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RESEARCH

Borrelia theileri infections in *Rhipicephalus annulatus* ticks from the north of Iran

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Abstract
Ticks serve as vectors and reservoirs of various *Borrelia* species, potentially causing diseases in humans and animals. Mazandaran, a fertile green land in northern Iran, provides ample grazing grounds for livestock and harbors at least 26 hard tick species. This study investigated *Borrelia* infection in hard ticks from forest areas in this region and compared their genetic identity with the species data in the GenBank database. A total of 2,049 ticks were collected manually from mammalian hosts or using dragging and flagging methods. These ticks were then grouped into 190 pools and 41 individuals based on host, species, developmental stage, and gender. A real-time PCR (qPCR) detected *Borrelia* DNA in 26 pools from female, male, and nymph of *Rhipicephalus annulatus* ($n=17$) and *Ixodes ricinus* ($n=9$) ticks and one individual female *Haemaphysalis punctata* tick. The generated partial *flaB* and *glpQ* sequences from qPCR-positive *Rh. annulatus* ticks exhibited the highest identities of 98.1-100% and 98.2% with *Borrelia theileri* and closely related undefined isolates. Additionally, in phylogenetic analysis, these sequences clustered within well-supported clades with *B. theileri* and the closely related undefined isolates from various geographic regions, confirming the presence of *B. theileri* in the north of Iran. Divergence in *B. theileri flaB* and *glpQ* sequences across various geographical areas suggests potential subspeciation driven by adaptations to different tick species. This divergence in our *flaB* sequences implies the possible introduction of *B. theileri*-infected ticks from different geographical origins into Iran.

Keywords *Rhipicephalus annulatus* ticks · *Borrelia theileri* · Molecular detection · Phylogenetic analysis · Mazandaran · Iran

Introduction

The genus *Borrelia* comprises arthropod-borne spirochetes that complete their life cycle in vertebrate hosts (Qiu et al. 2021). Phylogenetically, they are divided into three groups: Lyme Group (LG), Relapsing Fever Group (RFG), and Echidna-Reptile Group (REPG). Among these, only the LG and certain members of the RFG borreliae are known to cause infections

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in humans. The REPG borreliae represent a distinct monophyletic group primarily infecting amphibians and reptiles (Trevisan et al. 2021). Apart from the louse-adapted *Borrelia recurrentis*, most members of RFG pathogenic to humans are transmitted by argasid soft ticks (Cutler et al. 2017; Talagrand-Reboul et al. 2018). Additionally, *Ixodes* and other hard ticks maintain *Borrelia* species such as *Borrelia miyamotoi*, *B. lonestari*, *B. theileri*, and some undescribed species genetically grouped with RFG (Cutler et al. 2017; Furuno et al. 2017; Naddaf et al. 2020; Talagrand-Reboul et al. 2018).

In Iran, molecular analyses have identified the presence of at least two soft tick relapsing fever (STRF) borreliae: the well-established *B. persica* (Marti Ras et al. 1996; Naddaf et al. 2015; Shirani et al. 2016) and a complex group infecting *Ornithodoros* ticks, rodents, and humans with the highest resemblance to East African *Borrelia duttonii* and *B. recurrentis* (Ghasemi et al. 2021; Houmansadr et al. 2020; Naddaf et al. 2012, 2015, 2017). Recently, in north Iran, LG borreliae, *Borrelia afzelii, B. garinii, B. valaisiana*, *B. bavariensis*, and RFG *B. miyamotoi* were identified in *Ix. ricinus* ticks (Naddaf et al. 2020).

In the Mazandaran province of Iran, a diverse range of 26 hard tick species inhabit different geographical areas, i.e., coastal plains, mountains, and forests (Nabian et al. 2007; Nasibeh et al. 2010; Rahbari et al. 2007; Razmi et al. 2007; Vahedi Noori et al. 2015). In previous sampling efforts, our focus was on the coastal plains and mountains, during which we investigated 11 tick species for the presence of *Borrelia* in this province (Naddaf et al. 2020). In the present study, we extended our survey to include hard ticks from forest areas of this province. We examined *Borrelia* infection in these hard ticks and compared their genetic identity with the species and isolates available in the GenBank database.

Materials and methods

Study area

Mazandaran province, located in the north of Iran, is characterized by its lush green landscape, situated between the Caspian Sea and north of the Alborz Mountains.

According to the Köppen-Geiger classification, the climate in this region falls under type C, subdivision Csa, a Mediterranean climate with hot summers (Kottek et al. 2006; Raziei 2017). Mazandaran province encompasses a stretch of the predominantly temperate deciduous Hyrkanian Mixed Forests belt, which extends across the northern slopes of the Alborz Mountains to the southern shores of the Caspian Sea. These ancient forests, estimated to be around 20–50 million years old, serve as vital refugia for many Arctic Tertiary relict species (Leestmans 2005; Zohary 1973) (Fig. 1).

Collection and identification of ticks

Ticks were manually collected using fine stainless-steel tweezers from domestic animals, including cattle, goats, sheep, and dogs. Additionally, dragging and flagging methods were employed across the forest floor or atop vegetation in wooded areas within Amol, Babol, Savadkuh, and Sari Counties (Fig. 1). These regions are characterized by heavy rainfall and high humidity. Sampling was conducted in October 2021, during which the temperature ranged from 14 °C to 16 °C, with moisture levels from 50 to 70% in the study area. After

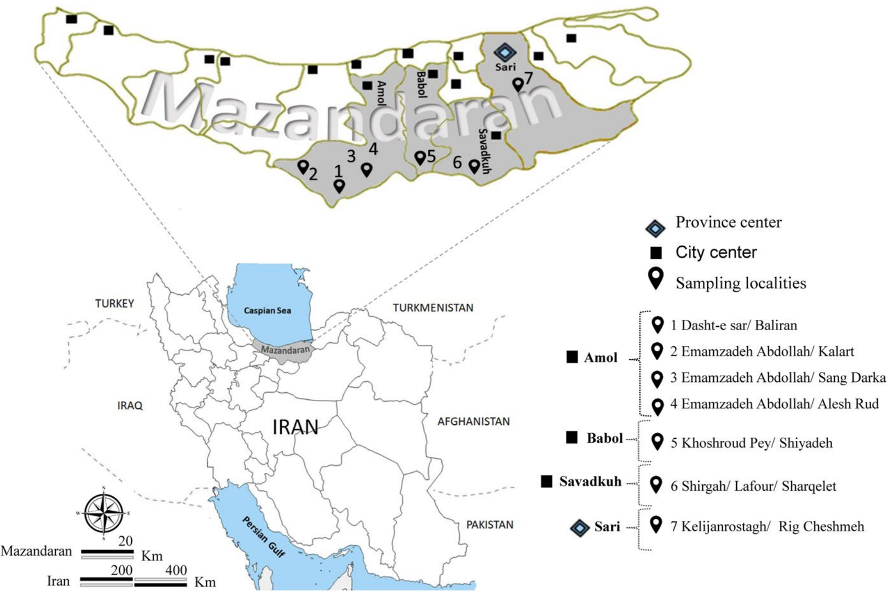

Fig. 1 Map showing the studied area in Mazandaran province (in grey), northern Iran

collection, ticks were transferred into sterile 50-ml Falcon tubes, and relevant data, including location, collection date, and coordinates, were recorded and stored in sealed Styrofoam containers lined with damp cotton pads. All collected ticks were transferred to the Parasitology Laboratory of the Amol Research Center, Pasteur Institute of Iran, where they were identified based on morphological features described in taxonomic keys (Apanaskevich and Horak 2005, 2008; Apanaskevich et al. 2010; Hosseini-Chegeni 2013; Hosseini-Chegeni et al. 2019; McCoy et al. 2014; Walker 2003; Walker 2000). The specimens were grouped according to developmental host, species, developmental stage (adult, nymph, and larvae), and gender and then transferred to the Bacteriology Laboratory, Pasteur Institute of Tehran, and stored at 4 °C until DNA extraction.

DNA extraction

Following the journey, only some female ticks survived, while all males and nymphs perished. The dead specimens were transferred to 70% ethanol. Ticks were washed twice with 75% ethanol and phosphate-buffered saline (PBS), as previously described by Jiao et al. (2021). Live ticks were dissected, and their midguts were extracted and preserved in PBS at 4 °C; dead specimens could not be dissected due to desiccation. DNA was extracted from individuals or pools of midgut and whole ticks, grouped by host, species, developmental stage, and gender. Forty-one ticks were tested individually: six live females, one larva, and 34 dead ticks comprising females, males, and nymphs. The remaining specimens were grouped into pools, ranging from 2 to 40 for adults and 2 to 160 for nymphs. Samples were homogenized in 2 ml tubes containing 300–800 µL PBS and 5-mm stainless steel beads (QIAGEN, Hilden, Germany) using a homogenizer (TissueLyser II, QIAGEN).

DNA extraction was then performed from 200 µL of the homogenized ticks using the potassium acetate procedure developed by Rodríguez (2014) and successfully deployed by others (Ghasemi et al. 2021; Naddaf et al. 2020). The optical density (OD) of DNA extracted from dead ticks ranged from 0.03 to 126.2 ng/µl, and from midguts ranged from 0.3 to 2620.3 ng/µl.

Real-time PCR (qPCR)

Detection of *Borrelia* in the whole genomic DNA extracted from ticks was conducted using primers and the probe specific for amplifying a 136-bp sequence of 16 S rRNA in the genus *Borrelia* (Table 1).

Results

Tick identification

In Total, 1,756 were collected by hand from 90 domestic animals, including 86 cattle, two goats, one sheep, and one dog. Additionally, 293 ticks were collected using dragging and flagging methods. Four species were identified, with *Ix. ricinus* being the most abundant ($n=1651$), followed by *Rhipicephalus annulatus* ($n=384$), *Haemaphysalis punctata* ($n=11$), and *Hyalomma marginatum* ($n=3$). Among the collected specimens, there were 319 nymphs of *Ix. ricinus* and 44 nymphs of *Rh. annulatus*, along with one *Rh. annulatus* larva (Table 2).

qPCR amplification

Of the 231 DNA samples, 26 pools comprising female, male, and nymphs of *Rh. annulatus* ($n=17$) and *Ix. ricinus* ($n=9$) ticks, along with one individual female *Ha. punctata* tick were positive for *Borrelia* DNA. The cycle thresholds (*Ct*) for the controls containing approximately 60’000, 6’000, 600, 60, and 6 bacteria DNA copy numbers were 24.5, 25.5, 26.5, 27.5, and 28.32, respectively. The *Ct* for the qPCR-positive samples ranged from

Table 1 The primers and probe used for qPCR, nested PCR, and single PCR amplification

Target marker	Primer/ Probe		Oligonucleotide (5’ to 3’)	Amplicon size (bp)	Reference
16 S rRNA	16SBOR-Fw		GGTCAAGACTGACGCTGAGTCA	136	Ornstein and Barbour (2006)
	16SBOR-Rev		GGCGGCCACTTAACACGTTAG		
	16SBOR-P		Fam-TCTACGCTGTAAACGATGCAC ACTTGG TG-BHQ-1		
flaB	Outer	132-Fw	TGGTATGGGAGTTTCTGG	774	Wodecka (2007)
		905-Rev	TCTGTCATTGTAGCATCTTT		
	Inner	220-Fw	CAGACAACAGAGGGAAAT	604	
		823-Rev	TCAAGTCTATTTGGAAAGCACC		
glpQ	Fw		TATGGCATAAACAACAAGGTA	453	Toledo et al. (2010)
	Rev		AATCTGTAAATAGACCATCTA		

Abbreviation Fw, Forward Primer; *Rev*, Reverse primer; *P*, Probe

Table 2 Details of tick species collected in Mazandaran province, Iran

Sampling method	Host	Tick species	Number of ticks				County/ City/ Village
			Female (live)	Male	Nymph	Larva	
Hand-collection	Cattle, goats, dogs, sheep	*Rh. annulatus*	140 (135)	67	26	0	Amol/ Dasht-e sar/ Baliran
	Cattle, goats, sheep	*Ix. ricinus*	7	2	0	0	
	Cattle	*Ha. punctata*	1	0	0	0	
Hand-collection	Cattle, goats	*Ix. ricinus*	720 (534)	252	21	0	Amol/ Emamzadeh Abdollah/ Kalart
	Cattle	*Rh. annulatus*	0	1	0	0	
		Hy. marginatum	0	3	0	0	
		Ha. punctata	5 (3)	2	0	0	
Dragging/ Flagging	NA	*Ix. ricinus*	1	2	160	0	Amol/ Emamzadeh Abdollah/ Kalart
Dragging/ Flagging	NA	*Ix. ricinus*	0	0	129	0	Amol/ Emamzadeh Abdollah/ Sang Darka
		Ha. punctata	0	1	0	0	
Hand-collection	Cattle	*Ix. ricinus*	65 (25)	11	1	0	Amol/ Emamzadeh Abdollah/ Alesh Rud
		Rh. annulatus	57 (11)	5	8	0	
Hand-collection	Cattle	*Ix. ricinus*	168 (101)	37	7	0	Babol/ Khoshroud Pey/ Shiyadeh
		Rh. annulatus	0	2	0	0	
Hand-collection	Cattle	*Ix. ricinus*	31 (8)	9	0	0	Savadkuh/ Shirgah/ Lafour/ Sharqelet
		Rh. annulatus	32	4	5	0	
Hand-collection	Cattle	*Ix. ricinus*	19 (13)	8	1	0	Sari/ Kelijan-rostagh/ Rig Cheshmeh
		Rh. annulatus	24	7	5	1	
		Ha. punctata	2 (1)	0	0	0	
		Total	1,272 (831)	413	363	1	2,049

Abbreviations Rh Rhipicephalus; *Ix* Ixodes; *Hy* Hyalomma; *Ha* Haemaphysalis

25.5 to 42.1 (Table 3). No measurable *Ct* values were determined for NTCs and other tick specimens

PCR amplification and sequencing

The qPCR-positive samples were further analyzed by amplifying and sequencing partial fragments of *flaB* and *glpQ* using the primers (Table 1) and amplification conditions described previously (Naddaf et al. 2020; Toledo et al. 2010). The 25 µL reactions included 2 µL (0.5–50 ng/µL) template DNA, 12.5 µL 2X Master Mix (Ampliqon, Denmark), 10 pm each of forward and reverse primers, and DDW to final volume. The *flaB* sequence was amplified by deploying a nested PCR assay with two sets of specific primers. The first amplification program included an initial denaturation at 95 °C for 15 min, followed by 40

Table 3 *Borrelia*-qPCR positive ticks/pools from Mazandaran province

No	Sample code	Ticks in pool	Species	Gender	County/ City/ Village	Host	qPCR (Ct)	flaB (*P*/*N*)	glpQ (*P*/*N*)
1	Ir-Maz 1	40 †	*Rh. annulatus*	Female	Amol/ Dasht-e sar/ Baliran	Cattle	33.3	P^s	P^s
2	Ir-Maz 2	12 †	*Rh. annulatus*	Female	Amol/ Dasht-e sar/ Baliran	Cattle	33.3	P^s	P^s
3	Ir-Maz 3	15 †	*Rh. annulatus*	Female	Amol/ Dasht-e sar/ Baliran	Cattle	35.0	P^s	P
4	Ir-Maz 1A4	10	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Cattle	26.6	N	N
5	Ir-Maz 1A5	4	*Rh. annulatus*	Nymph	Amol/ Dasht-e sar/ Baliran	Cattle	25.5	P^s	P^s
6	Ir-Maz 1A7	9	*Rh. annulatus*	Nymph	Amol/ Dasht-e sar/ Baliran	Cattle	25.5	P^s	P^s
7	Ir-Maz 1A8	9	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Cattle	29.3	P^s	N
8	Ir-Maz 1A10	1	*Ha. punctata*	Female	Amol/ Dasht-e sar/ Baliran	Cattle	39.1	N	N
9	Ir-Maz 1A11	5	*Rh. annulatus*	Female	Amol/ Dasht-e sar/ Baliran	Cattle	39.7	N	N
10	Ir-Maz 1B1	13	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Cattle	26.5	P	P
11	Ir-Maz 1B4	4	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Goat	40.8	N	N
12	Ir-Maz 1B7	3	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Dog	39.9	N	N
13	Ir-Maz 1B8	9	*Rh. annulatus*	Male	Amol/ Dasht-e sar/ Baliran	Sheep	40.9	N	N
14	Ir-Maz 1B11	16	*Ix. ricinus*	Male	Amol/ Emamzadeh Abdollah/ Kalart	Cattle	32.5	P^s	N
15	Ir-Maz 1C2	2	*Ix. ricinus*	Male	Amol/ Emamzadeh Abdollah/ Kalart	Dragging/ Flagging	36.5	N	N
16	Ir-Maz 1C3	160	*Ix. ricinus*	Nymph	Amol/ Emamzadeh Abdollah/ Kalart	Dragging/ Flagging	39.5	N	N
17	Ir-Maz 1C4	129	*Ix. ricinus*	Nymph	Amol/ Emamzadeh Abdollah/ Sang Darka	Dragging/ Flagging	38.8	N	N
18	Ir-Maz 1F11	3	*Ix. ricinus*	Female	Amol/ Emamzadeh Abdollah/ Alesh Rud	Cow	40.9	N	N
19	Ir-Maz 2A1	2	*Rh. annulatus*	Nymph	Amol/ Emamzadeh Abdollah/ Alesh Rud	Cattle	41.8	N	N
20	Ir-Maz 2C7	9	*Ix. ricinus*	Male	Babol/ Khoshroud Pey/ Shiyadeh	Cattle	42.0	N	N
21	Ir-Maz 2C9	2	*Rh. annulatus*	Female	Babol/ Khoshroud Pey/ Shiyadeh	Cattle	31.3	P^s	N
22	Ir-Maz 2D5	7	*Ix. ricinus*	Female	Babol/ Khoshroud Pey/ Shiyadeh	Cattle	30.7	N	N
23	Ir-Maz 2D9	4	*Rh. annulatus*	Male	Savadkuh/ Shirgah/ Lafour/ Sharqelet	Cattle	42.1	N	N
24	Ir-Maz 2E1	10	*Ix. ricinus*	Female	Savadkuh/ Shirgah/ Lafour/ Sharqelet	Cattle	40.9	N	N
25	Ir-Maz 2E9	2	*Ix. ricinus*	Male	Savadkuh/ Shirgah/ Lafour/ Sharqelet	Cattle	41.3	N	N

Table 3 (continued)

No	Sample code	Ticks in pool	Species	Gender	County/ City/ Village	Host	qPCR (Ct)	flaB (*P*/*N*)	glpQ (*P*/*N*)
26	Ir-Maz 2F3	3	*Rh. annulatus*	Nymph	Sari/ Kelijanrostagh/ Rig Cheshmeh	Cattle	33.5	N	N
27	Ir-Maz 2F10	2	*Rh. annulatus*	Nymph	Sari/ Kelijanrostagh/ Rig Cheshmeh	Cattle	33.5	N	N

Abbreviations Ct Cycle threshold; *P* Positive; *N* Negative; [s], Sequenced; †, midgut; *Rh, Rhipicephalus; Ix, Ixodes*; *Ha Haemaphysalis*

cycles at 95 °C for 30 s, annealing at 50 °C for 45 s, extension at 72 °C, and a final extension at 72 °C for 7 min. For the second round of amplification, 2 µL of 1/20 dilutions from the first PCR template were used. The program for the second PCR set was the same as before, except for the annealing temperature, which was increased to 54 °C. The *glpQ* amplification was programmed for an initial denaturation step at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 51.8 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. A reaction containing all reagents except DNA was included in all assays as a negative control.

PCR products were electrophoresed on 1.5% agarose gels stained with Green Viewer (Eco DNA Safe Stain, Kala Zist, Iran) alongside a molecular weight marker and visualized under ultraviolet radiation in a gel docking device (UVITEC Cambridge Gel Documentation System, UK). Amplicons were sequenced in both directions using the Sanger method with the same primers utilized for amplification at the Sequencing Department of the Pasteur Institute, Iran, employing an ABI-3500 Sequencer. The sequences were manually checked for ambiguities by the BioEdit Sequencing Alignment Editor software (version 7.2.5), and consensus sequences were obtained. The generated sequences were deposited in the GenBank database under the accession numbers OR037292- OR037295 for *glpQ* and OR037296-OR037302 for *flaB*.

BLAST and phylogenetic analysis

The generated sequences were compared with the *Borrelia* sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov), and similarities were obtained. DNA sequences were aligned using Clustal W multiple alignments (Thompson et al. 1994). Then, phylogenetic analysis was performed using MEGA 11.0.10 (Tamura et al. 2021), and evolutionary *flaB* and *glpQ* trees were constructed with obtained sequences along with similar ones sourced from the GenBank database as detailed in the supplementary material (Tables S1 and S2) using the maximum likelihood algorithm based on the best-fit model (Kumar et al. 2001), i.e., Tamura 3-parameter (T92) for the *flaB* and Tamura-Nei (TN93) for the *glpQ*. Bootstrap values were calculated from 1000 replications, and the trees were rooted using the midpoint approach.

PCR amplification and sequencing

Nine qPCR-positive samples yielded the expected 604-bp *flaB* band, eight from *Rh. annulatus* and one from *Ix. ricinus* ticks. Also, six representatives from *Rh. annulatus* exhibited the

453-bp *glpQ* amplicon. One *flaB* amplicon (Ir-Maz1B1) exhibited background sequencing noises, and two *glpQ* amplicons (Ir-Maz 3 and Ir-Maz 1B1) yielded short sequencing results and thus were excluded from further analysis (Table 3)

BLAST analysis

Among the seven *flaB* sequences from *Rh. annulatus* ticks, five (Ir-Maz1, Ir-Maz2, Ir-Maz3, Ir-Maz1A5, and Ir-Maz1A8) were identical and matched 99.8% and 99.3% with two other sequences (Ir-Maz1A7 and Ir-Maz2C9). The *flaB* sequences exhibited 98.7-99.6% similarity with *B. theileri* C5 (acc. no. MG601737), 98.5-100% with *B. theileri* CATMAR-HS (acc. no. MG564190), 98.1-98.5% with *B. theileri* KAT (acc. no. KF569936), and 98.5-98.9% with *B. theileri* W27 (acc. no. LC656218). They also matched 96.2-96.6% with *B. lonestari* (acc. no. U26705) and 89-89.3% with *B. miyamotoi* Ir-Maz57 (acc. no. MN958345). The only generated *flaB* sequence from *Ix. ricinus* ticks (Ir-Maz1B11) matched 100% with *B. miyamotoi* (acc. no. KX646199).

The four *glpQ* sequences from *Rh. annulatus* ticks were identical and matched 98.2% with *B. theileri* KAT (acc. no. KF569936), 92.9% with *B. lonestari* MO2002-V1 (acc. no. AY682922), and 92.6% with *B. miyamotoi* Yekat-18 (acc. no. CP037471).

Phylogenetic analysis

In both trees, the hard tick relapsing fever (HTRF) sequences, including those generated in this study, were grouped separately from soft tick relapsing fever (STRF) borreliae and *B. recurrentis*. Additionally, *B. miyamotoi* appeared in a distant clade separate from other HTRF borreliae. In the *flaB* tree, our generated sequences, *B. theileri* C5 from Brazil, *B. theileri* CATMAR-HS from Egypt, *B. theileri* KAT from Mali, *B. theileri* W27 from Zambia, and undefined isolates *Borrelia* sp. BR and *Borrelia* sp. T23, from Brazil and Madagascar, formed a separate clade that branched into four subclades, close to *B. lonestari* and distant from *Borrelia* sp. RSF2354, and *Borrelia* sp. Ir-Maz173, previously detected in *Rhipicephalus* ticks in Portugal and Iran. Six generated *flaB* sequences emerged as a sister taxon to *B. theileri* C5, and one grouped with *B. theileri* CATMAR-HS and *Borrelia* sp. BR and *Borrelia* sp. T23 from Brazil and Madagascar. *Borrelia theileri* KAT from Mali and *B. theileri* W27 from Zambia appeared in the third and fourth subclades (Fig. 2)

Discussion

Borrelia theileri, a causative agent of bovine borreliosis, was initially identified in South Africa in *Rhipicephalus* ticks by Arnold Theiler over a century ago (Theiler 1904). This spirochete has since been reported in various domestic animals, including cattle, goats, sheep, and horses across Africa, North and South America, and Australia (Callow 1967; Cutler et al. 2012; Uilenberg et al. 1988). Transmission of *B. theileri* occurs through the infective bites of *Rhipicephalus* (*Boophilus*) ticks, such as *Rhipicephalus microplus*, *Rh. annulatus*, *Rh. evertsi*, and *Rh. decoloratus*, to domestic ruminants (Qiu et al. 2021).

This spirochete typically induces a mild febrile disease in cattle, sometimes leading to hemoglobinuria and anemia (Callow 1967). Nevertheless, *B. theileri* remains one of the

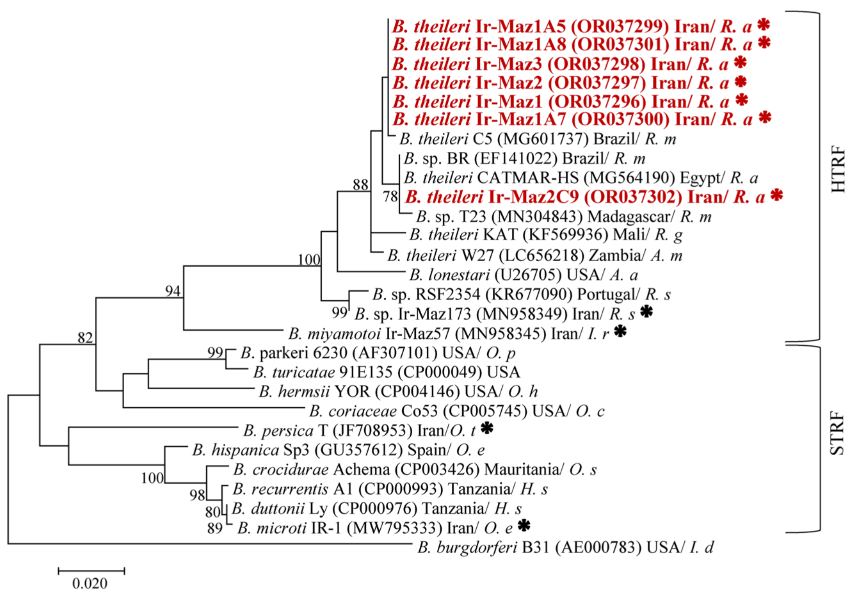

Fig. 2 The phylogenetic tree constructed based on the analyzed *flaB* partial sequence. The sequences generated in this study are in red and marked with asterisks. Black asterisks show sequences from Iran reflected in previous reports. Numbers at nodes refer to bootstrap probabilities; support values < 70 are not shown here. The scale bar indicates 0.02 estimated nucleotide substitutions per site. *Borrelia* spp. origin: *R. a, Rhipicephalus annulatus*; *R. m, Rhipicephalus microplus*; *R. g, Rhipicephalus geigyi*; *A. m, Aepyceros melampus*; *A. a, Amblyomma americanum*; *R. s, Rhipicephalus sanguineus* s.l; *I. r, Ixodes ricinus*; *O. p, Ornithodoros parkeri*; *O. h, Ornithodoros hermsi*; *O. c, Ornithodoros coriaceus*; *O. t, Ornithodoros tholozani*; *O. e, Ornithodoros erraticus*; *O. s, Ornithodoros sonrai*; *H. s, Homo sapiens* In the *glpQ* tree, our sequences appeared as a sister group alongside the only available sequence from *B. theileri* KAT (acc. no. KF569938), sharing a common ancestor with *B. lonestari* MO2002-V1 (acc. no. AY682922) (Fig. 3)

least characterized pathogenic *Borrelia* transmitted by ticks (McCoy et al. 2014). Currently, no isolates of this species are available, and efforts to culture this spirochete have been unsuccessful (McCoy et al. 2014; Smith et al. 1985). Only a limited number of DNA sequences are available to distinguish this spirochete from closely related species harbored by hard ticks (McCoy et al. 2014).

In the present study, we report the presence of *B. theileri* in *Rh. annulatus* ticks from Iran for the first time. *Rhipicephalus annulatus* is a one-host tick, meaning once the larva hatches and finds a host, it typically develops to the next life stages on the same animal (D'Amico et al. 2017; Wang et al. 2017). We initially aimed to study *Borrelia* infection alongside blood sources in individual ticks. However, the low infection rate observed in early assays led us to pool the specimens. We identified *B. theileri* DNA in all pooled life stages of *Rh. annulatus* ticks collected from cattle. However, not all ticks from a single host were infected, implying the ticks might have acquired the infection during a short period of spirochetemia. *Borrelia* DNA was also detected in unfed adults and nymphs, suggesting transstadial transmission.

Our *flaB* sequences displayed the highest identity with *B. theileri* isolates from Brazil, Egypt, Mali, and Zambia. In phylogenetic analysis, these sequences formed a distinct

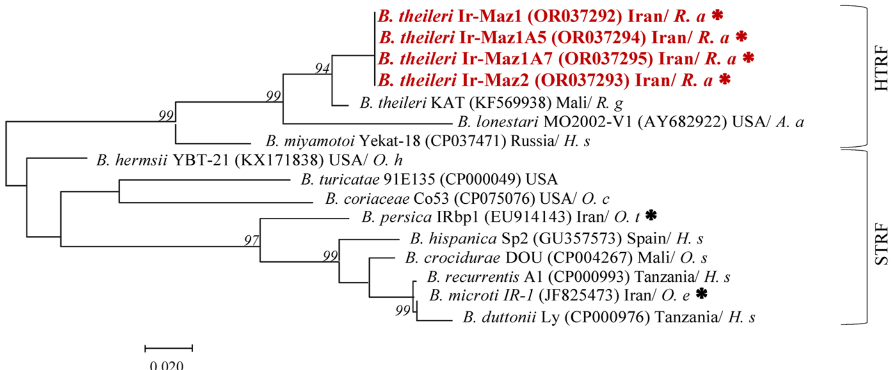

Fig. 3 The phylogenetic tree based on the analyzed *glpQ* partial sequence. The sequences generated in this study are in red and marked with asterisks. Black asterisks show sequences from Iran reflected in previous reports. The numbers at the nodes refer to the bootstrap probabilities; support values < 70 are not shown here. The scale bar indicates 0.02 estimated nucleotide substitutions per site. *Borrelia* spp. origin: *H.s*, *Homo sapiens*; *O. e*, *Ornithodoros erraticus*; *O. s*, *Ornithodoros sonrai*; *O.c*, *Ornithodoros coriaceus*; *O. h*, *Ornithodoros hermsi*; *A. a*, *Amblyomma americanum*; *R. g, Rhipicephalus geigyi*; *R. a*, *Rhipicephalus annulatus*

clade along with *B. theileri* and closely related isolates from Brazil and Madagascar, further branching into four subclades (Fig. 2). Similarly, in the *glpQ* tree, our sequences and *B. theileri* from *Rh. geygi* in Mali diverged into two subclades (Fig. 3). This divergence suggests potential subspeciation in *B. theileri*, possibly driven by adaptations to different tick species infesting vertebrate hosts across diverse geographical areas. *Borrelia* sp. Ir-Maz173, previously identified in *Rhipicephalus* ticks in northern Iran (Naddaf et al. 2020), exhibited a considerable genetic similarity of 96.9-97.2% with our *flaB* sequences. However, in phylogenetic analysis, this isolate appeared as a sister taxon alongside an isolate from Portugal, suggesting a somewhat distant relationship from *B. theileri* (Fig. 2).

Our qPCR also detected *Borrelia* in other DNA samples obtained from *Ix. ricinus* and *Ha. punctata* tick specimens. However, despite low *Ct* values, we could not amplify the *flaB* and *glpQ* genes from these specimens. Previous reports have also documented the higher sensitivity of qPCR compared to conventional PCRs (Gil et al. 2005; Naddaf et al. 2020; Ornstein and Barbour 2006). Notably, DNAs extracted from the midguts of ticks (samples Ir-Maz1, IrMaz2, and Ir-Maz3) had higher *Ct* values in qPCR, yet yielded sharper *flaB* and *glpQ* bands and superior sequencing results (Table 3).

Various tick species infest livestock in Mazandaran province (Nabian et al. 2007; Nasibeh et al. 2010; Rahbari et al. 2007; Razmi et al. 2007; Vahedi Noori et al. 2015); however, we only captured four species, possibly due to limited sampling time and a focus on forest areas. *Ixodes ricinus* was the most collected species, similar to our previous survey in Mazandaran province (Naddaf et al. 2020).

Borrelia theileri DNA has also been identified in *Ornithodoros* ticks (Safdie et al. 2010) and human head lice (Amanzougaghene et al. 2016). Soft ticks may acquire this agent during the spirochetemia or via co-feeding transmission and subsequently test positive in PCR assays (Filatov et al. 2023). However, infection in head lice remains controversial. Head

lice are very specific to humans and could have only received the agent via feeding on *B. theileri*-infected individuals, which makes the scenario more dilemmatic.

Iran is home to several veterinary ticks-borne infectious diseases like anaplasmosis, babesiosis, and tularemia manifested by fever, anemia, and jaundice (Esmaeili et al. 2019; Haghi et al. 2017; Hosseini-Vasoukolaei et al. 2014; Mirahmadi et al. 2022). Hence, despite displaying similar but mild symptoms (Abanda et al. 2019), screening for bovine borreliosis could offer a broader insight into circulating infectious diseases in livestock. *Rhipicephalus annulatus* has been documented in Lorestan, Mazandaran, Golestan, and Gilan provinces in Iran (Davari et al. 2017; Nabian et al. 2007; Ronaghi et al. 2015; Saidi et al. 2016; Telmadarraiy et al. 2010; Ziapour et al. 2017) as well as in neighboring countries such as Turkey, Iraq, and Pakistan (Ghafar et al. 2020; Jalil and Zenad 2016; Koc et al. 2015). Further investigation of ticks, livestock, and wild animals in various regions of Iran and neighboring countries can enhance our understanding of *B. theileri* and its relationship with other closely related species that infect *Rhipicephalus* ticks.

Conclusions

In this study, we have uncovered the presence of *B. theileri* in *Rh. annulatus* ticks in the north of Iran. The high identity of generated partial *flaB* and *glpQ* sequences with *B. theileri*, along with their clustering within well-supported clades with this species and closely related isolates from various geographical origins, offer conclusive evidence for the occurrence of *B. theileri* in this area. The observed divergence in *B. theileri flaB* and *glpQ* sequences from multiple geographical regions suggests potential subspeciation driven by adaptations to different tick species. This divergence in our *flaB* sequences implies the possible introduction of *B. theileri*-infected ticks from different geographical origins into Iran. As *B. theileri* has not been previously detected in domestic animals in Iran, monitoring this infection could enhance our understanding of febrile diseases in livestock on a broader scale.

Reactions were performed in 20 µL containing 4 µL (~50 nM) of template DNA, 10 µL of Master Mix 2X (Amplicon, Denmark), 500 nM of each primer, 200 nM of the probe, and deionized-distilled water (DDW) to the final volume. Amplifications were performed using an ABI StepOnePlus Real-Time PCR system (Applied BioSystems, USA). The amplification protocol included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. DDW was included as a no-template control (NTC) in all assays to ensure the reagents were free of DNA contamination. *Borrelia burgdorferi* sensu stricto DNA (AmpliRun® Borrelia DNA) was used in the control reactions with approximately 60,000, 6,000, 600, 60, and 6 bacteria DNA copies per reaction to determine the limit of detection, as previously described by Naddaf et al. (2020).

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Author contributions SRN and MR conceived and designed the study; SPZ and SRN participated in sample collection and identification; MM, SRN, MR, and AAS performed laboratory experiments and analyzed data.

SRN, MM, MR, and SPZ contributed to writing and revising the manuscript. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate The ethical committee of the Pasteur Institute of Iran approved this project (approval ID: IR.PII. REC.1400.051).

Consent for publication All authors who participated in the study concurred with the submission and subsequent revisions submitted by the corresponding authors.

Competing interests The authors declare no competing interests.

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