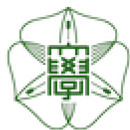

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Molecular identification and genetic characterization of tick-borne pathogens in sheep and goats at two farms in the central and southern regions of Malawi

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20 **Abstract**

21 Tick-borne diseases (TBDs) caused by pathogens belonging to the genera *Anaplasma, Ehrlichia,*
22 *Babesia* and *Theileria* in small ruminants are widespread in the tropical and sub-tropical countries.
23 The epidemiology of tick-borne pathogens (TBPs) in small ruminants is less understood compared to
24 those infecting cattle in general. This study was carried out to investigate and characterize TBPs in
25 sheep and goats using molecular tools. A total of 107 blood samples from sheep (n = 8) and goats (n =
26 99) were collected from animals that were apparently healthy from two farms in the central and the
27 southern regions of Malawi. The V4 hypervariable region of the 18S ribosomal RNA gene (rDNA)
28 and the V1 hypervariable region of the 16S rDNA polymerase chain reaction (PCR) assays were used
29 for detection of tick-borne piroplasms and Anaplasmataceae, respectively. Almost the full-length 18S
30 rDNA and the heat shock protein (*groEL*) gene sequences were used for genetic characterization of the
31 piroplasms and Anaplasmataceae, respectively. The results showed that 76.6 % of the examined
32 animals (n = 107) were positive for at least one TBP. The overall co-infection with at least two TBPs
33 was observed in forty-eight animals (45 %). The detected TBPs were *Anaplasma ovis* (65 %), *Ehrlichia*
34 *ruminantium* (4%), *Ehrlichia canis* (2%), *Babesia* strain closely related to *Babesia gibsoni* (1%),
35 *Theileria ovis* (52 %), *Theileria mutans* (3%), *Theileria separata* (2%), *Anaplasma* sp. (1%) and
36 *Theileria* sp. strain MSD-like (17 %). To the authors knowledge this is the first molecular study of
37 TBPs in sheep and goats in Malawi. These results have therefore provided a significant milestone in
38 the knowledge of occurrence of TBPs in sheep and goats in Malawi, which is prerequisite to proper

39 diagnosis and control.

40

41 **Keywords**: Tick-borne pathogens; Goats; Sheep; Malawi; Molecular identification; Genetic

42 characterization

43

44 **1. Introduction**

45 Ticks transmit a wide range of pathogens, which include bacteria (spirochetes and Rickettsiales),

46 parasites (protozoa and nematodes) and viruses (Crowder et al., 2010; de la Fuente et al., 2008; Matjila

47 et al., 2008). Tick-borne diseases (TBDs) are some of the limiting factors in the development of small

48 ruminant production worldwide where vector ticks are present (Yin and Luo, 2007). Some of the

49 economically important TBDs of small ruminants include anaplasmosis, babesiosis, ehrlichiosis and

50 theileriosis (Uilenberg, 1995; Friedhoff, 1997).

51 Anaplasmosis in small ruminants is caused by *Anaplasma ovis* which is usually subclinical but may be

52 characterized with a low-grade fever with minimal impact on the animal wellbeing (Cabezas-Cruz et al.,

53 2019). However, under stressful conditions and other secondary infections clinical disease may occur

54 (Renneker et al., 2013). Some highly pathogenic strains associated with high mortality rates of 40– 50 %

55 in small ruminants such as *An. ovis* Haibei strain have also been documented (Lu, 1997). Furthermore,

56 *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, *Anaplasma*

57 *marginale*, *Anaplasma centrale* and *Anaplasma bovis* which infect cattle have also been reported in

58 sheep and goats (da Silva et al., 2018; Yousefi et al., 2017; Ben Said et al., 2015; Liu et al., 2012).
59 However, their clinical and economical significance in small ruminants is not well understood.
60 Ehrlichiosis in small ruminants is caused by *Ehrlichia ruminantium, Ehrlichia ovina* and *Ehrlichia* sp.
61 Omatjenne (Bilgic et al., 2017). *Ehrlichia ruminantium* is economically the most important species in
62 small ruminants and the one commonly diagnosed in sub-Saharan Africa (Rango et al., 2019; Rango et
63 al., 2018). It is mainly transmitted by the vector ticks *Amblyomma variegatum* and *Amblyomma*
64 *hebraeum* which are widespread in Africa and southern Africa, respectively (Walker et al., 2003).
65 Babesiosis is principally caused by *Babesia ovis* and *Babesia motasi* (Liu et al., 2007), which are the
66 most pathogenic species in small ruminants. Recently another pathogenic species *Babesia* sp.
67 Mymensingh was reported in sheep and goats from Vietnam (Sivakumar et al., 2020). Other species
68 include *Babesia taylori*, *Babesia foliata* and non-pathogenic *Babesia crassa* (Hashemi-Fesharki and
69 Uilenberg, 1981). Theileriosis in small ruminants is caused by *Theileria lestoquardi*, *T. ovis*, *Theileria*
70 *recondita*, *T. separata*, *Theileria luwenshuni* (=*Theileria* sp. China 1), *Theileria uilenbergi* (=*Theileria*
71 sp. China 2) and *Candidatus* Theileria sp. (OT1 and OT2) (Yin et al., 2007; Luo and Yin, 1997).
72 Among these, *T. lestoquardi*, *T. luwenshuni* and *T. uilenbergi* have been reported to be pathogenic in
73 sheep and goats while *T. ovis* and *T. separata* are non-pathogenic (Luo and Yin, 1997; Uilenberg, 1981).
74 In Malawi, sheep and goats are kept in all the three geographical regions (northern, central and
75 southern). The 2018 national housing and population census showed that 0.4 % and 17.8 % of the total
76 3,984,981 households own sheep and goats, respectively (NSO, 2018). Small ruminants especially

77 goats play a vital role in improving the socioeconomic standards as proceeds from goat sells account
78 for about 61.2 % of the total household income from livestock (Kaumbata et al., 2020). Ticks are
79 widespread throughout the country and this pose a serious threat to livestock production (Chintsanya
80 et al., 2004; Musisi and Kamwendo, 1996). Several tick species that are known to transmit TBPs,
81 namely *Am. variegatum, Hyalomma truncatum, Rhipicephalus appendiculatus, Rhipicephalus*
82 *microplus, Rhipicephalus decoloratus, Rhipicephalus bursa, Rhipicephalus simus,* and *Rhipicephalus*
83 *sanguineus* sensu lato have all been reported in Malawi (Walker et al., 2003). The most diagnosed
84 TBDs in cattle in Malawi are theileriosis and anaplasmosis, which is based on clinical presentation of
85 the disease, basic parasitological examination, postmortem findings and serology (DAHLD, 2018).
86 The aim of this study was to detect and genetically characterize TBPs in small ruminants in Malawi
87 using molecular techniques.
88
89 **2. Materials and methods**
90
91 **2.1 Ethical consideration**
92 DNA blood samples from sheep (n = 8) and goats (n = 99) were used in this study. This was a parallel
93 study of our initial study (Chatanga et al., 2020) which was approved by Ministry of Agriculture,
94 Irrigation and Water Development (MoAIWD) in Malawi through the Department of Animal Health
95 and Livestock Development (DAHLD) reference number 10/15/32/D and permission for sampling

96 was obtained from Lilongwe University of Agriculture and Natural Resources (LUANAR) and
97 Mikolongwe Veterinary Station.
98

99 **2.2 Study area and sample collection**

100 The blood samples were collected at Bunda student farm of LUANAR in Lilongwe district in the
101 central region and Busa farm at Mikolongwe Veterinary Station in Chiradzulu district in the southern
102 region (Supplementary Figure S1). Approximately 5 mL of blood was collected from the external
103 jugular vein into EDTA vacutainer tubes. The sampled animals were grouped into two, the young
104 (less than 1 year old) and the adult (more than 1 year old) based on the records at the two farms. DNA
105 was extracted from 200 μl whole blood using the Quick Gene DNA whole blood kit S (DB-S) (Kurabo
106 Industries Ltd., Osaka, Japan) according to the manufacturers' instructions. The extracted DNA was
107 stored at -20°C until required for use.
108

109 **2.3 PCR and sequencing**

110 A polymerase chain reaction (PCR) assay targeting the V4 hypervariable region (approximately 460 -
111 540 bp) of 18S ribosomal RNA gene (rDNA) was used for screening of *Babesia* and *Theileria* species
112 using reverse line blot (RLB) PCR assay as described by Gubbels et al. (1999). To characterize
113 *Babesia*- and *Theileria*-positive samples, we amplified almost the full length of the 18S rDNA

114 (approximately 1,500 bp) using nested BTH PCR assays as described by Masatani et al. (2017).

115 However, in 18 samples that were positive for *Theileria* spp. by RLB PCR, but for which the BTH

116 PCR assays failed, *Theileria* genus-specific nested PCR assays were used to amplify approx. 1,421 bp

117 of the 18S rDNA as described by Heidarpour Bami et al. (2009).

118 The screening for *Anaplasma* and *Ehrlichia* species was done using EHR PCR assay targeting the V1

119 hypervariable region (approximately 345 bp) of the 16S rDNA as described by Parola et al. (2000).

120 All samples that were positive for *Anaplasma* and *Ehrlichia* spp. were characterized using a *groEL*

121 gene PCR (Selmi et al., 2019; Qiu et al., 2018). A major surface protein 4 (*msp4*) gene PCR was also

122 used to further characterize *An. ovis* detected in this study as it has been reported that the *msp4* gene

123 has high resolution in characterization of *An. ovis* than the *groEL* gene (de la Fuente et al., 2002;

124 Selmi et al., 2019).

125 The primers used in this study, their annealing temperatures and expected amplicon size are listed in

126 Supplementary Table S1 (Gubbels et al., 1999; Parola et al., 2000; Masatani et al., 2017; Heidarpour

127 Bami et al., 2009; Rar et al., 2010; Liz et al., 2000; Gofton et al., 2016; de la Fuente et al., 2003). All

128 PCR reactions were conducted in a 25 μl reaction mixture containing 0.5 μl of Tks Gflex DNA

129 Polymerase 1.25 units/ μl (TaKaRa Bio Inc., Shiga, Japan), 200 nM of each primer, 1.0 μl of template

130 DNA and molecular grade water. The cycling conditions and amplicons electrophoresis and

131 visualization under UV light was done as described by Chatanga et al. (2020).

132 The amplicons from all 2nd BTH, 2nd *Theileria* spp., 2nd *groEL* and *msp4* PCR assays were purified

133 using ExoSAP-IT (USB Corporation, Cleveland, OH) according to manufacturer's instructions, and

134 sequenced using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems,

135 Foster City, CA, USA) utilizing an ABI Prism 3130x genetic analyser (Applied Biosystems). Sequence

136 editing was conducted using ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan). The

137 sequences generated in this study were submitted to the DNA Data Bank of Japan (DDBJ) under the

138 accession numbers LC553508 to LC553515 for 18S rDNA, LC553516 to LC553531 for the *groEL*

139 gene and LC553532 to LC553542 for the *msp4* gene.

140

141 **2.4 Data analysis**

142 Alignments of the consensus nucleotide sequences generated from the amplified DNA fragments were

143 made using ClustalW in Molecular Evolutionary Genetics Analysis (MEGA version 7) (Kumar et al.,

144 2016). To further characterise the sequences obtained in this study, maximum likelihood (ML)

145 phylogenetic trees were constructed using MEGA version 7 software with other sequences deposited

146 in GenBank. Kimura 2-parameter model was used to generate the 18S rDNA and *msp4* gene trees

147 while General Time Reversible (GTR+G) model was used to generate *groEL* gene tree with 1,000

148 bootstrap values. Chi-square statistics was used to determine the correlation between TBP detection

149 rate with regard to study site, age, breed, species and sex of the animals, where the level of significance

150 was set at $p < 0.05$.

151

152 **3. Results**

153 **3.1. PCR-positive**

154 Table 1 shows the PCR-positive rates of *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* spp. From the
155 tested animals, seven sheep (88 %) and 72 goats (73 %) were infected with one of either *Theileria* or
156 *Babesia* spp., while for *Anaplasma* and *Ehrlichia* spp. it was found that eight sheep (100 %) and 74 goats
157 (75 %) were infected with either of the pathogens. *Theileria ovis* infection rate in sheep was 88 % while
158 it was 49 % in goats. Those TBPs that were detected only in goats were *T. mutans* (3%), *T. separata*
159 (2%), *Theileria* sp. strain MSD-like (18 %) and *Babesia gibsoni*-like strain (1%). The *An. ovis* infection
160 rates in sheep and goats were 100 % and 62 %, respectively. An uncharacterized *Anaplasma* sp. was
161 detected in one goat (1%), *E. ruminantium* was detected in three sheep (38 %), and one goat (1%) while
162 *E. canis* was only detected in two goats (2%).

163 Mixed infection of *Theileria* spp. and *Anaplasma* or *Ehrlicha* spp. were observed in 48 animals (45 %).
164 Infection with three TBPs; *T. ovis*, *An. ovis and E. ruminantium* was detected in four animals (4%), while
165 infection with two TBPs, *T. ovis and An. ovis* was detected in 36 animals (34 %). A mixed infection of
166 *An. ovis* and *Theileria* sp. strain MSD-like was detected in eight animals (7%).

167

168 **3.2 Statistical analysis**

169 There was a statistically significant correlation between TBP positive detection rate and age which was
170 higher in older animals (85 %) than young animals (54 %) (X^2 = 11.2668; df = 1; p = 0.000789).

171 However, there were no statistically significant correlations between the TBPs positive detection rates

172 and the breed, study site, species and sex of the animals (X^2 = 4.091; *df* = 2; *p* = 0.251801), (X^2 =

173 1.6977; *df* = 1; *p* = 0.192588), (X^2 = 0.6544; *df* = 1; *p* = 0.418544) and (X^2 = 0.0072; *df* = 1; *p* =

174 0.932563), respectively (Supplementary Table S2).

175

176 **3.3 Phylogenetic analysis**

177 To compare our sequences of *Babesia* sp. and *Theileria* spp. with those deposited in the GenBank we

178 generated a ML tree based on almost full length of the 18S rDNA (Figure 1). The results showed that

179 our *Theileria* sequences clustered with *T. ovis* (S1, S2 and G18)*, T. separata* (G57), *T. mutans* (G5)

180 and *Theileria* sp. strain MSD-like (G9). Interestingly, we obtained *Babesia* strain sequence (G50) that

181 was closely related to *B. gibsoni* (JX962780) reported from a pig in China (Figure 1).

182 The tree for *groEL* gene showed that some of our sequences clustered with of *An. ovis*, while one

183 sequence of *Anaplasma* sp. (G29) clustered separately and had only 79% identity with *An.*

184 *phagocytophilum* (HQ629909; AY529489). We also obtained sequences for *E. canis* (G46 and G77)

185 and *E. ruminantium* (G18) which shared 100 % and 97 % identities with *E. canis* FL strain (U96731)

186 and *E. ruminantium* Kiswani and Welgevonden strains (DQ647004; U13638), respectively (Figure 2).

187

188 **4. Discussion**

189 *Anaplasma ovis* infection has been reported in several domestic and wild animal species in Africa (Ben

190 Said et al., 2018). The overall prevalence of 65 % in this study shows high infection rate which is
191 comparable to those reported in other sub-Saharan African countries such as 36.3 % in South Africa
192 (Ringo et al., 2018) and 34.2 % in Kenya (Ringo et al., 2019). This shows that *An. ovis* is an important
193 TBP in the region especially with the common finding of mixed infections with other TBPs which
194 enhances the occurrence of clinical disease and complicates its diagnosis and control (Bilgic et al., 2017).
195 Despite the sampling sites being more than 500 km apart most of the *An. ovis* sequences obtained from
196 the two study sites generally clustered closely in the *groEL* tree (Figure 2). This is suggestive that closely
197 related strains of *An. ovis* are circulating in small ruminants in Malawi, as also reported in Kenya (Rango
198 et al., 2019). However, we observed that some *msp4* sequences clustered in different clades in ML tree
199 (Supplementary Figure S2), which may suggest the presence of divergent *An. ovis* in Malawi, as
200 observed by Selmi et al. (2019).
201 The *E. ruminantium*-positivity rate of 4% is similar to that found in other studies in sub-Saharan African
202 countries, in which generally low prevalences of 14.3 % in South Africa (Ringo et al., 2018) and 7.9 %
203 in Kenya (Ringo et al., 2019) were reported in small ruminants. We did not observe any clinical signs of
204 ehrlichiosis in the animals during the sampling period which is in accordance with other studies that
205 have reported that the occurrence of clinical disease is mainly dependent on the pathogenicity of the *E.*
206 *ruminantium* strain and the breed or species of the infected animals (Ahmadu et al., 2004; Steyn and
207 Pretorius, 2020). *Ehrlichia canis* which is a pathogen of dogs was detected in goats in this study.
208 Infection of *E. canis* in both domestic and wild ruminants has been documented previously (Li et al.,

209 2016; Qiu et al., 2016; Zhang et al., 2015). This finding emphasizes the need to apply molecular
210 techniques that can detect a wider range of pathogens when screening for TBPs as species specific
211 methods may result in failure to detect novel infections.

212 We detected a *Babesia* strain that is closely related to *Babesia gibsoni* in goat based on the almost full-
213 length sequence of 18S rDNA. This finding supports previous studies that have reported *B. gibsoni*
214 infection in non-canine species such as cattle, goat, sheep and donkey (Li et al., 2015). However, species-
215 specific BgTRAP gene, cytochrome *B* gene and *p18* gene primers for *B. gibsoni* failed to amplify our
216 sample (G50) (data not shown). This suggests that our sample may not be infected with *B. gibsoni*
217 reported from dogs but a closely related *Babesia* strain. This may also suggest that small ruminants are
218 infected with a previously uncharacterized strain of *Babesia* closely related to *B. gibsoni*. In this study,
219 *B. ovis* and *B. motasi*, the most pathogenic *Babesia* spp. in small ruminants, were not detected.

220 *Theileria ovis* and *T. separata* the causative agents of theileriosis in small ruminants have been detected
221 in this study. Furthermore, we detected *T. mutans* and *Theileria* sp. closely related to *Theileria* sp. strain
222 MSD reported in cattle (Chae et al., 1999) for the first time in small ruminants. *Theileria lestoquardi,*
223 the causative agent of malignant theileriosis in small ruminants, was not detected in this study, which
224 might be attributed to the limited sample size employed here. A further survey with a larger sample size
225 needs to be carried out to confirm its absence.

226 Our observation of the infection rate being higher in older animals compared to young animals may be
227 due to prolonged exposure of the older animals to vector ticks. The higher prevalence of anaplasmosis

228 and theileriosis in sheep and goats is in accordance with the reports from the department of animal health

229 and livestock development in Malawi (DAHLD, 2018). This study has also shown that despite the

230 absence of the vector ticks on the sampled animals due to recent application of acaricides the animals

231 were still infected with TBPs. This shows that absence of the ticks on the animals may not imply that

232 the animals are not infected with TBPs.

233

234 **5. Conclusions**

235 In this study, the presence of *An. ovis*, uncharacterized *Anaplasma* sp., *E. canis*, *E. ruminantium*, *T.*

236 *ovis*, *T. separata*, *T. mutans*, *Theileria* sp. strain MSD-like, and *Babesia gibsoni*-like strain infection

237 in sheep and goats has been confirmed using molecular techniques for the first time in Malawi. We

238 have also observed mixed infections which may complicate the proper diagnosis and treatment of

239 TBPs. This information is important for the development and monitoring of the disease's diagnosis,

240 prevention, management and evaluation of control measures. We have also observed new host range

241 of some pathogens which further highlights the need to conduct more studies on TBPs in a variety of

242 hosts in Malawi.

243

244 **Author contributions**

245 **Elisha Chatanga**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology,

246 Visualization, Writing - original draft.**Ryo Nakao**: Conceptualization, Funding acquisition,

247 Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.**Henson**

248 **Kainga**: Resources, Writing - review & editing.**Emmanuel Maganga**: Resources, Writing - review &

249 editing.**Kyoko Hayashida**: Resources, Supervision, Writing - review & editing.**Chihiro Sugimoto**:

250 Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.**Ken**

251 **Katakura**: Resources, Supervision, Writing - review & editing.**Nariaki Nonaka**: Resources,

252 Supervision, Writing - review & editing.

253 **Declaration of Competing Interest**

254 The authors report no declarations of interest.

255

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262

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433 **Figure legends**

434

435 **Figure 1. The maximum likelihood phylogenetic tree of the *Theileria* spp. and *Babesia* sp.** 436 **detected in sheep and goats.** The analysis was based on almost the full-length sequences of 18S

437 rDNA (1400-1500 bp) which was constructed using the Kimura 2 parameter model. *Plasmodium*

438 *falciparum* was used as an outgroup. All bootstrap values > 50 % from 1000 replications are shown

439 on the interior branch nodes and the sequences obtained in this study are indicated in bold. The

440 sample IDs are in the parenthesis after accession number where S and G represent sheep and goat,

441 respectively where the sample was collected.

442

443 **Figure 2. The maximum likelihood phylogenetic tree of the *Anaplasma* spp. and *Ehrlichia* spp.**

444 **detected in sheep and goats.** The analysis was based on partial sequences of *groEL* gene (1000-

445 1100 bp) which was constructed using the General Time Reversible (GTR) +G model. *Rickettsia*

446 *rickettsii* was used as an outgroup. All bootstrap values > 50 % from 1000 replications are shown on

447 the interior branch nodes and the sequences obtained in this study are indicated in bold. The sample

448 IDs are in the parenthesis after accession number where S and G represent sheep and goat,

449 respectively where the sample was collected.

450

451 **Supplementary Figure S1. (A) The location of Malawi on the map of Africa. (B) Map of Malawi**

452 **showing the sites where blood samples were collected.**

453

454 **Supplementary Figure S2. The maximum likelihood phylogenetic tree of the *Anaplasma ovis***

455 **detected in sheep and goats.** The analysis was based on almost the full-length sequences of *msp4*

456 gene (808 bp) which was constructed using the Kimura 2 parameter model. *Anaplasma marginale*

457 was used as an outgroup. All bootstrap values > 50 % from 1000 replications are shown on the

458 interior branch nodes and the sequences obtained in this study are indicated in bold. The sample IDs

459 are in the parenthesis after accession number where S and G represent sheep and goat, respectively

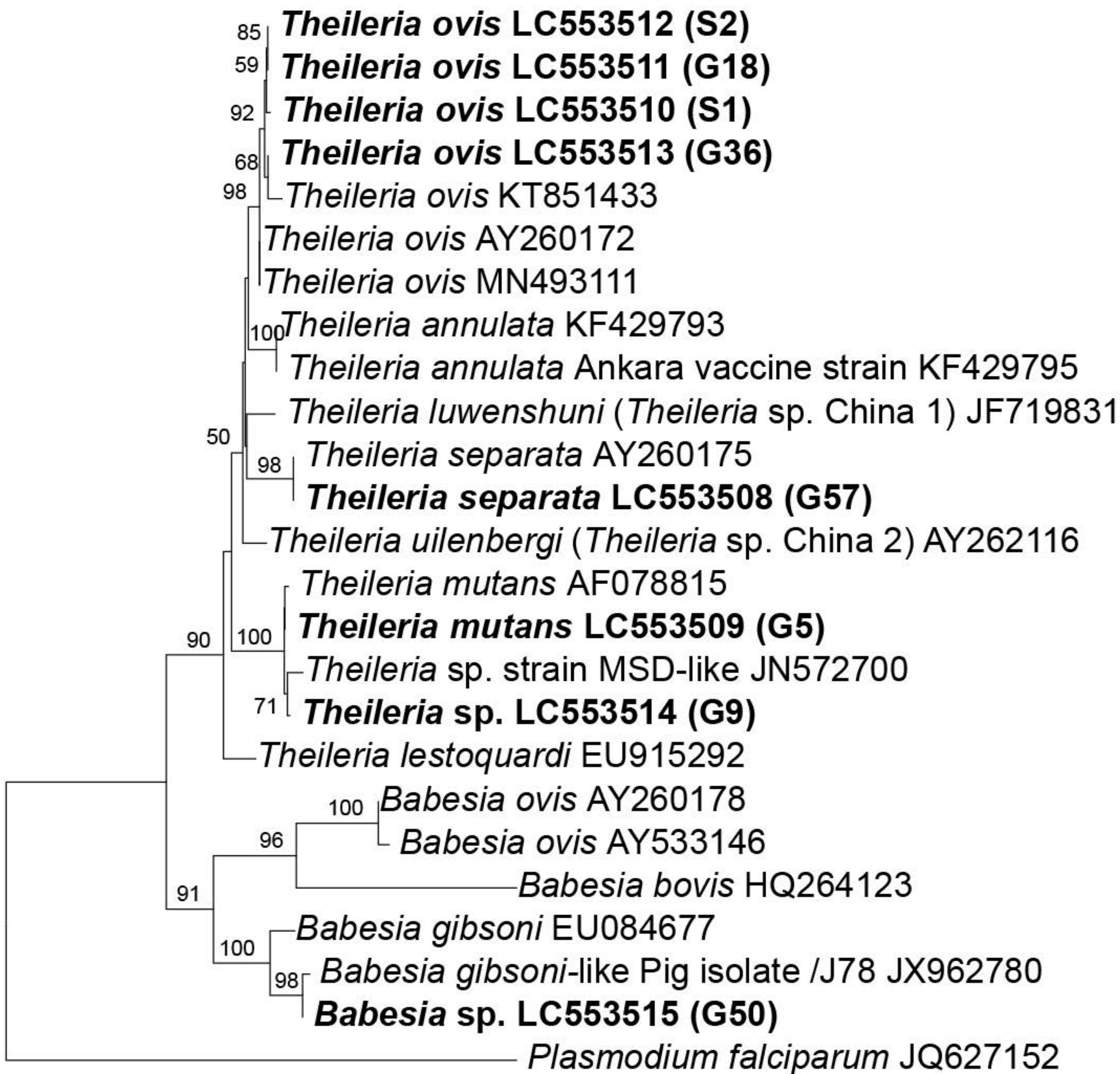
460 where the sample was collected.

Table 1. Tick-borne pathogens (TBPs) detected in sheep and goats in this study.

Study site	Animal spp.	*Theileria ovis*	*T. mutans*	*T. separata*	*Theileria* sp. strain MSD-like	*Babesia gibsoni*-like strain	*A. ovis*	*Anaplasma* sp.	*E. ruminantium*	*E. canis*	*T. ovis* + *A. ovis* + *E. ruminantium*	*T. ovis* + *A. ovis*	*Theileria* sp. strain MSD-like + *A. ovis*	Co-infection♣
Bunda Student Farm	Goats (n = 44)	27 (61)	1 (2)	0	13 (30)	0	35 (80)	1 (2)	1 (2)	0	1 (2)	17 (39)	5 (11)	23 (52)
	Sheep (n = 8)	7 (88)	0	0	0	0	8 (100)	0	3 (38)	0	3 (38)	4 (50)	0	7 (88)
Busa Farm	Goats (n = 55)	21 (38)	2 (4)	2 (4)	5 (9)	1 (2)	26 (47)	0	0	2 (4)	0	15 (27)	3 (5)	18 (33)
Total		55 (51)	3 (3)	2 (2)	18 (17)	1 (1)	69 (65)	1 (1)	4 (4)	2 (2)	4 (4)	36 (34)	8 (7)	48 (45)

Theileria ovis, T. mutans, T. separata and *Babesia gibsoni*-like strain were characterized using BTH PCR while *Theileria* sp. strain MSD-like was characterized using *Theileria* genus-specific PCR assay. *Anaplasma ovis, Anaplasma* sp., *Ehrlichia ruminantium* and *E. canis* were characterized using *groEL* PCR assay. The number of samples positive for each species and the percentages in brackets.

♣ Represent those animals that were positive for more than 1 TBP



Theileria ovis LC553512 (S2)
Theileria ovis LC553511 (G18)
Theileria ovis LC553510 (S1)
Theileria ovis LC553513 (G36)
Theileria ovis KT851433
Theileria ovis AY260172
Theileria ovis MN493111
Theileria annulata KF429793
Theileria annulata Ankara vaccine strain KF429795
Theileria luwenshuni (Theileria sp. China 1) JF719831
Theileria separata AY260175
Theileria separata LC553508 (G57)
Theileria uilenbergi (Theileria sp. China 2) AY262116
Theileria mutans AF078815
Theileria mutans LC553509 (G5)
Theileria sp. strain MSD-like JN572700
Theileria sp. LC553514 (G9)
Theileria lestoquardi EU915292
Babesia ovis AY260178
Babesia ovis AY533146
Babesia bovis HQ264123
Babesia gibsoni EU084677
Babesia gibsoni-like Pig isolate /J78 JX962780
Babesia sp. LC553515 (G50)
Plasmodium falciparum JQ627152
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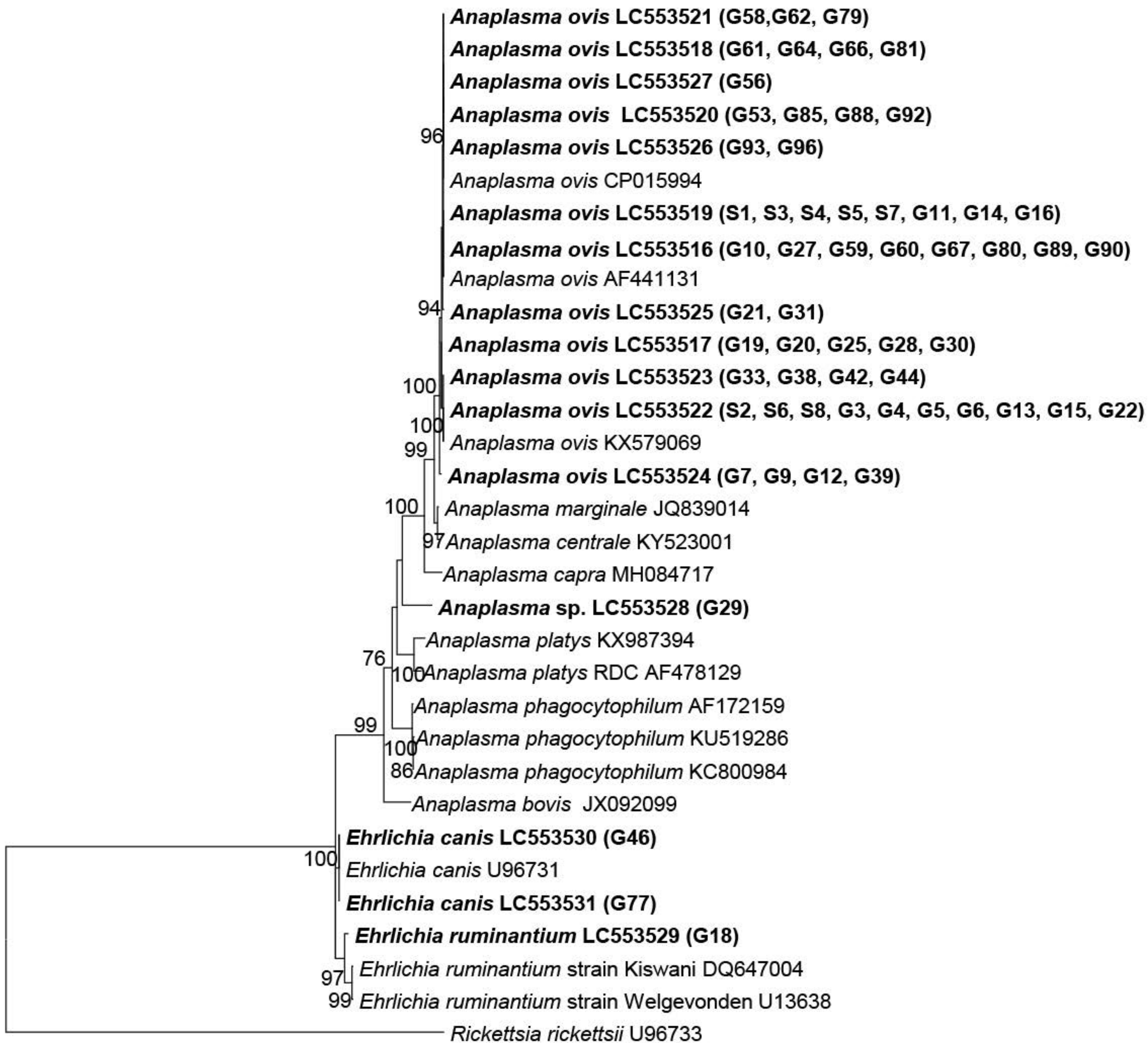

Anaplasma ovis LC553521 (G58,G62, G79)
Anaplasma ovis LC553518 (G61, G64, G66, G81)
Anaplasma ovis LC553527 (G56)
Anaplasma ovis LC553520 (G53, G85, G88, G92)
Anaplasma ovis LC553526 (G93, G96)
Anaplasma ovis CP015994
Anaplasma ovis LC553519 (S1, S3, S4, S5, S7, G11, G14, G16)
Anaplasma ovis LC553516 (G10, G27, G59, G60, G67, G80, G89, G90)
Anaplasma ovis AF441131
Anaplasma ovis LC553525 (G21, G31)
Anaplasma ovis LC553517 (G19, G20, G25, G28, G30)
Anaplasma ovis LC553523 (G33, G38, G42, G44)
Anaplasma ovis LC553522 (S2, S6, S8, G3, G4, G5, G6, G13, G15, G22)
Anaplasma ovis KX579069
Anaplasma ovis LC553524 (G7, G9, G12, G39)
Anaplasma marginale JQ839014
Anaplasma centrale KY523001
Anaplasma capra MH084717
Anaplasma sp. LC553528 (G29)
Anaplasma platys KX987394
Anaplasma platys RDC AF478129
Anaplasma phagocytophilum AF172159
Anaplasma phagocytophilum KU519286
Anaplasma phagocytophilum KC800984
Anaplasma bovis JX092099
Ehrlichia canis LC553530 (G46)
Ehrlichia canis U96731
Ehrlichia canis LC553531 (G77)
Ehrlichia ruminantium LC553529 (G18)
Ehrlichia ruminantium strain Kiswani DQ647004
Ehrlichia ruminantium strain Welgevonden U13638
Rickettsia rickettsii U96733
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94
100
100
99
100
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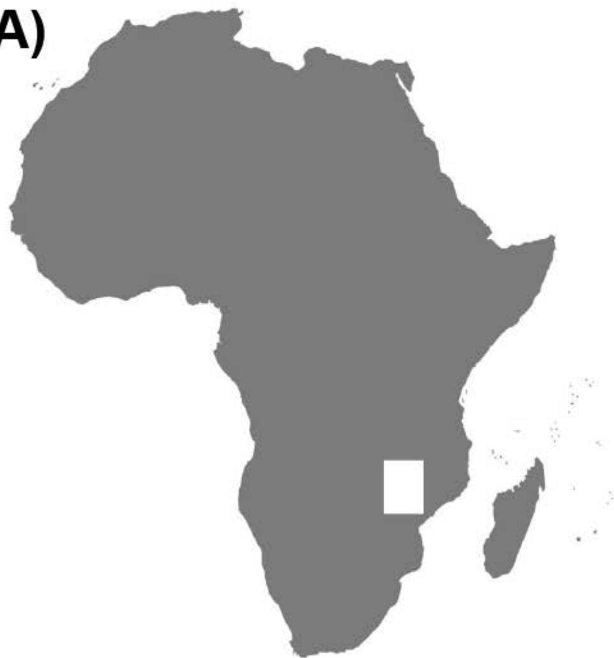
76
100
99
100
86
100
97
99
0.50

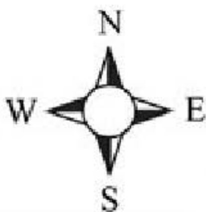


Study site

Major city

Regional boundary

International boundary



100 km

(B)

UNITED REPUBLIC OF TANZANIA

ZAMBIA

NORTHERN

Mzuzu

MALAWI

CENTRAL

Lilongwe

Bunda student farm

MOZAMBIQUE

Busa farm

Blantyre

SOUTHERN

Anaplasma ovis KR608305 Sheep Spain
***Anaplasma ovis* LC553539 (G28, G80)**
***Anaplasma ovis* LC553541(S1, S2, S3, S6, G21)**
***Anaplasma ovis* LC553534 (G19, G37, G40)**
***Anaplasma ovis* LC553536 (G31)**
Anaplasma ovis KM285220 Sheep Tunisia
Anaplasma ovis KY807128 Goat China
Anaplasma ovis JQ621903 Sheep Iran
Anaplasma ovis AY702923 Sheep Italy
Anaplasma ovis KY659324 Sheep Tunisia
Anaplasma ovis LC126849 Cervus elaphus Portugal
Anaplasma ovis MF360029 Sheep Kenya
Anaplasma ovis MF945973 Sheep Burkina Faso
59
Anaplasma ovis MF360026 Sheep Kenya
Anaplasma ovis KY511046 Sheep China
53
Anaplasma ovis JF714148 Cattle Italy
Anaplasma ovis KY659322 Goat Tunisia
Anaplasma ovis MF071307 Sheep China
***Anaplasma ovis* LC553540 (G44)**
95
***Anaplasma ovis* LC553532 (G20, G27, G34, G53, G56, G59, G60, G61, G66, G68, G85)**
***Anaplasma ovis* LC553542 (S2, S5, S7, S8, G26, G42, G58)**
***Anaplasma ovis* LC553533 (G51, G62)**
61
***Anaplasma ovis* LC553538 (G96)**
Anaplasma ovis KY091899 Goat Iran
64
***Anaplasma ovis* LC553535 (G64, G81, G92)**
***Anaplasma ovis* LC553537 (G79)**
Anaplasma marginale KX989519 Cattle India

0.01

Supplementary Table S1. List of primers used in this study.

Primer name	Primer sequence (5′ to 3′)	Target gene	PCR type*	Product size (bp)	Annealing temperature (°C)	Reference
RLB_F	GAGGTAGTGACAAGAAATAACAATA	18S rDNA of *Babesia* & *Theileria* spp.	Single PCR	460-540	55	Gubbels et al., 1999
RLB_R	TCTTCGATCCCCTAACTTTC					
EHR_F	GGTACCYACAGAAGAAGTCC	16S rDNA of Anaplasmataceae	Single PCR	345	61	Parola et al., 2000
EHR_R	TAGCACTCATCGTTTACAGC					
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA of *Babesia* & *Theileria* spp.	1st PCR	1400- 1600	55	Masatani et al., 2017
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	18S rDNA of *Babesia* & *Theileria* spp.	2nd nPCR	1400- 1600	55	Masatani et al., 2017
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
Thei 1F	AACCTGGTTGATCCTGCCAG	18S rDNA of *Theileria* spp.	1st PCR	1412-1421	50	Heidarpour Bami et al., 2009
Thei 1R	AAACCTTGTTACGACTTCTC					
Thei 2F	TGATGTTCGTTTYTACATGG	18S rDNA of *Theileria* spp.	2nd nPCR	1412-1421	55	Heidarpour Bami et al., 2009
Thei 2R	CTAGGCATTCCTCGTTCACG					
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* gene of Anaplasmataceae	1st PCR	1300	54	Rar et al., 2010
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* gene of *Anaplasma* spp.	2nd nPCR	1256	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* gene of *Ehrlichia* spp.	2nd nPCR	1100	50	Gofton et al., 2016
groEL_rev2	GCCGACTTTTAGTACAGCAA					
MSP43	CCGGATCCTTAGCTGAACAGGAATCTT	*msp4* gene of *Anaplasma ovis* & *A. marginale*	Single PCR	851	60	de la Fuente et al., 2003
MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC					

Abbreviations: PCR = Polymerase Chain Reaction; nPCR: nested Polymerase Chain Reaction; F = Forward; R = Reverse; bp = base pairs

* The PCR was either single (PCR) or nested PCR (nPCR)

Supplementary Table S2. Tick-borne pathogens (TBPs) detection rate based on PCR with regard to the host attributes.

Attribute	No. of sheep and goats	No. infected with at least 1 TBP (%)	*p-value*
Study site			0.192588
Bunda student farm	52	37 (71)	
Busa farm	55	45 (82)	
Age			0.000789*
< 1 year	28	15 (54)	
>1 year	79	67 (85)	
Sex			0.932563
Male	9	7 (78)	
Female	98	75 (76)	
Species			0.418544
Goats	99	74 (75)	
Sheep	8	8 (100)	
Breed			0.251801
Saanen (goats)	18	14 (78)	
Boar (goats)	55	45 (82)	
Crossbreed (goats)	26	15 (58)	
Dorper (sheep)	8	8 (100)	

Abbreviation: PCR = Polymerase chain reaction. *Chi-square analysis determined the significant difference between variables (<0.05).