

1 **Molecular identification and characterization of Rickettsia spp. and other tick-**
2 **borne pathogens in sheep and their ticks from Birjand, Iran**

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23 **Abstract:**

24 **Introduction**

25 Tick-borne pathogens (TBPs) pose significant threats to both animal and human
26 health worldwide. Among these pathogens, *Babesia*, *Theileria*, and *Rickettsia*
27 species are of particular concern because of their prevalence in sheep and their
28 potential zoonotic impact.

29 **Objective**

30 The objective of this study was to investigate the prevalence of major tick-borne
31 pathogens in sheep and their associated tick species collected from different
32 geographical locations in eastern Iran using molecular approaches.

33 **Materials and Methods**

34 This cross-sectional study was conducted from January to June 2025. Blood
35 samples were collected from 100 heads of sheep, and ticks were simultaneously
36 removed from the animals in the study areas. A total of 95 ticks were
37 morphologically identified, including *Hyalomma marginatum* (42%, n = 40),
38 *Dermacentor marginatus* (36.8%, n = 35), and *Rhipicephalus sanguineus* (21%, n
39 = 20). Both sheep blood samples and tick specimens were screened for tick-borne
40 pathogens using molecular techniques.

41 **Results**

42 The most frequently detected pathogen in sheep blood samples was *Rickettsia* spp.,
43 identified in 5% (n = 5) of the samples, followed by *Theileria ovis* detected in 4%.
44 Overall, 7.3% (7/95) of the tick DNA pools tested positive for protozoan pathogens.
45 Sequencing analysis revealed infection of ticks with *Rickettsia conorii* (3%),
46 *Babesia ovis* (3%), and *T. ovis* (1%).

47 **Conclusion**

48 The results of this study demonstrate the presence of multiple tick-borne pathogens
49 in sheep and their associated tick species in eastern Iran. These findings emphasize
50 the need for continuous surveillance and the implementation of appropriate control

51 measures to reduce the risk of tick-borne diseases in animal populations and
52 potentially in humans

53 **Keywords:** Sheep, Polymerase chain reaction, Tick-borne pathogens, Iran

54 **1. Introduction**

55 The blood-feeding behavior of many arthropods, especially ticks and fleas, allows
56 them to act as important vectors for a wide range of viral, bacterial, and protozoan
57 pathogens that affect both animal and human health [1]. Some microorganisms can
58 survive within ticks throughout their developmental stages through transstadial
59 transmission, which supports their continued presence in tick populations [2].
60 Tick-borne diseases are a major concern for public and veterinary health
61 worldwide. Among the different tick genera involved in pathogen transmission,
62 Rhipicephalus species are particularly important due to their broad geographic
63 distribution and their ability to carry several infectious agents. In Iran, sheep are
64 commonly infested with Rhipicephalus ticks, which may contribute to the
65 transmission of pathogens of veterinary relevance as well as those with zoonotic
66 potential [3].

67 Recent studies have indicated that ticks can transmit a variety of microorganisms,
68 including Ehrlichia, Anaplasma, hemotropic Mycoplasma, Babesia, and Theileria
69 species [4]. Infected animals may develop clinical outcomes ranging from
70 subclinical infection to severe or fatal disease. The use of molecular diagnostic
71 methods has greatly improved the detection of tick-borne pathogens. Traditional
72 diagnostic techniques, such as microscopic examination and serological tests, often
73 show limited sensitivity and specificity, particularly when pathogen levels are low
74 or when mixed infections are present [5]. In comparison, molecular methods such
75 as polymerase chain reaction (PCR) and quantitative PCR (qPCR) provide more
76 reliable detection and allow accurate identification of closely related pathogens [5].
77 However, information on the prevalence and diversity of tick-borne pathogens in
78 sheep and their associated ticks in Iran is still limited [6]. Therefore, the present
79 study aimed to investigate the occurrence of major tick-borne pathogens in sheep
80 and their associated ticks in Iran using molecular methods.

81 **2. Materials and methods:**

82 2.1. Study area

83 The study was conducted from January to June 2025 in different locations in eastern
84 Iran. The research was carried out at the Islamic Azad University, Science and
85 Research Branch, Iran. The cattle included in the study had no history of deworming
86 or treatment with ectoparasiticides.

87 **2.3. DNA extraction**

88 Genomic DNA was extracted from blood and tick samples using a Blood DNA
89 Extraction Kit and the G-spin™ Genomic DNA Extraction Kit (iNtRON
90 Biotechnology, South Korea), respectively, according to the manufacturer's
91 instructions. The extracted DNA samples were stored at -20 °C until further
92 analysis. A total of 95 tick specimens were selected for DNA extraction. To
93 minimize the possibility of amplifying host blood DNA, visibly engorged ticks
94 were excluded from molecular analysis.

95 Prior to DNA extraction, ticks were removed from ethanol and air-dried at room
96 temperature. Each tick was then placed in a sterile Petri dish and bisected using a
97 sterile blade. One half of each specimen was used for DNA extraction, while the
98 remaining half was preserved for future analyses. Molecular identification and
99 confirmation of tick species were performed by PCR amplification and sequencing
100 following the methodology described by Latrofa et al. (2013) [9]. The quality and
101 concentration of the extracted DNA were evaluated using agarose gel
102 electrophoresis and a NanoDrop spectrophotometer.

103 **2.4. PCR amplification**

104 Polymerase chain reaction (PCR) was used to detect tick-borne pathogens (TBPs)
105 in DNA samples obtained from sheep and ticks. Positive PCR products were
106 subsequently subjected to DNA sequencing for accurate pathogen identification.
107 Primers targeting conserved genomic regions of *Rickettsia* spp. and
108 *Babesia/Theileria* spp. were used in the assays. Details of the target genes, primer
109 sequences, PCR conditions, and corresponding references are provided in Table 1.

110 Following amplification, PCR products were analyzed by agarose gel
 111 electrophoresis to confirm the presence of expected DNA fragments. A 1.5%
 112 agarose gel was prepared, and DNA bands were stained using ethidium bromide or
 113 SYBR Safe. Five microliters of each PCR product were mixed with loading dye
 114 and loaded into the gel wells. Electrophoresis was performed at 100 V for 30–
 115 45 min, and the bands were visualized under ultraviolet light. The sizes of the
 116 amplified products were determined by comparison with a DNA ladder to confirm
 117 pathogen identity.

118 Primer sequences and PCR conditions used for tick species, *Rickettsia* spp.,
 119 *Babesia/Theileria* spp. identification.

Assay	Target	Primer	size	References
Rickettsia spp.	16S	TAAGGAGGTAATCCAGCC	1482	(23)
	rRNA	CCTG GCTCAGAACGAA		
Babesia spp.	18S	ATTGACGGAAGGGCACCACC	390	(24)
	rRNA	GATGCAGACTGCTGGTTACCC		
Theileria	18S	GTA GGG CTA ATA CAT GTT CGA GAC	121	(25)
	rRNA	CTT C		
		TGA TAC ATC GCA TCC GAA GAC		
Mitochondrial	16S	CTGCTCAATGATTTTTTAAATTGCTGTGG	350	(24)
16S ribosomal DNA	rDNA	TTACGCTGTTATCCCTAGAG	450	

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121 **2.5.Sequencing**

122 Following the successful amplification of target genes through PCR, the resulting
 123 PCR products were purified using a commercial DNA purification kit (e.g., Qiagen
 124 QIAquick PCR Purification Kit) to remove excess primers, nucleotides, and
 125 enzymes. The purified DNA was then subjected to Sanger sequencing, which was
 126 performed by a commercial sequencing facility. To confirm the identity of the
 127 sequenced products, the obtained sequences were subjected to a Basic Local

128 Alignment Search Tool (BLAST) search against the National Center for
129 Biotechnology Information (NCBI) database.

130 **2.6.Ethical Considerations**

131 All sampling procedures were conducted in accordance with ethical guidelines for
132 animal research. Informed consent was obtained from wners prior to blood
133 collection, and all efforts were made to minimize discomfort and stress to the
134 animals during the sampling process.

135 **3- RESULT**

136 From the total 95 ticks collected, 40 (42 %) were morphologically identified as
137 *Hyalomma marginatum*. and 35 (36.8%) as *Dermacentor marginatus*, and 20 (21%)
138 *Rhipicephalus sanguines*. A total of two *H. marginatum*, five *D. marginatus*, and
139 three *R. sanguines* were further studied on 16S rDNA mitochondrial genes for
140 molecular identification. From the sequence analysis Table 2 of the 16S rDNA
141 mitochondrial gene of the two *H. marginatum* ticks, both showed to be identical,
142 sharing 99.5 identity with *H. marginatum* (PP937568.1) from Egypt. The 16S
143 rDNA analysis of the *D. marginatus* ticks, showed that all five were identical,
144 sharing 100 identity with an Kazakhstan *D. marginatus* (OR486023.1). Analysis
145 for the 16S rDNA mitochondrial gene of three ticks also showed that all were
146 identical, sharing 98.9% with *R. sanguines* (MK732015.1) from Portugal. From the
147 total of 95 ticks studied, three (3.1%) were found positive for rickettsiae using the
148 16srRNA assay, all in *R. sanguines* ticks. Further characterization of the 16srRNA
149 sequences showed an identity between 99.8 with *R. conorii* (NR_074480.2). When
150 screening for *Babesia* genera using the 18S rRNA gene only three tick (*H.*
151 *marginatum*) (3.1%) amplified a product of the expected size. The analysis of 18S
152 rRNA sequence obtained showed 100% identity with *Babesia ovis* (MN560046.1).
153 When screening for *Theileria* genera using the 18S rRNA gene only one tick (*R.*
154 *sanguineus*) (1%) amplified a product of the expected size. The analysis of 18S
155 rRNA sequence obtained showed 100% identity with *T. ovis* (KT851426.1).
156 Regarding blood specimens, five sample was found positive for rickettsiae, by using
157 the 16SrRNA PCR assays. From the 100 blood specimens tested for *Theileria*
158 protozoan parasites four (4%) presented amplified products of the expected size.

159 The amplified sequences of rickettsiae and *Theileria* were same as the tick isolated.
 160 GenBank accession numbers of tick sequences obtained in this study are:
 161 PV490755 (*Hyalomma marginatum*), PV490754 (*Dermacentor marginatus*),
 162 PV490756 (*Rhipicephalus sanguines*). GenBank accession numbers of *Rickettsia*
 163 sequences obtained in this study is: PV490810 (*R. conorii*).

164 Table2.Tick-borne pathogens present in sheep blood and tick specimens

	<i>R. conorii</i>	<i>B. ovis</i>	<i>T. ovis</i>
sheep blood	5	0	4
Adult ticks	3	3	1

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166 4- DISCUSSION

167 In the present study, molecular methods were employed to detect *Rickettsia*,
 168 *Babesia*, and *Theileria* species in blood samples obtained from apparently healthy
 169 sheep as well as from their associated tick specimens. Morphological examination
 170 revealed that sheep in Iran were predominantly infested with *Hyalomma*
 171 *marginatum*, *Dermacentor marginatus*, and *Rhipicephalus sanguineus*. The
 172 findings showed that 10% of sheep with heavy tick infestations were positive for
 173 tick-borne pathogens (TBPs), indicating a notable level of pathogen exposure
 174 among animals in the study area. This prevalence is slightly higher than that
 175 reported in a previous Iranian study, which documented a TBP infection rate of 8%
 176 [10]. Among the detected agents, *Rickettsia conorii* was the most frequently
 177 identified pathogen, being found in 5% of sheep blood samples and 3% of pooled
 178 tick DNA samples. This result differs from earlier reports in Iran that described a
 179 considerably higher overall prevalence of *Rickettsia* spp. (24.9%; 95% CI: 20.28–
 180 29.52) [11]. The pronounced level of tick infestation observed in the current study
 181 suggests a high degree of environmental contamination in the sampled regions,
 182 which likely increases the risk of sheep exposure to infected ticks, as also noted by

183 previous studies [12]. These observations emphasize the need for effective control
184 measures directed at both livestock and their ectoparasites [13,14]. The 18S rRNA
185 gene of *Theileria* and *Babesia* was detected exclusively in *R. sanguineus* and *H.*
186 *marginatum* ticks collected from sheep, respectively. Partial sequencing of the 18S
187 rRNA gene from positive tick samples demonstrated a high degree of sequence
188 similarity with *Theileria ovis* and *Babesia ovis* sequences available in the GenBank
189 database. Both *T. ovis* and *B. ovis* are well-known tick-borne pathogens affecting
190 sheep and other domestic ruminants, including sheep and goats, as well as wild
191 ruminant species [15]. Theileriosis and babesiosis are among the most important
192 parasitic diseases of livestock, causing substantial economic losses worldwide
193 [16,17]. Previous investigations have examined the distribution of bovine
194 theileriosis in eastern and northern regions of Iran and have also reported molecular
195 characterization and phylogenetic analysis of the 18S rRNA gene from *Theileria*
196 and *Babesia* isolates recovered from domestic animals in these areas [18].
197 Molecular techniques, particularly PCR amplification followed by DNA
198 sequencing, are widely accepted as reliable methods for epidemiological studies
199 and phylogenetic analysis of tick-borne pathogens, especially piroplasmids [19]. In
200 the present study, PCR targeting the 18S rRNA gene was applied for the detection
201 of *Theileria* and *Babesia* DNA. Broad-range PCR assays directed at this gene,
202 together with partial sequencing, have previously enabled the identification of
203 several known and novel *Babesia* and *Theileria* species [20]. In contrast, the
204 relatively low prevalence of *B. ovis* observed in this study is comparable with
205 findings from Egypt and Tunisia, where prevalence rates of 8.0% [21] and 3.0%
206 [22] were reported, respectively.

207 Unlike previous studies conducted in Iran, which reported a prevalence of
208 babesiosis in animal up to 42% [23], no cases were observed in the present study.
209 In the present study, the prevalence of *Babesia spp* in ticks collected from sheep
210 being 3% while being zero in the sheep. This discrepancy could be due to several
211 reasons: The infected ticks did not successfully transmit the *Babesia* parasite to the
212 sheep during the feeding process; The sheep may have had some level of immunity
213 against *Babesia* that prevented them from becoming infected even when exposed

214 to infected ticks and the tick population collected for the study may not have been
215 actively feeding on the cattle, leading to a lower likelihood of transmission [24].

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218 **CONCLUSION**

219 This study verifies the presence of multiple vector-borne pathogens (VBPs) in
220 sheep in east of Iran, likely due to their significant exposure to arthropod vectors
221 like ticks. While the implementation of regular preventive measures may be
222 hindered by a lack of financial resources, public-private partnerships could enhance
223 efforts to reduce the risk of VBP transmission, particularly those of zoonotic
224 significance. This study concludes that *R. sanguineus* and *H. marginatum* are the
225 primary vectors responsible for babesiosis and theileriosis in east of Iran.

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229 **Ethical approval**

230 All animal owners were interviewed for sampling and consents were taken. This
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236 **Conflict of interest**

237 The authors have no relevant financial or non-financial interests to disclose.

238 **Author Contributions**

239 Study concept and design:P.T

240 Acquisition of data:,M.S
241 Analysis and interpretation of data: S.SH &R.S
242 Drafting of the manuscript: S.SH &R.S
243 Critical revision of the manuscript for important
244 intellectual content: R.S
245 Statistical analysis: M.S, P.T
246 Administrative, technical, and material support:P.T

247 **Data Availability Statement:**

248 The data that support the findings of this study are
249 available upon request from the corresponding author

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254 REFERENCES

255 1. Nuttall PA. Tick saliva and its role in pathogen transmission. *Wiener klinische*
256 *Wochenschrift*. [2023;135\(7\):165–76.](#)

257 2. Kalmar Z, Cozma V, Sprong H, Jahfari S, D’Amico G, Mărcuțan DI, et al.
258 Transstadial transmission of *Borrelia turcica* in *Hyalomma aegyptium* ticks. *PLoS*
259 *One*. [2015;10\(2\):e0115520.](#)

260 3. Mostafavi SM, Khalili M, Akhtardanesh B, Nourollahifard SR, Esmaeili S.
261 *Rickettsia* spp. in *Rhipicephalus sanguineus sensu lato* ticks collected from stray
262 dogs in Kerman city, Iran. *Ticks and Tick-borne Diseases* [2022;13\(5\):101985.](#)

263 4. Alieva E, Bondarenko E, Maliy K, Shvalov A, Verbenets E, Gafarova M. The
264 role of *Rhipicephalus sanguineus* ticks parasitizing dogs in the spread of tick-borne

- 265 rickettsial pathogens in the city of Sevastopol. *New microbes and new infections*
266 [2020;36:100704.](#)
- 267 5.Huynh LN, Diarra AZ, Pham QL, Le-Viet N, Berenger J-M, Ho VH, et al.
268 Morphological, molecular and MALDI-TOF MS identification of ticks and tick-
269 associated pathogens in Vietnam. *PLoS Neglected Tropical Diseases*
270 [2021;15\(9\):e0009813.](#)
- 271 6.Fathi A, Nabavi R, Noaman V, Sarani A, Saadati D, Ben Said M, et al. Molecular
272 identification, risk factor assessment, and phylogenetic analysis of tick-borne
273 pathogens in symptomatic and asymptomatic cattle from South-Eastern Iran.
274 *Experimental and Applied Acarology* [2024;92\(3\):479-506.](#)
- 275 7.Lorusso V, Dantas-Torres F, Lia RP, Tarallo V, Mencke N, Capelli G, et al.
276 Seasonal dynamics of the brown dog tick, *Rhipicephalus sanguineus*, on a confined
277 dog population in Italy. *Medical and Veterinary Entomology* [2010;24\(3\):309-15.](#)
- 278 8.Nava S, Beati L, Venzal JM, Labruna MB, Szabó MP, Petney T, et al.
279 *Rhipicephalus sanguineus* (Latreille, 1806): Neotype designation, morphological
280 re-description of all parasitic stages and molecular characterization. *Ticks and tick-*
281 *borne diseases* [2018;9\(6\):1573-85.](#)
- 282 9.Latrofa MS, Dantas-Torres F, Annoscia G, Cantacessi C, Otranto D. Comparative
283 analyses of mitochondrial and nuclear genetic markers for the molecular
284 identification of *Rhipicephalus* spp. *Infection, Genetics and Evolution*
285 [2013;20:422-7.](#)
- 286 10.Iatta R, Sazmand A, Nguyen V-L, Nemati F, Ayaz MM, Bahiraei Z, et al.
287 Vector-borne pathogens in dogs of different regions of Iran and Pakistan.
288 *Parasitology Research* [2021:1-10.](#)
- 289 11. Qorbani A, Khalili M, Nourollahifard S, Mostafavi E, Farrokhnia M, Esmaeili
290 S. Diversity of Rickettsia species in collected ticks from Southeast Iran. *BMC*
291 *Veterinary Research* [2024;20\(1\):279.](#)

- 292 12. Otranto D, Dantas-Torres F, Weigl S, Latrofa MS, Stanneck D, Decapariis D,
293 et al. Diagnosis of *Hepatozoon canis* in young dogs by cytology and PCR. *Parasites*
294 & *vectors* 2011;4:1-6.
- 295 13. Otranto D, Mendoza-Roldan JA, Beugnet F, Baneth G, Dantas-Torres F. New
296 paradigms in the prevention of canine vector-borne diseases. *Trends in*
297 *Parasitology* 2024.
- 298 14. Rakhshanpour A, Malmasi A, Mohebali M, Nabian S, Mirhendi H, Zarei Z, et
299 al. Transmission of *Leishmania infantum* by *Rhipicephalus sanguineus* (Acari:
300 Ixodidae) in dogs. *Iranian journal of parasitology* 2017;12(4):482.
- 301 15. Majidiani H, Nabavi R, Ganjali M, Saadati D. Detection of *Theileria annulata*
302 carriers in Holstein–Friesian (*Bos taurus taurus*) and Sistani (*Bos taurus indicus*)
303 cattle breeds by polymerase chain reaction in Sistan region, Iran. *Journal of*
304 *parasitic diseases* 2016;40:1184-8.
- 305 16. Almazán C, Scimeca RC, Reichard MV, Mosqueda J. Babesiosis and
306 theileriosis in North America. *Pathogens* 2022;11(2):168.
- 307 17. Rashid M, Akbar H, Rashid I, Saeed K, Ahmad L, Ahmad AS, et al. Economic
308 significance of tropical theileriosis on a Holstein Friesian dairy farm in Pakistan.
309 *The Journal of parasitology* 2018;104(3):310-2.
- 310 18. Soosaraei M, Haghi MM, Etemadifar F, Fakhar M, Teshnizi SH, Hezarjaribi
311 HZ, et al. Status of theileriosis among herbivores in Iran: A systematic review and
312 meta-analysis. *Veterinary world* 2018;11(3):332.
- 313 19. Soares JF, Giroto A, Brandão PE, Da Silva AS, Franca RT, Lopes ST, et al.
314 Detection and molecular characterization of a canine piroplasm from Brazil.
315 *Veterinary Parasitology* 2011;180(3-4):203-8.
- 316 20. Adjou Moumouni PF, Aboge GO, Terkawi MA, Masatani T, Cao S,
317 Kamyngkird K, et al. Molecular detection and characterization of *Babesia bovis*,
318 *Babesia bigemina*, *Theileria* species and *Anaplasma marginale* isolated from cattle
319 in Kenya. *Parasites & vectors* 2015;8:1-14.

- 320 21. Mahmoud HY, Shahat MS, Fereig RM, Ali AO, Emeish WF, Soliman AM, et
321 al. Molecular detection and characterization of *Anaplasma marginale* and *Babesia*
322 *canis vogeli* infecting dogs in Luxor, Egypt. *Scientific Reports* 2024;14(1):16888.
- 323 22. Bouattour A, Chabchoub A, Hajjaji I, M'ghirbi Y. Hepatozoon *canis* and
324 *Babesia vogeli* infections of dogs in Tunisia. *Veterinary Parasitology: Regional*
325 *Studies and Reports* 2021;23:100512.
- 326 23. Razmi G. A review on *Babesia* spp. and tick vectors in animals in Iran. *Iranian*
327 *Journal of Veterinary Science and Technology* 2022;14(2):1-0.
- 328 24. Fesseha H, Mathewos M, Eshetu E, Tefera B. Babesiosis in cattle and ixodid
329 tick distribution in Dasenech and Salamago Districts, southern Ethiopia. *Scientific*
330 *reports* 2022; 12(1):6385.

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