination with the highest frequency was *T. mutans* + *T. velifera* at 12.0% (*n* =
281 23). In triple infections, the highest frequency was observed in the combination *T. mutans* +
282 *T. parva* + *T. velifera* at 7.9% (*n* = 15). For quadruple infections, the highest frequency was
283 observed in the combination *A. marginale* + *T. mutans* + *T. parva* + *T. velifera* at 2.6% (*n* =
284 5).

285 Table 3. Number of TBPs co-infections observed in the studied cattle in Malawi.

Number of pathogens in co-infection	Study site						Overall *n* = 191
	Kasungu *n* = 26	Nkhotakota *n* = 37	Katete *n* = 33	Likasi *n* = 34	LUANAR *n* =33	Mikolongwe *n* = 28	
2	7 (27%)	14 (38%)	10 (30%)	8 (24%)	13 (39%)	2 (7%)	54 (28.3%)
3	12 (46%)	13 (35%)	7(21%)	17 (50%)	13 (39%)	13 (46%)	75 (39.3%)
4	1 (4%)	1 (3%)	5 (15%)	4 (12%)	1 (3%)	6 (21%)	18 (9.4%)
5	0	1 (3%)	0	0	2 (6%)	1 (4%)	4 (2.1%)
6	0	0	0	0	1 (3%)	0	1 (0.5%)
Total	20 (77%)	29 (78%)	22 (67%)	29 (85%)	30 (91%)	22 (79%)	152 (79.6%)

286 *n* = number of samples, number of samples positive and percentage is provided parenthesis.

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The summary of the pathogen combinations per sampling site has been provided in Table S1. Statistical analysis showed both positive and negative correlations between the co-infecting pathogens examined in this study. Statistically significant correlation that was statistically significant was observed between *Anaplasma* species infection with *A. bovis* ($p = < 0.001$) and *A. platys*-like ($p = 0.026$). This study also showed statistically significant correlation between co-infection with *T. mutans* and *T. taurotragi* ($p = 0.01$) and *T. mutans* with *T. velifera* ($p = < 0.001$) Table 4.

316 Table 4. Association between co-infections of TBPs in the studied cattle in Malawi.

	A. marginale	*A. platys*-like	*Anaplasma* sp.	*Ehrlichia* sp.	*B. bigemina*	*T. mutans*	*T. parva*	*T. taurotragi*	*T. velifera*
A. bovis	C: 1, (0.5) r = -0.008 95% CI: -0.15-0.134 *p* = 0.914	C: 2, (1.0) r = 0.106 95% CI: -0.037-0.244 *p* = 0.146	C: 3, (1.6) r = 0.294 95% CI: 0.159-0.419 *p* = < 0.001*	NCO	NCO	C: 5, (2.6) r = 0.079 95% CI: -0.064-0.219 *p* = 0.277	C: 1, (0.5) r = -0.047 95% CI: -0.188-0.096 *p* = 0.519	C: 1, (0.5) r = 0.034 95% CI: -0.109-0.175 *p* = 0.645	C: 3, (1.6) r = 0.025 95% CI: -0.117-0.166 *p* = 0.73
A. marginale		NCO	C: 1, (0.5) r = -0.122 95% CI: -0.259-0.021 *p* = 0.094	NCO	C: 1, (0.5) r = 0.011 95% CI: -0.132-0.152 *p* = 0.884	C: 34, 17.8) r = -0.035 95% CI: -0.176-0.107 *p* = 0.63	C: 12, (6.3) r = -0.029 95% CI: -0.17-0.114 *p* = 0.693	C: 7, (3.7) r = 0.056 95% CI: -0.086-0.197 *p* = 0.439	C: 21, (11.0) r = -0.025 95% CI: -0.166-0.117 *p* = 0.731
A. platys-like			C: 6, (3.1) r = 0.162 95% CI: 0.02-0.297 *p* = 0.026*	NCO	C: 1, (0.5) r = 0.035 95% CI: -0.108-0.176 *p* = 0.633	C: 26, (13.6) r = 0.031 95% CI: -0.112-0.172 *p* = 0.674	C: 12, (6.3) r = 0.049 95% CI: -0.094-0.189 *p* = 0.505	C: 4, (2.1) r = -0.002 95% CI: -0.144- 0.14 *p* = 0.973	C: 20, (10.5) r = 0.136 95% CI: -0.007-0.272 *p* = 0.061
Anaplasma sp.				1, (0.5) r = -0.074 95% CI: -0.214-0.069 *p* = 0.309	NCO	C: 16, (8.4) r = 0.104 95% CI: -0.039-0.242 *p* = 0.154	C: 4, (2.1) r = -0.066 95% CI: -0.266-0.077 *p* = 0.364	C: 4, (2.1) r = 0.097 95% CI: -0.046-0.235 *p* = 0.183	C: 9, (4.7) r = 0.004 95% CI: -0.138-0.146 *p* = 0.96

Ehrlichia sp.	NCO	C: 19, (9.9) r = -0.073 95% CI: -0.213-0.069 *p* = 0.313	C: 6, (3.1) r = -0.129 95% CI: -0.266-0.013 *p* = 0.076	C: 3, (1.6) r = -0.024 95% CI: -0.165-0.119 *p* = 0.744	C: 14, (7.3) r = -0.004 95% CI: -0.146-0.138 *p* = 0.955
B. bigemina		C: 3, (1.6) r = -0.023 95% CI: -0.164-0.119 *p* = 0.752	C: 1, (0.5) r = -0.026 95% CI: -0.168-0.116 *p* = 0.717	NCO	C: 3, (1.6) r = 0.066 95% CI: -0.076-0.206 *p* = 0.362
T. mutans			C: 57, (29.8) r = 0.087 95% CI: 0.056-0.0.226 *p* = 0.232	C: 25, (13.1) r = 0.187 95% CI: 0.046-0.32 *p* = 0.01∗	C: 96, (50.2) r = 0.344 95% CI: 0.213-0.464 *p* = <0.001∗
T. parva				9, (4.7) r = 0.02 95% CI: -0.122-0.162 *p* = 0.778	31, (16.2) r = -0.056 95% CI: -0.196-0.087 *p* = 0.444
T. taurotragi					NCO

C = Number of co-infections and percent related to the total cattle population examined (191 specimens), NCO = no co-infection observed, R = correlation coefficient of infections between pathogens in cattle in Malawi, 95% CI = 95% confidence intervals for R, ∗ A *p*-value of ≤0.05 was considered to be significant.

320 **3.3 Phylogenetic analysis**

321 To determine the phylogenetic relationship of the detected pathogens with those
322 reported elsewhere phylogenetic trees were constructed as shown in figures 3, 4, and 5 for
323 *Babesia* and *Theileria* 18S rDNA, *Anaplasma* and *Ehrlichia* species *gltA* and *groEL* genes,
324 respectively. The *Babesia* and *Theileria* sequences obtained in this study clustered with
325 homologous sequences available in the GenBank (Figure 3). We observed divergence in the
326 clustering of *T. mutans* sequences obtained in this study. This finding suggests that there are
327 at least two strains of *T. mutans* circulating in cattle in Malawi. The *T. parva* sequences
328 obtained in this study clustered together with sequences of *T. parva* Muguga cocktail vaccine
329 strains (MG952923, MG952924 and MG952925) even from non-vaccinated cattle.

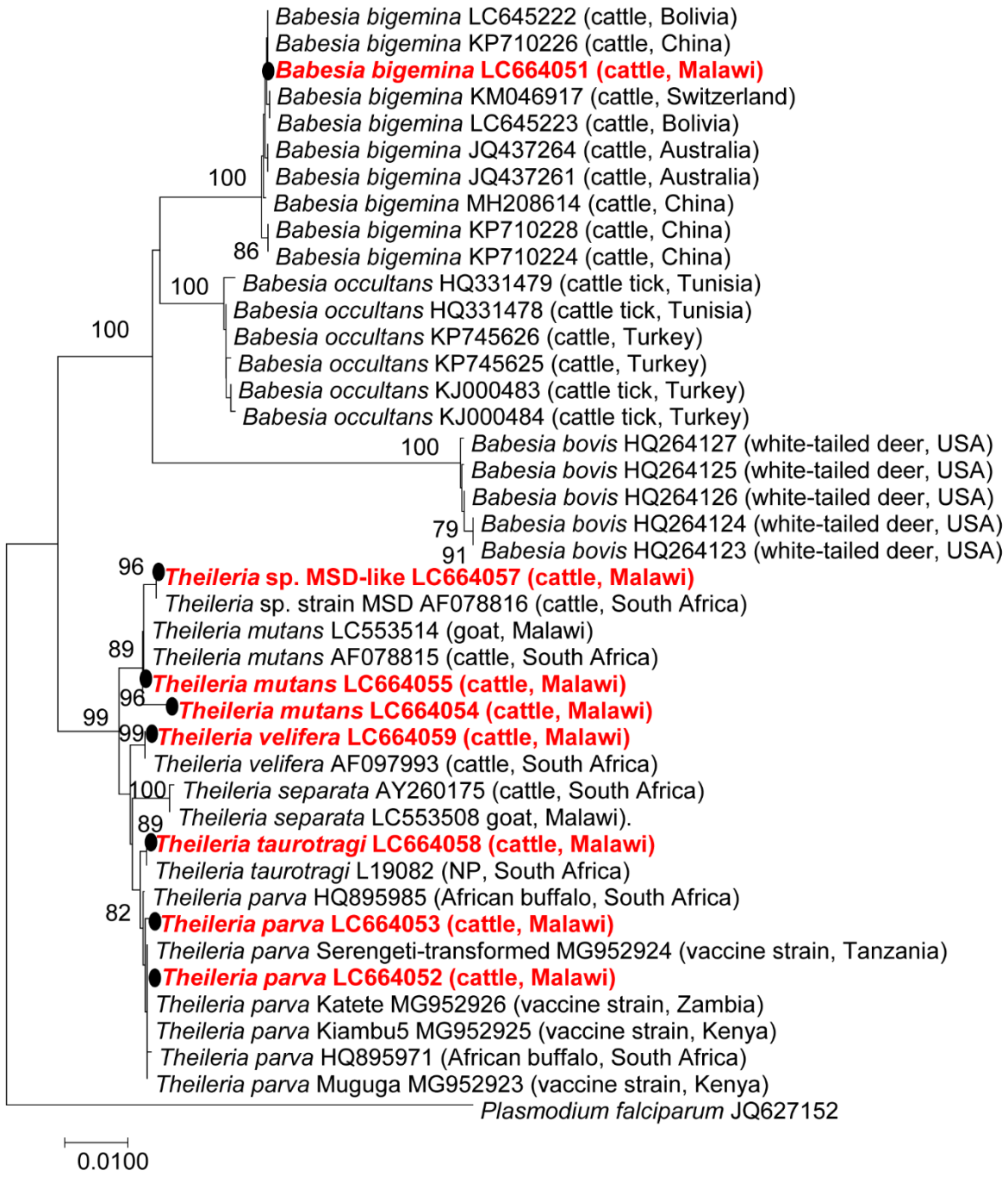

Figure 3. The maximum likelihood phylogenetic tree of the *Babesia* and *Theileria* species detected in cattle in Malawi. The analysis was based on partial sequences of 18S rDNA which was constructed using the Kimura 2 parameter model. *Plasmodium falciparum* was used as outgroup. All bootstrap values > 75% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in red. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.10 is the genetic distance of each sequence from the root of the tree.

338 *Anaplasma marginale* sequences obtained in this study clustered with other
339 homologous sequences obtained from the GenBank. The *A. bovis* sequences obtained from all
340 the five samples were identical and shared 100% identity with homologous sequence of *A.*
341 *bovis* (MH255914) reported from cattle in China. The phylogenetic trees of both the *gltA* and
342 *groEL* genes phylogenetic trees showed that the *A. platys*-like sequences obtained in this
343 study clustered with other *A. platys*-like sequences from ruminants and associated ticks but
344 separated from those reported from dogs or dog ticks (Figures 4 and 5). The obtained
345 sequences of *Ehrlichia* species showed high degree of divergence as evidenced by the
346 branching of their clade. Some sequences had 98-100% identity with homologous sequence
347 of *E. minasensis* (JX629807) isolated from *R. microplus* tick in Brazil. However, the
348 sequences of *Ehrlichia* species clustered separately from those of *E. ruminantium* (Figures 4
349 and 5).

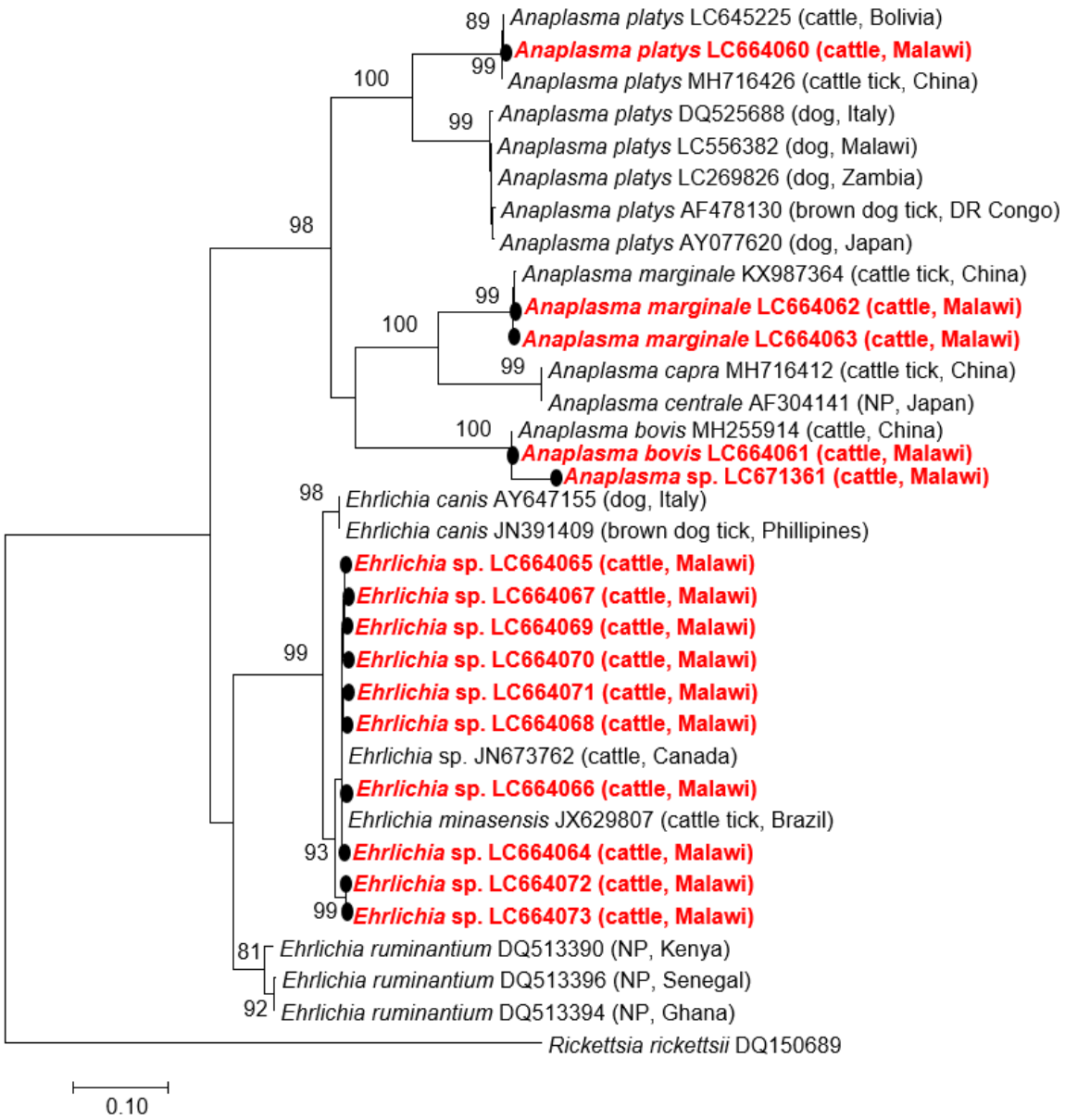

Figure 4. The maximum likelihood phylogenetic tree of *Anaplasma* and *Ehrlichia* species detected in cattle in Malawi. The analysis was based on partial sequences of *gltA* gene which was constructed using the Tamura 2 parameter model. *Rickettsia rickettsii* was used as outgroup. All bootstrap values > 75% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in red. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.10 is the genetic distance of each sequence from the root of the tree.

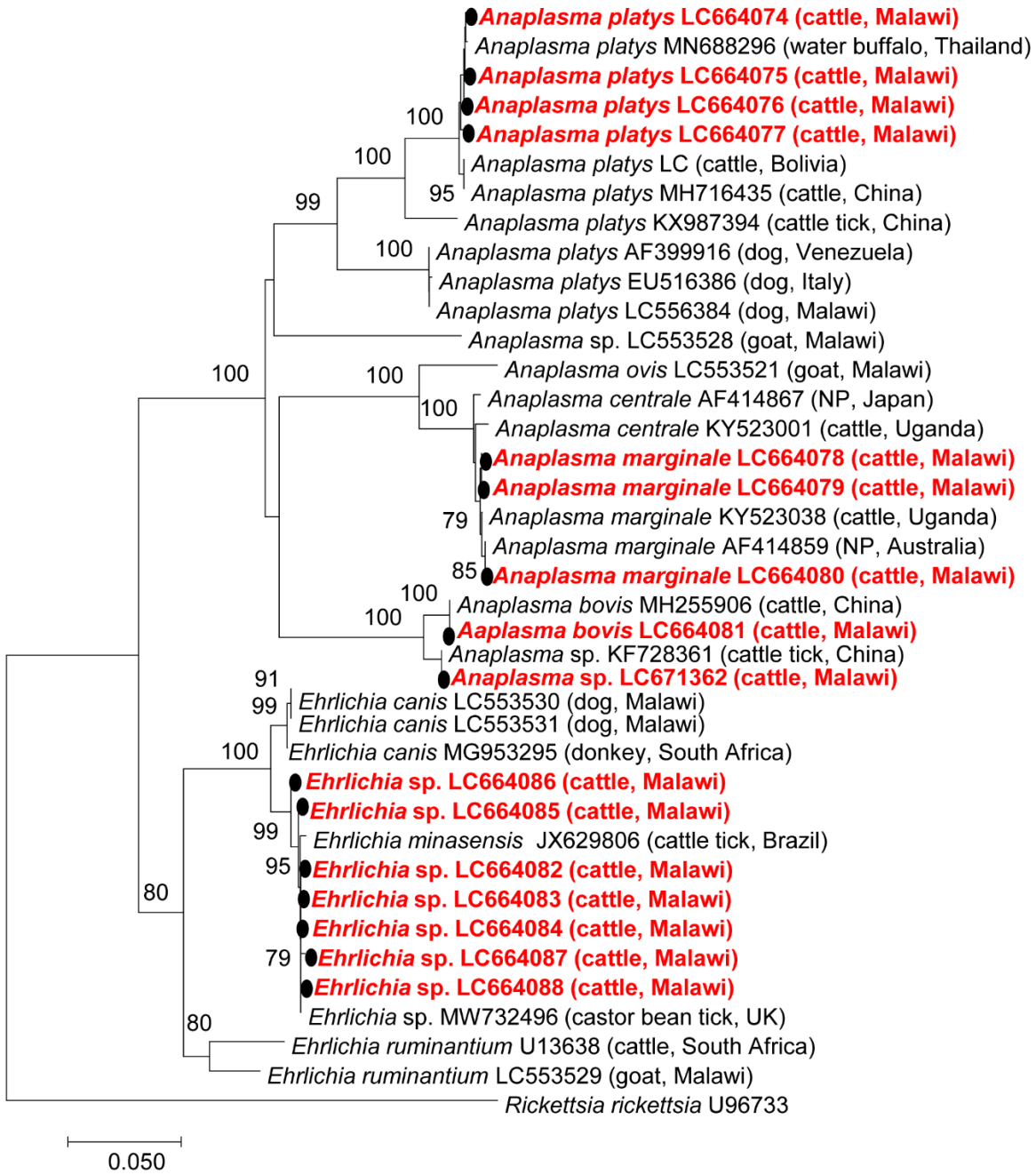

Figure 5. The maximum likelihood phylogenetic tree of the *Anaplasma* and *Ehrlichia* species detected in cattle in Malawi. The analysis was based on partial sequences of *groEL* gene which was constructed using the Tamura 2 parameter model. *Rickettsia rickettsii* was used as outgroup. All bootstrap values > 75% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.050 is the genetic distance of each sequence from the root of the tree.

368 **4. Discussion**

369 Tick-borne Anaplasmataceae and piroplasms in cattle have been investigated using
370 molecular techniques in the neighboring countries of Malawi, such as Mozambique (Martins
371 et al., 2010, 2008; Alfredo et al. 2005), Tanzania (Ringo et al., 2020, 2018; Swai et al., 2009),
372 and Zambia (Tembo et al., 2018; Simuunza et al., 2011; Makala et al., 2003). However, in
373 Malawi, the epidemiological data on TBPs of cattle is mainly based on basic blood smear and
374 serological examinations. This is the first study that has investigated tick-borne
375 Anaplasmataceae and piroplasms in cattle in Malawi using molecular techniques. DNA
376 nucleotide sequence analysis has shown that cattle in Malawi were infected with *A. bovis*, *A.*
377 *marginale*, *A. platys*-like, uncharacterized *Anaplasma* species, uncharacterized *Ehrlichia*
378 species*, B. bigemina*, *T. mutans*, *T. parva*, *T. taurotragi, T. velifera* and *Theileria* sp. MSD like
379 strain.

380 The 18S rDNA multiplex PCR assay designed in this study for the identification of
381 *Babesia* and *Theileria* species would be a useful resource in the resource limited settings
382 without the use of sequencing technology in the diagnosis of tick-borne piroplasms especially
383 when co-infections are highly prevalent. This assay may also be used to screen for the targeted
384 *Babesia* and *Theileria* species before animals are introduced in a new area. Multiplex PCR
385 assays have the advantage of being relatively cost effective compared to the sequencing
386 technologies such as next generation sequencing (NGS) (Qiu et al., 2018). However, the major
387 limitation of multiplex PCR assay is that only targeted pathogens can be detected. This does
388 not allow the detection of novel or non-characterized pathogens. On the other hand, although
389 NGS technology is expensive, it has the advantage of being able to detect non-target pathogens
390 (Squarre et al., 2020).

391 *Anaplasma marginale* which causes bovine anaplasmosis was detected in samples from
392 all the six sampling sites. The observed prevalence of 24.6% is comparable to 25.7% reported

393 in Zambia (Tembo et al., 2018). However, it is higher than 10.2%, 7.4% and 18.0% reported
394 in Tanzania (Ringo et al., 2020, 2018; Swai et al., 2007) and 9.4% reported in Mozambique
395 (Martins et al., 2010). The *A. bovis* was detected at a prevalence of 2.9%. Although it is
396 considered non-pathogenic, it has been reported to cause mild disease in calves (Park et al.,
397 2018), and could be fatal when there is co-infection with other tick-borne pathogens (Jurković
398 et al., 2020).

399 *Anaplasma platys*-like infection in cattle with a prevalence of 13.6% is being reported
400 for the first time in southern Africa. *Anaplasma platys* is known to be pathogenic in dogs where
401 it cause infectious cyclic thrombocytopenia. The clinical disease in dogs is characterized by
402 lethargy, anorexia, fever, weight loss and bleeding disorder (Skyes & Foley, 2014). The
403 prevalence of 13.6% reported herein is higher than 3.5% reported in Tunisia in North Africa
404 (Ben Said et al., 2017). However, it is lower than 44.7% reported in Kenya in East Africa
405 (Peters et al., 2020). This finding suggests that either the pathogen is expanding its geographical
406 distribution or that it was previously neglected. The phylogenetic trees of the *gltA* and *groEL*
407 genes showed that *A. platys*-like isolates from cattle, water buffalo and associated ticks
408 clustered separately from the isolates from dogs and associated ticks as previously reported
409 (Ogata et al., 2021). This is evident because dogs are specifically infected with the pathogenic
410 *A. platys* while ruminants are infected with genetically related strains (*A. platys*-like).

411 In this study, *Ehrlichia* species have been detected at a prevalence of 16.2%. All
412 sequences obtained were closely related to sequence of *E. minasensis* (JX629807) isolated from
413 *R. microplus* tick which was previously reported to be closely related to *Ehrlichia canis*
414 (Cabezas-Cruz et al., 2012). This finding is also in accordance with the study conducted in
415 Kenya by Peters et al. (2020), where all sequences of *Ehrlichia* species detected were closely
416 related to *E. minasensis*.

417 However, *E. ruminantium* which causes bovine ehrlichiosis (heartwater) was not
418 detected in this study, although it has been reported with prevalence ranging from 0.33% to
419 45.3% in neighboring Zambia (Tembo et al., 2018; Simmunza et al., 2011). The failure to detect
420 the pathogen in this study may be either due to the predisposition of *E. ruminantium* to infect
421 vascular endothelial cells but not circulating cells which limits rickettsemia and chances of
422 being detected in peripheral blood (Deem, 2008).

423 The prevalence of *B. bigemina* of 2.6% reported in this study is within the same range
424 as 3.3% (Tembo et al., 2018) and 5.5% (Simuunza et al., 2011) reported in Zambia and 5.1%
425 (Ringo et al., 2020) reported in Tanzania. However, in this study, *B. bovis* which is the most
426 pathogenic *Babesia* species was not detected in the examined animals. This is in contrast to
427 7.7% (Tembo et al., 2018) and 15.5% (Simuunza et al., 2011) reported in Zambia and 4.5%
428 reported in Tanzania (Ringo et al., 2020). The negative results of *B. bovis* may be due to low
429 prevalence of the pathogen in the sampled areas or due to small sample size used in this study.

430 *Theileria parva* which causes East Coast fever (ECF) was one of the pathogens reported
431 with the highest frequency in this study. The detection of *T. parva* at all the six sampling sites
432 including the previously non-endemic region indicates that the pathogen has expanded its
433 geographical distribution in Malawi. The prevalence in this study (33.0 %) is comparable to
434 34.4% and 41.2% reported in Tanzania (Ringo et al., 2020; 2018) but higher than 24.4% and
435 0.3% reported in Zambia (Simuunza et al., 2011; Tembo et al., 2018). However, because in
436 some sampling sites in the central region the Muguga cocktail vaccine is used to control ECF
437 some positive samples may be due to the vaccinations. The phylogenetic analysis also showed
438 that the sequences of *T. parva* from Malawi clustered together with sequences obtained from
439 the components of the Muguga cocktail vaccine (Muguga, Kiambu-5 and Serengeti
440 transformed) deposited in GenBank even those from animals without vaccination history.
441 Furthermore, the obtained sequences of *T. parva* clustered with the sequence of Katete vaccine

442 strain from Zambia. Since the stocks of the Muguga cocktail vaccine are originally from Kenya
443 and Tanzania, the findings of this study may suggest that the strains of *T. parva* circulating in
444 cattle in eastern and southern Africa are closely related to one another and the vaccine strains.
445 However, more detailed studies are required to support this hypothesis as the marker used (18S
446 rDNA) is too conserved amongst the *T. parva* strains.

447 There was high infection rates of *T. mutans* and *T. velifera* at 73.8% and 53.4%,
448 respectively. These rates are higher than 38.1% and 3.4% reported in Tanzania (Ringo et al.,
449 2020) and 54.5% and 51.5% in Zambia (Tembo et al., 2018), respectively. *Theileria mutans*
450 has been reported to be the most prevalent tick-borne pathogen in both Tanzania (Ringo et al.,
451 2020, 2018) and Zambia (Tembo et al., 2018; Simuunza et al., 2011). The *T. mutans* sequences
452 obtained in this study clustered in two clades, one with *T. mutans* (AF078815) isolated from
453 cattle in South Africa and another with *Theileria* sp. MSD like (AF078816) a strain of *T.*
454 *mutans* (Chaisi et al., 2013) reported from cattle in South Africa. This shows that there are at
455 least two strains of *T. mutans* circulating in cattle in Malawi. The finding of closely related
456 strains reported in South Africa may suggest that most strains circulating in cattle in southern
457 Africa are closely related. Although *T. mutans* infection in cattle is considered less pathogenic,
458 it has been reported that mixed infection with *T. parva* increases the pathogenicity of *T. parva*
459 (Morrison, 2010).

460 *Theileria taurotragi* which is considered less pathogenic in cattle but when clinical
461 disease occurs it has been reported to present with clinical signs like those of ECF was also
462 detected in cattle in Malawi. The phylogenetic tree of the *18S rDNA* supports the close
463 relatedness with *T. parva* as they cluster in the same clade. The vector tick for both *T. parva*
464 and *T. taurotragi* is the brown ear tick *R. appendiculatus* (Walker et al., 20003; Ringo et al.,
465 2020). The positive detection rate of 12.6% reported in this study is lower than 30.9% reported

466 in Tanzania (Ringo et al., 2020) and 27.5% reported in Zambia (Simuunza et al., 2011).
467 However, it is comparable to 11.4% reported by another study in Zambia (Tembo et al., 2018).

468 The detection of co-infections in 152 animals (79.6%) with both Anaplasmataceae and
469 piroplasms is an important finding as it complicated the proper diagnosis and treatment of the
470 animals that show clinical disease as some combinations need different treatment regimen. The
471 co-infection rate reported in this study was higher than those reported in neighboring Tanzania
472 of 36.4% (Ringo et al., 2020) but lower than those reported in Zambia of 52.9% and 75.6% by
473 Simuunza et al. (2011) and Tembo et al. (2018), respectively. Mixed infections with TBPs that
474 are individually less pathogenic than other species within the same genus have been reported
475 to cause clinical disease (Jurković et al., 2020). However, another study has suggested that this
476 may be beneficial to the animals as the less pathogenic species may help to generate immunity
477 against the more pathogenic species by heterologous protection (Woolhouse et al., 2015). In
478 this study, although there were many combinations of mixed infections, only four combinations
479 were statistically significant suggesting that these pathogens have a high likelihood of being
480 detected when there is co-infection and may be transmitted by same tick species. *Theileria*
481 *mutans* and *T. velifera* co-infection correlation in cattle was also reported in Zambia (Tembo
482 et al., 2018). However, in this study, there was no co-infection correlation between *T. parva*
483 and the causative agents of benign theilerioses (*T. mutans*, *T. taurotragi* and *T. velifera*) in this
484 study as also previously reported in Zambia (Tembo et al., 2018). This finding shows that the
485 challenge to TBPs is widespread in the southern Africa and collaborative efforts to develop
486 novel methods to control ticks and tick-borne diseases are warranted.

487 The high co-infection rate may have resulted from the examined animals being infested
488 with different tick species which are vectors of different pathogens detected in this study at the
489 same time (Foughali et al., 2021). The transmission of different pathogens by the same tick
490 species such as *T. parva* and *T. taurotragi* which are both transmitted by *R. appendiculatus*

may also play a role in the high co-infection rate (Ringo et al., 2020). Since there is no data on tick's specificity, further studies focusing on tick tropism to understand the vector competence of the ticks infesting cattle in Malawi are required.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Author contributions

R.N. and E.C. conceived and designed the experiments; R.N. and C.S. obtained funding; E.C, E.M, collected samples; E.C., G.P., A.E.A., performed the experiments; E.C. and W.M.A.M., analyzed the data; E.C. Writing- Original draft preparation; K.H., C.S., K.K., N.N. and R.N. Supervision; K.H., K.K., N.N., C.S. and R.N. Writing- Reviewing and Editing. All authors approved the final version of the manuscript.

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