



Title	Potential role of dogs as sentinels and reservoirs for piroplasms infecting equine and cattle in Riyadh City, Saudi Arabia
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1 **Potential role of dogs as sentinels and reservoirs for piroplasms infecting equine and**
2 **cattle in Riyadh City, Saudi Arabia**

3

4 Running title: Dogs as reservoirs for equine/bovine piroplasms

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32

33 **Summary**

34 Canine tick-borne diseases have been considered emerging and re-emerging threats,
35 given their increasing global prevalence. In this molecular survey, we aimed to detect and
36 identify common tick-borne pathogens in dogs from Riyadh city in Saudi Arabia. Initially,
37 the study included 36 dogs visiting private veterinary clinics. PCRs targeting the 18S
38 ribosomal RNA gene (rDNA) of haemoparasites (*Babesia*, *Theileria* and *Hepatozoon*)
39 and the 16S rDNA of Anaplasmataceae were performed. The results showed that 26
40 (72.2%) dogs were infected by some of the haemoparasites under investigation. The
41 sequencing analysis of the amplicons confirmed the infections due to two parasite species
42 *Theileria equi* and *Theileria velifera*. Further examination of guard dogs kept in the horse
43 stables of the Riyadh Municipality revealed that the majority of the tested dogs (65.2%:
44 30 out of 46) were infected with either of the parasites. In addition, the genotypes of all
45 the parasites in these dogs were identical to those of the parasites in the dogs from the
46 veterinary clinics. Thus, it can be concluded that dogs are infected with these

47 haemoparasites and serve as a reservoir for both *T. equi* and *T. velifera* in the study area;
48 however, the clinical implication of this finding is to be studied.

49

50 **Keywords:** Dogs; *Theileria equi*; *Theileria velifera*

51

52

53 **Introduction**

54 Together with the remarkable increase in the amount of DNA sequence data in open
55 repositories, molecular diagnostic techniques have allowed the sensitive and accurate
56 detection of tick-borne pathogens. This has evoked an ever-growing interest in
57 evolutionary biologists to retrieve and use such data to identify orthologous sequences
58 and depicting phylogenetic inferences in an attempt to identify species and/or genotypes
59 more accurately. Thus, studying and controlling an infectious disease implies the need
60 for the knowledge of all factors involved in its transmission.

61 Ticks (Acari: Ixodida) are haematophagous ectoparasites of terrestrial and semi-aquatic
62 mammalian, avian, and reptilian species, which affect domestic animals and wildlife
63 (Dantas-Torres *et al.*, 2013; Barker and Walker, 2014; Panetta *et al.*, 2017). They are
64 important vectors for human and animal diseases, and their global distribution contributes
65 to the increase in the incidence of emerging and re-emerging tick-borne diseases
66 worldwide (Guglielmone *et al.*, 2013; de la Fuente *et al.*, 2017). The prevalence of vector-
67 borne diseases in a population closely reflects the distribution and density of the vectors
68 (Vascellari *et al.*, 2016).

69 *Theileria* spp. and *Babesia* spp. have been reported as a major constraint for the
70 production of small ruminants and large animals in Saudi Arabia (Alanazi *et al.*, 2012,

71 2014; Al-Khalifa *et al.*, 2009; Mostafa and Bin Dajem, 2014). However, limited
72 information about canine vector-borne diseases in Saudi Arabia is available, with only
73 two case reports demonstrating the presence of endemic infections with *Ehrlichia canis*
74 and *Dirofilaria repens* (Sacchini *et al.*, 2007; Tarello, 2003).

75 This study was initially set up to screen for canine haemoparasites or bacteria from the
76 family Anaplasmataceae in dogs admitted to the private veterinary clinics. The results
77 indicated the presence of equine and bovine haemoparasites in dogs. With this in mind,
78 we also investigated the dogs that live together with horses to understand the role of dogs
79 as a reservoir of these parasites.

80

81

82 **Materials and methods**

83 **Study areas**

84 The investigation was conducted in Riyadh city, Saudi Arabia. Riyadh city is the capital
85 of Saudi Arabia, with the following geographical positions: latitude 24°–08° north and
86 longitude 47°–18° east. It has an area of about 1,798 km² and was reported to be inhabited
87 by approximately seven million people in 2016 (General Authority for Statistic, 2016).
88 Riyadh city has a very hot summer, with temperatures reaching up to 49°C or more, and
89 an average temperature of 43°C. Winters are cold with windy nights. The overall climate
90 is arid, with very little annual rainfall (22.6 mm); the relative humidity ranges from 10%
91 to 42% throughout the year (The General Authority of Meteorology and Environmental
92 Protection (GAMEP), Saudi Arabian Government website: <http://www.pme.gov.sa>).

93 **Dogs**

94 Initial investigation included 36 dogs (19 male and 17 female) who visited two private
95 veterinary clinics in Riyadh City. Clinical symptoms, including fever (cut-off value \geq
96 39.5°C), diarrhoea, weakness, emaciation, reddish eyes and haematouria, were recorded
97 by the veterinarians. Second part of this study was conducted on 46 guard dogs (21 male
98 and 25 female) who were kept at 37 horse stables in Riyadh municipality. These dogs
99 were apparently healthy and did not show any clinical signs.

100 **Blood and DNA extraction**

101 A volume of 2-5 ml blood of the cephalic vein were drawn from each dog into EDTA
102 vacuum tubes (BD Vacutainer® Tube, Gribbles Pathology, VIC, Australia) and
103 subsequently dispatched to the Laboratory of Parasitology, Shaqra University, for DNA
104 extraction. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue kit
105 (Qiagen, Hilden, Germany), eluted in 50 μl of elution buffer as per manufacturer's
106 instruction, and stored at -20°C prior to use.

107 **PCR and sequencing**

108 PCR detecting *Babesia*, *Theileria*, and *Hepatozoon* parasites (BTH-PCR1) was carried
109 out to amplify the parasite's 18S ribosomal RNA gene (rDNA) using BTH 18S 1st F: 5'-
110 GTGAAACTGCGAATGGCTCATTAC-3' and BTH 18S 1st R: 5'-
111 AAGTGATAAGGTTACAAAACCTTCCC-3' for primary amplification. This was
112 followed by the nested BTH-PCR2 using BTH 18S 2nd F: 5'-
113 GGCTCATTACAACAGTTATAGTTTATTTG-3' and BTH 18S 2nd R: 5'-
114 CGGTCCGAATAATTCACCGGAT-3' for secondary amplification as described
115 previously (Masatani *et al.*, 2017). PCR detecting members from the Anaplasmataceae
116 family was conducted to amplify bacterial 16S rDNA using EHR16SD: 5'-
117 GGTACCYACAGAAGAAGTCC-3' and EHR16SR: 5'-

118 TAGCACTCATCGTTTACAGC-3' (Parola *et al.*, 2000). PCR reactions were performed
119 in a 25 µl-reaction mixture containing 12.5 µl of 2 × Gflex PCR Buffer (Mg²⁺, dNTP
120 plus) (TaKaRa Bio Inc., Shiga, Japan), 0.5 µl of Tks Gflex DNA Polymerase (1.25
121 units/µl) (TaKaRa Bio Inc.), 200 nM of each primer, 1.0 µl of template DNA or 5-fold
122 diluted first PCR product, and water. The reaction conditions were 95°C for 3 min and 40
123 cycles of 95°C for 30 s, annealing temperature of 55°C for 30 s and extension at 68°C for
124 90 s, followed by a final extension at 68°C for 5 min. The PCR products were subjected
125 to electrophoresis in a 1.2% agarose gel stained with Gel-Red™ (Biotium, Hayward, CA).
126 The PCR products were purified by using the NucleoSpin Gel and PCR Clean Up Kit
127 (Takara Bio Inc.). Cycle sequencing reactions were performed using the nested primers
128 and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems,
129 Foster City, CA, USA) and analysed on an ABI Prism 3130 x genetic analyser (Applied
130 Biosystems) according to the manufacturers' instructions.

131 **Sequence data analysis**

132 Sequences obtained were manually edited using the ATGC software version 9.1
133 (GENETYX Corporation, Tokyo, Japan). The obtained sequences were compared with
134 those available in public databases using nucleotide BLASTn at the NCBI website
135 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was conducted by using
136 MEGA version 7.0 (Kumar *et al.*, 2016). Sequences were aligned with closely related
137 sequences retrieved from the GenBank using MUSCLE algorithms implemented in
138 MEGA (Kumar *et al.*, 2016). A neighbour-joining method was used to construct rooted
139 phylogenetic tree with 1,000 bootstrap replicates. The sequences obtained in the present
140 study were submitted to the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>) under

141 accession numbers LC431545–LC431547 for *T. equi* and LC431548–LC431551 for *T.*
142 *velifera*.

143 **Statistical analysis**

144 To understand the association of parasite infections with clinical symptoms mentioned
145 above, age and sex of dogs, we performed multivariate logistic regression analysis using
146 statistical software R version 3.1.2. Possible multicollinearity between the variables was
147 assessed by calculating variance inflation factor (VIF). Since multicollinearity was
148 observed among "weakness", "emaciation" and "reddish eyes" (VIF: >10), we excluded
149 the variable "weakness" from the model. We then calculated the odds ratio and its 95%
150 confidence interval for each association. We also conducted a likelihood-ratio test to
151 evaluate the significance of each variable in the model.

152

153 **Results**

154 **Dogs admitted to the private veterinary clinics**

155 BTH PCR showed positive results for haemoparasite infections in 26 dogs (72.2%), while
156 PCR for detecting members of the Anaplasmataceae family yielded negative results in
157 case of all dogs (Table 1). All positive samples were further subjected to direct Sanger
158 sequencing. Sequence alignment and BLAST analysis revealed that 7 and 19 samples
159 were *T. equi* and *T. velifera*, respectively. Almost entire 18S rDNA sequence was
160 obtained from 13 samples, resulting in 3 and 4 different genotypes for *T. equi* and *T.*
161 *velifera*, respectively (Table 2). Three genotypes of *T. equi* were divided into two clusters
162 in a phylogenetic tree (Figure 1). *T. equi* genotype 1 was found in 2 samples with 100%
163 similarity to the sequences of *T. equi* reported from Saudi Arabia (KJ801922-KJ801937),
164 Turkey (MG569904-5), Israel (KX227620- KX227630), Brazil (KJ573370), and USA

165 (JX177673). *T. equi* genotypes 2 and 3 (1 sample each) had 98% identity with *T. equi*
166 available in the database. Likewise, *T. velifera* further resulted into 4 genotypes, all of
167 which were clustered into one single clade in a phylogenetic tree (Figure 1). Genotype 1
168 was the most prevalent and detected in 4 samples, followed by genotype 2 (n = 3) and
169 genotypes 3 and 4 (1 sample each) (Table 2). The alignment of the sequences obtained
170 from this study is provided in supplementary Figure S1. There were one or two nucleotide
171 differences observed between *T. velifera* genotypes.

172 Table 3 indicates the number of dogs positive for infection by each parasite and the results
173 of the clinical observations. Clinical signs obtained from the private clinics showed that
174 a majority of the dogs (n = 31) had pyrexia (body temperature above 39.5°C). Diarrhoea
175 was also common in the tested dogs. All the dogs were administered with 120 mg/mL of
176 imidocarb dipropionate (Imizol, Schering Plough Animal Health), and the dogs with
177 severe haematological disorder were administered with Phenamidine Isethionate B. Vet.
178 C 5% m/v by subcutaneous injection (0.3 ml per kg body mass). A statistical analysis did
179 not find any association between the parasite infection status and clinical symptoms, age,
180 and sex of the dogs (Wald test, $P > 0.05$), except that *T. equi* infection was found to be
181 associated with age (Wald test, $P < 0.05$) (Supplementary Table S1).

182 **Dogs kept in horse stables of Riyadh Municipality**

183 BTH PCR showed positive results for haemoparasite infections in 30 dogs (65.2%) (Table
184 1). None of the samples yielded positive results for members of the Anaplasmatataceae
185 family by PCR. Sequencing analysis of the amplified products identified that 8 and 22
186 were infected with *T. equi* and *T. velifera*, respectively. A total of 21 samples yielded
187 almost entire 18S rDNA sequences, which resulted in 2 different genotypes for both *T.*

188 *equi* (genotypes 1 and 2) and *T. velifera* (genotypes 1 and 2) (Table 3). All four genotypes
189 were identical to those found in the dogs obtained from the veterinary clinics.

190

191 **Discussion**

192 It is generally acknowledged that dogs play an important role in transmitting tick-borne
193 diseases by: (i) carrying ticks with a broad host range, (ii) acting as a domestic reservoir
194 for certain nidicolous ticks, and (iii) possibly carrying ticks at all life stages that are not
195 attached to the host or that may have been interrupted during feeding (Otranto et al., 2015;
196 Dantas-Torres and Otranto D, 2016).

197 The results of the current study provide molecular evidence for the presence of *T. equi*
198 and *T. velifera*, which are known to be equine and bovine parasites belonging to the genus
199 *Theileria*. In the recent past, *T. equi* was detected in clinically ill dogs in Croatia (Beck *et*
200 *al.*, 2009) and South Africa (Rosa *et al.*, 2014); although these studies reported only one
201 and two cases of *T. equi* infections, respectively, the present study indicated a high
202 prevalence of *T. equi* in the tested dogs. Moreover, we detected *T. velifera* in a total of 41
203 dogs (Table 1). To the best of our knowledge, a direct detection of this parasite in canine
204 blood has not yet been reported. This parasite was recently detected in ticks (*Dermacentor*
205 *marginatus*, *Haemaphysalis parva*, *Haemaphysalis sulcata*, and *Rhipicephalus*
206 *sanguineus*) collected from sheep and dogs in Greece by the reverse line blot (RLB) assay
207 (Chaligiannis *et al.*, 2018). The presence of *T. equi* and *T. velifera* in dogs is not surprising,
208 since these dogs share the same habitat with other domestic animals. Collectively, our
209 study provides evidence for not excluding the dogs from the epidemiology of the
210 infections caused by these parasites.

211 Comparison of parasite genotypes in terms of location showed that some genotypes were
212 shared between parasites from dogs brought to the clinics and those from dogs in the
213 horse stables. This fact suggests that dogs might transmit parasites to horses in *Theileria*-
214 free regions, which results in the expansion of *Theileria*-endemic areas. This may also
215 warrant the testing of dogs travelling to disease-controlled areas or countries not only for
216 traditionally recognized dog parasites, but also for other horse and cattle piroplasms. In
217 the present study, genotyping was conducted on the sequences of 18S rDNA, where only
218 a small number of nucleotide differences were observed between genotypes. Moreover,
219 PCR amplicons were sequenced directly without cloning, which might have masked the
220 presence of multiple genotypes in a single animal. To better understand the transmission
221 of these parasites between animals, further studies employing highly polymorphic
222 markers are required.

223 The brown dog tick (*R. sanguineus*), one of the most widely distributed ticks worldwide
224 and a vector of many pathogens affecting dogs, is also a vector for equine piroplasmiasis
225 (Scoles and Ueti, 2015). The same tick species also infests cattle and horses (Schoeman,
226 2009). In the study areas, there are several cattle farms near the horse stables. Although a
227 direct physical contact between cattle and dogs was not confirmed, it is possible that the
228 dogs entered the farms and acquired the ticks, since the dogs roam freely. Though several
229 tick species including *R. sanguineus* have been recorded in Riyadh (Al-Khalifa *et al.*,
230 1986; Alanazi *et al.*, 2018), no information regarding the vector of the *Theileria* spp. in
231 dogs in Saudi Arabia is available. Further studies should include the surveys on ticks to
232 understand the lifecycle of the parasites in the tested areas.

233 Dogs appeared to be susceptible to *T. equi* and *Theileria* spp. infection, but systematic
234 investigations on the clinical impacts of these infections, for which pale mucous

235 membranes, bleeding, lethargy, thrombocytopenia, anaemia, and myelofibrosis are the
236 main clinical manifestations, are still relatively rare (Criado *et al.*, 2006; and Rosa *et al.*,
237 2014). Infection of dogs with cattle piroplasms is not uncommon. For instance, *T.*
238 *annulata* has been reported from an asymptomatic dog in Spain and Iran (Bigdeli *et al.*,
239 2012; Criado *et al.*, 2006). These findings led us to agree with the assumption that some
240 piroplasm species may lack host specificity or that distinct yet undiscovered piroplasm
241 species closely related to those already recognised may exist, as these parasites were
242 merely classified based on phenetic relationships rather than at deep molecular
243 characterization levels.

244 The current study showed no clear association between *Theileria* infection and clinical
245 outcomes in dogs, suggesting that healthy dogs might carry *Theileria* spp. In fact, guard
246 dogs kept at the horse stables were apparently healthy and did not show any clinical signs
247 of infections. This unclear association may also be related to the study design whereby
248 all the dogs tested in the statistical analysis visited clinics. Limitations of the current
249 statistical analysis includes a lack of data on possible variables such as breed and weight
250 of dogs. Further studies are essential for understanding the association between *Theileria*
251 infection and clinical outcomes in dogs. In addition, a diagnostic protocol to detect
252 asymptomatic infection of *Theileria* in dogs is yet to be established.

253

254 **Conclusion**

255 The current study has confirmed that *T. equi* and *T. velifera* are highly prevalent in dogs
256 in Riyadh city, and we presume that dogs can be potential reservoirs for these parasites,
257 which primarily infect equines and cattle. Further investigation is required to determine
258 the potential biological or ecological vectors of these pathogens both under experimental

259 and field conditions. Moreover, further experimentation is also needed to confirm the
260 clinical signs of infection in dogs, although some literatures have already provided
261 circumstantial evidences supporting this claim.

262

263

264 **Ethics approval and consent to participate**

265 Sampling procedure was reviewed and approved by the Ethical Committee of the
266 Department of Biological Science at Faculty of Science and Humanities, Shaqra
267 University, Kingdom of Saudi Arabia (Approval no. SH 03-2018). Informed consent was
268 sought from animal owners.

269

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277

278 **Conflict of interest statement**

279 The authors declared no potential conflicts of interest with respect to the research,
280 authorship, and/or publication of this article.

281

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405

406 **Figure Legend**

407 **Figure 1. Phylogenetic tree of 18S rDNA of haemoparasites detected in dogs in the**
408 **private veterinary clinics and the horse stables using a maximum likelihood. All**
409 bootstrap values from 1,000 replications are shown on the interior branch nodes. The
410 sequences obtained in the present study are shown in bold. GenBank/EMBL/DDBJ
411 accession numbers are given after the species name.

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Tables

Table 1. Results of PCR and sequencing for haemoparasite and Anaplasmatataceae infections in dogs in the private veterinary clinics and the horse stables.

Location	No. tested	No. positive for <i>T. equi</i>	No. positive for <i>T. velifera</i>	No. positive for Anaplasmatataceae
Private veterinary clinics	36 (19/17) [†]	7 (4/3)	19 (11/8)	0
Horse stables	46 (21/25)	8 (4/4)	22 (10/12)	0
Total	82 (40/42)	15 (8/7)	41 (21/20)	0

[†](male/female)

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14 **Table 2. Parasite genotypes detected in dogs in the private veterinary clinics and the horse stables.**

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Location	<i>T. equi</i>			<i>T. velifera</i>			
	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 4
Private veterinary clinics	2	1	1	4	3	1	1
Horse stables	5	1	0	10	5	0	0
Total	7	2	1	14	8	1	1

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24 **Table 3. Clinical information and parasite species detected in dogs admitted to the private veterinary clinics.**

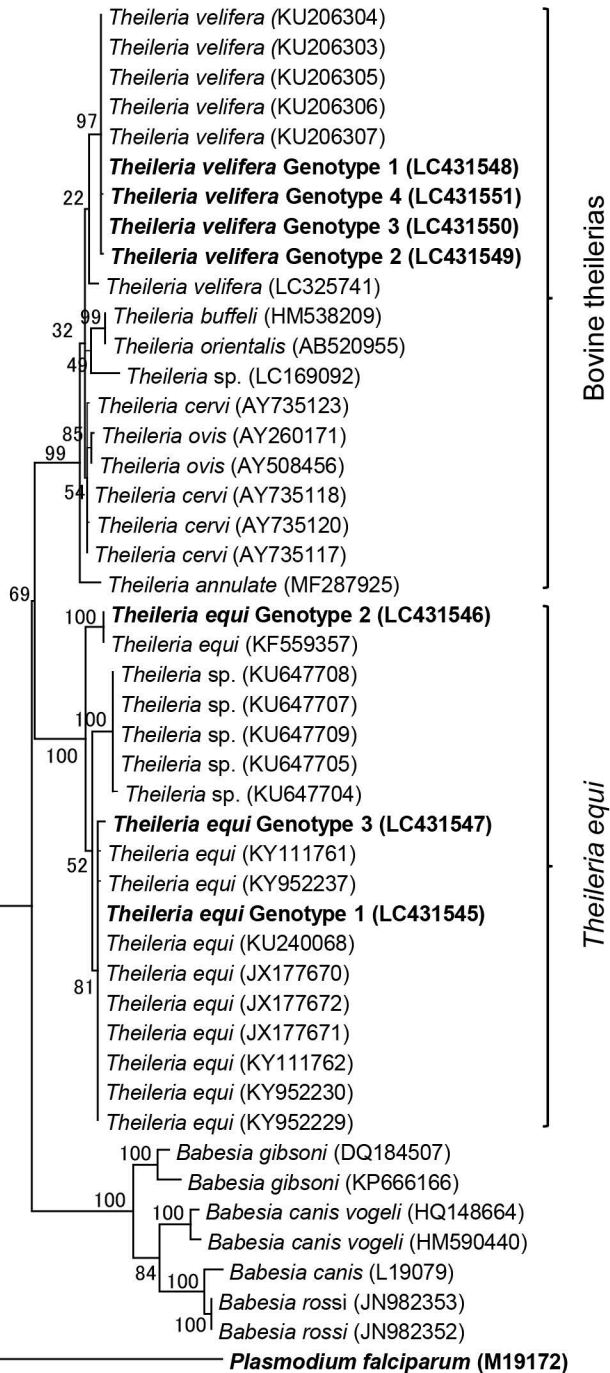
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Parasite	Symptom					
	Fever	Diarrhoea	Weakness	Emaciation	Reddish eyes	Haematuria
<i>T. equi</i> (n = 7)	7	5	2	1	1	0
<i>T. velifera</i> (n = 19)	17	10	4	2	1	1
Negative (n = 10)	7	4	1	1	0	0
Total	31	19	7	4	2	1

27

Figure 1.



0.05

Supplementary Data

Supplementary Table S1. Odds ratios for parasite infections with age, sex and clinical symptoms.

	<i>T. velifera</i> (95% CI)	<i>T. equi</i> (95% CI)
Age	1.41 (0.85, 2.34)	0.24 [†] (0.08, 0.76)
Sex	0.76 (0.18, 3.18)	0.80 (0.07, 8.60)
Fever	1.67 (0.22, 12.45)	40821294.65 (0.00, ∞)
Diarrhoea	1.88 (0.34, 10.32)	2.86 (0.15, 55.61)
Emaciation	0.68 (0.07, 6.90)	1.46 (0.04, 47.61)
Reddish eyes	1.53 (0.07, 35.94)	10.43 (0.04, 2512.73)
Haematouria	17643787.38 (0.00, ∞)	0.00 (0.00, ∞)

[†] denotes p-value < 0.05 with likelihood ratio test.

CI, confidence interval.

Supplementary Figure S1. Alignment of 18S rRNA sequences of *T. velifera* and *T. equi*.

