Original Article

Detection and genetic identification of *Borrelia lusitaniae* in questing *Ixodes inopinatus* tick from Tunisia

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ABSTRACT

Background: Until now, there has been limited information on the prevalence and the phylogeny of *Borrelia burgdorferi* sensu lato in *Ixodes* ticks in Tunisia, particularly in *Ixodes inopinatus*.
Methods: The present study aimed to determine the prevalence and the phylogeny of *B. burgdorferi* s.l., in coexisted *I. ricinus* and *I. inopinatus* ticks collected from Northern Tunisia. One hundred questig ticks were collected during winter 2020 by tick-dragging method in Beja gouvernorate located in the north of Tunisia. Real-time PCR panel targeting *B. burgdorferi* s.l. 23S rRNA gene were performed. Positive DNA samples were subjected to conventional PCRs targeting 457 bp fragment of the *Borrelia* sp. flagellin (*fla*) gene using primers FlaF/FlaR. The identified *Borrelia* sp. isolate underwent partial sequence analysis to determine genospecies and evaluate their phylogenetic position.
Results: The study revealed a prevalence rate of 28% (28/100) for *B. burgdorferi* sensu lato in the *Ixodes* ticks. The prevalence rates across tick species and genders did not show significant variations ($p > 0.05$). Interestingly, the study underlines the coexistence of *I. inopinatus* and *I. ricinus* sharing the same geographic areas in Northern Tunisia. Furthermore, DNA of *B. lusitaniae* was detected in *I. inopinatus* ticks for the first time in Tunisia. Revealed *B. lusitaniae* bacterium is similar to previously identified strains in Mediterranean region, but distinct from those isolated exclusively from countries of Eastern and Central Europe, such as Serbia, Romania, and Poland. This study highlights the prevalence of *B. burgdorferi* s.l. in *I. ricinus/I. inopinatus* ticks, and reveals *B. lusitaniae* in *I. inopinatus* ticks for the first time in Tunisia.
Conclusion: These findings suggest the involvement of *I. inopinatus* as a potential vector of this pathogenic genospeciess in Tunisia. This may help understanding the ecology of *Ixodes* ticks, the natural infection and the transmission dynamics of *Borrelia* species in this country.

1. Introduction

Borrelia is a genus of spirochetal bacteria that relies on arthropod vectors, mainly *Ixodes* ticks, and mammalian and/or avian hosts for its survival and transmission [1]. Taxonomically, *Borrelia* species were divided into two groups showing different levels of pathogenicity. The first group, known as the *Borrelia burgdorferi* sensu lato complex, includes agents responsible for Lyme disease and the second group associates species causing the relapsing fever [2].

Lyme disease causing-bacteria were highly adapted to different micro-climates, animal hosts and arthropod vectors which lead to its genetic diversity and its wide geographic distribution [3]. Bacteria of *Borrelia* genus manage to survive through resistance genes selection and regulation which promote an enzootic model of natural infection and make control and eradication more difficult [4].

It is a proven fact that the *Ixodes ricinus* complex and closely related hard ticks (Acari: *Ixodidae*) are highly incriminated in the transmission of *Borrelia* spp. between

animals and to humans [5,6]. Entomological investigations carried out in Tunisia, Algeria, and Morocco prouved the large distribution of *Ixodes ricinus* ticks specifically in cooler and humid areas [7].

Recent phylogenetic researches reported the occurrence of new tick species called *Ixodes inopinatus*, and sharing the same geographic areas of *I. ricinus* but remarkably extended for sub-humid and dry Mediterranean regions of Algeria, Morocco, Portugal, Spain, and Tunisia [8,9]. In Tunisia, researches focused on this topic have been developed in the last years but data still limited regarding phylogeny and its geographic distribution. *Ixodes ricinus* ticks, the primary carriers of *B. burgdorferi* s.l. in Europe, are abundant in the humid regions of Tunisia [7,10] and are commonly infected with *Borrelia lusitaniae* [11–14] that predominates in Mediterranean countries such as Portugal, Morocco, and Italy [14–16].

Numerous studies confirmed the zoonotic potential of *B. lusitaniae* [17,18]. It was detected in human patients' samples with suspected Lyme borreliosis [19,20]. However, the pathogenicity of *B. lusitaniae* remains controversial to be limited to some genotypes [19]. Two strains of *B. lusitaniae* were isolated from human clinical cases, underscoring the importance of studying this pathogen [18].

The aim of our study is to determine the presence of *B. burgdorferi* s.l. in *I. ricinus* and *I. inopinatus* ticks, and to identify the infecting species through sequencing and determine its phylogenetic position.

2. Materials and methods

2.1. Study regions, tick collection, and diagnosis

A total of 100 ticks were collected from the vegetation in Amdoun district, located in the governorate of Beja, northern Tunisia, during winter 2020. The Amdoun district falls within a lower humid bioclimatic area. Specimens of ticks were obtained by dragging method from the vegetation. Morphological identification of ticks was conducted based on the established taxonomic keys published by Walker et al. [21]. Ticks were identified at the genus and species levels by amplifying and sequencing a

specific region of the mitochondrial 16S rRNA gene. Ticks were individually preserved in a dedicated tube containing 70% ethanol and stored at a temperature of −20°C for further analysis and testing.

2.2. Total DNA extraction and tick DNA amplification

Identified ticks were immersed in distilled water during 10 minutes, dried on sterile filter paper, and individually crushed using the TissueLyser LT (Qiagen, Hilden, Germany). DNA was extracted from 100 μL of the homogenized tick sample in accordance with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) instructions. The quality and quantity of the extracted DNA were assessed using Qubit® dsDNA assays (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). To confirm the efficiency of the extraction process, polymerase chain reaction (PCR) amplification of the tick-specific 16S rRNA mitochondrial gene was carried out. The primers TQ16S+1F and TQ16S-2R, as described by Black and Piesman [22] (Table 1), were employed for this purpose. The DNA extracts were eluted in a final volume of 100 μL and stored at −20°C until further use.

2.3. Real-time PCR detection of Borrelia burgdorferi *sensu lato*

DNA samples were subjected to identify the presence of *B. burgdorferi* s.l. using complex-specific primers and a TaqMan probe, following the methodology outlined by Courtney et al. [23] (Table 1). The screening targeted a 75-bp fragment within the *B. burgdorferi* s.l. 23S rRNA gene. Real-time PCR was conducted using Premix Ex Taq™ (Perfect Real Time) (Takara, Mirus Bio, Madison, WI, USA) in a quantitative thermal cycler system (7500/7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed in a simplex format under optimized reaction conditions, utilizing Bb23Sf and Bb23Sr primers at a concentration of 700 nM each, Bb23Sp-FAM probe at a concentration of 175 nM, and 2 μL of template DNA. The cycling conditions involved an initial activa-

Table 1

Primers and probes used in the detection of *Borrelia burgdorferi* sensu lato and the species identification and characterization of *Ixodes inopinatus* and *Borrelia lusitaniae*.

Target tick and bacteria	Target gene	Primers and probes	Primer sequences (5′-3′)	Fragment size (bp)	References
Tick	Mitochondrial 16S rRNA	TQ16S+1F TQ16S-2R	CTGCTCAATGATTTTTTAAATTGCTGTGG ACGCTGTTATCCCTAGAG	324	Black and Piesman [22]
B. burgdorferi s.l.	23S rRNA	Bb23Sf Bb23Sr Bb23Sp	CGAGTCTTAAAAGGGCGATTTAGT GCTTCAGCCTGGCCATAAATAG 6FAM-AGATGTGGTAGACCCGAAGCCGAGTG-6TAMRA	75	Courtney et al. [23]
Borrelia spp.	*fla*	FlaF FlaR	AACACACCAGCATCACTTTCAGG GATTWGCRTGCGCAATCATTGCC	457	Richter et al. [24]

tion of the Taq DNA polymerase at 95°C for 15 minutes, followed by 45 cycles consisting of 1 minute denaturation at 95°C, and 1 minute annealing-extension at 60°C. Each run included negative and positive controls to ensure the accuracy and reliability of the results obtained.

2.4. Statistical analysis

The prevalence rates were calculated with exact confidence intervals (CI) at a 95% level. To compare the prevalence rates of *B. burgdorferi* s.l. among different categories for each risk factor, the χ^2 test and Fisher's exact test, available in Epi Info 6.01 software (CDC, Atlanta), were employed. Statistical significance was determined using a threshold value of 0.05. Additionally, a chi-square Mantel-Haenszel test was conducted to account for any potential confounding factors.

2.5. Single PCRs and sequencing for Borrelia and Ixodes species identification

For *Borrelia* genospecies identification, PCRs were conducted targeting the flagellin (*fla*) gene using primers FlaF and FlaR, which amplify a 457 bp fragment as described by Richter et al. [24]. Tick-specific primers were also used as previously described (Table 1). Positive PCR products were selected and purified using the GF-1 Ambi Clean kit (Vivantis, Oceanside, CA, USA) following the manufacturer's instructions. Purified DNA amplicons were then subjected to bidirectional sequencing using the Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI3730XL automated DNA sequencer (Macrogen Europe, Amsterdam, The Netherlands).

The resulting chromatograms were analyzed using Chromas Lite Version 2.01 software (http://www.technelysium.com.au/chromas_lite.html). Raw sequences from both the forward and reverse strands were obtained to ensure maximum data accuracy. The complementary strands of each sequenced product were manually assembled using DNAMAN software (Version 5.2.2; Lynnon Biosoft, Quebec, QC, Canada), with primer regions automatically removed. To assess sequence similarities, multiple sequence alignments were performed using the CLUSTAL W method. BLAST analysis was conducted to compare the obtained sequences with previously reported sequences (http://blast.ncbi.nlm.nih.gov/). Genetic distances between operational taxonomic units were calculated using the maximum composite likelihood method [25], and neighbor-joining trees [26] were constructed. The statistical support for internal branches of the trees was evaluated through bootstrapping with 1000 iterations [27].

3. Results

3.1. Ticks identification and distribution

Collected ticks were identified according to species and gender levels. A total of 60 specimens were molecularly identified as Ixodes inopinatus, while 40 were classified as Ixodes ricinus (unpublished data). Regarding gender, 39 specimens were considered as males and 61 were females.

3.2. Molecular prevalence of Borrelia burgdorferi sensu lato

Overall, 28.0% (28/100) ticks were revealed positive for B. burgdorferi s.l. pathogens. Regarding tick species, an apparent absence of a noteworthy link was noted between tick species and the presence of bacterial pathogens ($p = 0.41$, Table 2). Out of 60 I. inopinatus tick specimens, 15 (25%) showed positive result for B. burgdorferi s.l. However, I. ricinus ticks underlined slightly higher positive rate estimated at 32.5% (13/40). According to tick gender, no significant association was noted with prevalences of 38.4% (15/39) and 21.3% (13/61), respectively, for males and females ($p = 0.06$, Table 2).

3.3. BLAST analysis and phylogenetic study

All tick specimens testing positive by Real-time PCR underwent subsequent PCR targeting the *fla* gene, followed by sequencing. The amplification resulted in weak bands, likely attributed to the low bacterial load in the positive tick specimens. Consequently, successful sequencing was achieved for only one sample. The analysis of partial *fla* and mitochondrial 16S rRNA sequences (456 and 275 bp, respectively) from a selected *B. burgdorferi* s.l. bacterium and its associated infected *Ixodes* tick specimen revealed that the tick species is *I. inopinatus* (GenBank accession number OR253784) and the infecting *Borrelia* genospecies is *B. lusitaniae* (GenBank accession number OR248148).

Identified *I. inopinatus* specimen showed a genetic similarity of 99.64% to the Tick GenoMi_Ir176 isolate obtained from a questing *I. inopinatus* specimen collected in the north of Tunisia (GenBank accession number OP375401). According to the alignment of the partial mitochondrial 16S rRNA fragment isolated from *I. inopinatus* in this study and those of *I. inopinatus* specimens and other genetically related *Ixodes* species found in GenBank, a relatively heterogeneous cluster of *I. inopinatus* was generated but remained genetically close to *I. ricinus* cluster. The present specimen was classified within this cluster along with other specimens previously found in vegetation in northern Tunisia (GenBank accession number OP375401) and collected from a migratory bird in Italy (GenBank accession number MW173342) (Fig. 1).

Table 2

Molecular prevalence rates of Borrelia burgdorferi sensu lato according tick species and gender.

Factors	Classes	Number	Positive	Rate (95% CI)[1]	p-value (Khi)[2]
Tick species	*Ixodes inopinatus*	60	15	25.0±0.101	0.41 (0.66)
	Ixodes ricinus	40	13	32.5±0.174	
Tick gender	Male	39	15	38.4±0.013	0.06 (3.44)
	Female	61	13	21.3±0.092	
Total		100	28	28.0±0.064	

[1]: Confidence Interval of 95%; [2]: calculated according to different risk factors.
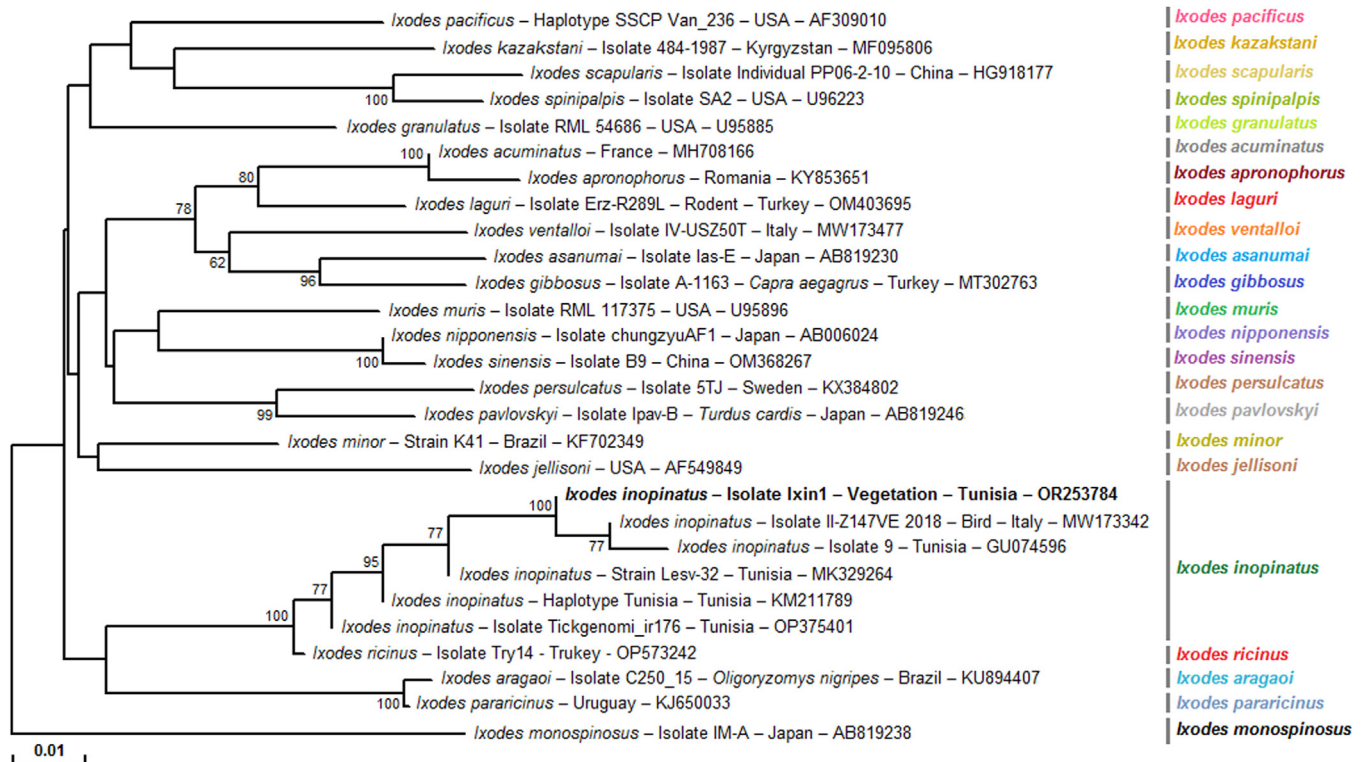
Fig. 1. Neighbor-joining tree based on the alignment of partial mitochondrial 16S rRNA sequence (275 bp) of *Ixodes inopinatus* obtained in this study with selected sequences representative of other *Ixodes* spp. isolates. Numbers over the branches indicate the percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1000 replicates, percentages greater than 50% were exclusively represented). The Tunisian isolate obtained in this study was written in bold letters. One *Ixodes monospinosus* 16SrRNA partial sequence was added as an out-group.

The partial sequence analysis of our *B. lusitaniae* bacterium revealed a genetic similarity of 98.03% to Strain PotiB2 of *B. lusitaniae* isolated from Portugal (GenBank accession number DQ111036), differing in 9 nucleotide positions, all of which are synonymous substitutions. Compared to other *Borrelia* isolates identified worldwide, the phylogenetic analysis reported phylogeographic distribution. In particular, in the phylogenetic tree, *B. lusitaniae* cluster is subdivided into two subclusters. One subcluster included isolates and strains from Mediterranean countries such as Tunisia and Portugal, while the other subcluster consisted exclusively of isolates from countries of Eastern and Central Europe like Serbia, Romania, and Poland. *B. lusitaniae* isolated in this study was classified within the first subcluster, along with two Portuguese strains, PotiB1 and PotiB2 (GenBank accession numbers DQ111035 and DQ111036, respectively) (Fig. 2).

4. Discussion

The present study reported the presence of *I. inopinatus* and *I. ricinus* sharing the same geographic areas. This sympatric occurrence of these two tick species has been previously described in Tunisia [28,29], as well as in other countries such as Algeria, Spain, and Turkey [30–32]. These recent reports underlined the progressive extension of *I. inopinatus* territory in several geographic regions with different bioclimate conditions.

In our study, we detected the presence of *B. burgdorferi* s.l. in *I. ricinus* and *I. inopinatus* ticks in northern Tunisia. However, the overall infection rate (28%) observed is lower compared to other sampling sites in Tunisia (30.5%, 34%) [11,12] and Morocco (47.8%) [14]. A lower average prevalence in Europe has been reported and is estimated at 12.3% [33]. The variations in *B. burgdorferi* s.l. prevalence rates among different regions
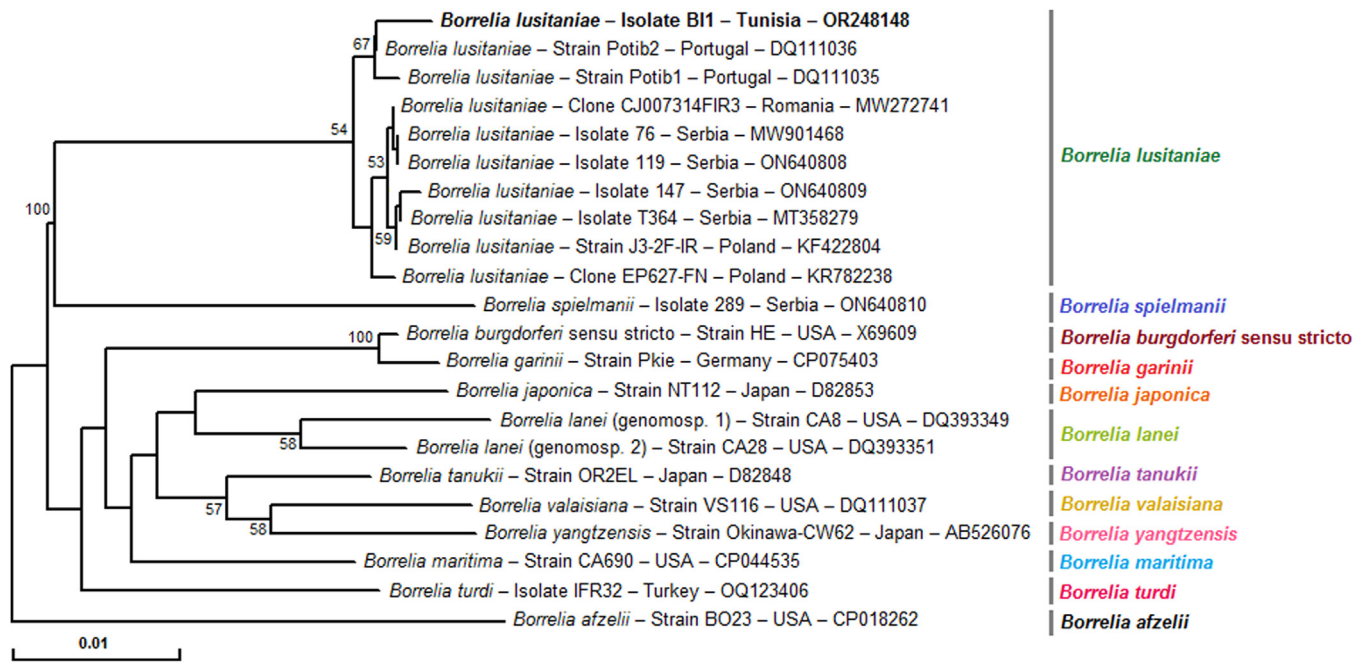
Fig. 2. Phylogenetic tree inferred with partial sequences (456 bp) of the *fla* gene of *Borrelia burgdorferi* s.l. complexe isolated from *Ixodes* sp. ticks found in GenBank using the neighbor-joining method. Bootstrap values (1000 replicates) are indicated in each node (only percentages greater than 50% are shown). The novel *B. lusitaniae* isolate obtained in the present study is indicated with bold letters. One *Borrelia afzelii fla* partial sequence was added as an out-group.

within the same country could be attributed to various factors, including sampling seasons, bioclimatic zones, molecular assay techniques, and also environmental conditions influencing the abundance and activity of ticks, mainly represented by vegetation, landscape structure, occurrence and density of hosts, use of grazing, and management practices [34,35].

The detection of *B. burgdorferi* s.l. species DNA was higher in *I. ricinus* (32.5%) compared to *I. inopinatus* (25%), suggesting that *B. burgdorferi* s.l. infection may be maintained in the studied area through the combined activity of *I. ricinus* and *I. inopinatus*. Consistent with previous reports, *B. lusitaniae* remains the exclusive genospecies within *B. burgdorferi* s.l. complex isolated from *I. ricinus* ticks collected in Tunisia [12,13]. Similar findings were observed in Portugal, where a high *Borrelia* infection rate was identified in *I. ricinus* ticks, with *B. lusitaniae* being the predominant genospecies [15]. These observations suggest a decrease in genospecies diversity of *B. burgdorferi* s.l. toward the southwestern margin of its Old-World subtropical range [15].

Ixodes inopinatus, a tick species closely related to *I. ricinus*, was first described in 2014 [8] and has been reported in Tunisia, Morocco, Algeria, Spain, Portugal, Turkey, Austria, and Germany [9,28,30-32,36,37]. Previous studies have indicated a divergent clade of *I. ricinus* in North Africa [38], suggesting that *I. inopinatus* specimens may be misclassified as *I. ricinus* in the past [8,36]. The geographic distribution of *Ixodes* ticks is influenced by various climatic and ecological factors, including temperature, humidity, and plant cover, which impact their emergence and activities [39,36]. Notably, species like *I. inopinatus* and *I. ricinus* can infest animals and humans both naturally and accidentally, suggesting a potential for interaction or simple coexistence within preferred geographic areas [28]. Genetic analysis has shown distinct clustering of *I. ricinus* and *I. inopinatus* sequences, with variations in their 16S rDNA sequences allowing for their differentiation [8,32,37].

The results of the blast analysis and phylogenetic study offer valuable insights into the genetic relationships among our *B. burgdorferi* s.l. bacterium, the associated *Ixodes* tick specimen, and other related species documented in GenBank. Analysis of partial *fla* and mitochondrial 16S DNA sequences identified *I. inopinatus* as the tick species involved in the carriage of *B. lusitaniae* revealed in the present study.

The significant genetic similarity of 99.64% between our *I. inopinatus* specimenand the Tick GenoMi_Ir176 isolate from Tunisia [40] suggests a close relationship and possible proximity of sampling sites. These findings raise intriguing questions about the distribution and prevalence of *I. inopinatus* in various regions of Tunisia. Further investigations could delve into the ecological factors contributing to the presence and spread of this tick species, as well as the potential implications for *Borrelia* transmission within the country. Additionally, the presence of an *I. inopinatus* specimen from vegetation in northern Tunisia [40] and another from a migratory bird in Italy [41] within the same cluster as our specimen indicates a broader distribution of genetically related ticks and potentially *Borrelia* strains across Mediterranean regions.

These findings suggest potential avenues for tick dispersal and the transmission of *Borrelia* across geographical boundaries.

Regarding our *B. lusitaniae* bacterium, its genetic similarity of 98.03% to the PotiB2 strain from Portugal [24] signifies a close relationship and warrants further investigation into the prevalence and genetic diversity of *B. lusitaniae* in different populations within the Mediterranean region. Comparative analysis reveals the presence of a relatively diverse cluster of *B. lusitaniae*, which can be further subdivided into two distinct subclusters. This subdivision implies the existence of separate genetic lineages within *B. lusitaniae* and raises questions about the factors influencing their geographic divergence [42–44].

Ixodes inopinatus is sympatric and morphologically and ecologically similar to *I. ricinus*. Tick infections with *Borrelia* and other pathogens may occur not only through infected animal hosts but also through infected specimens via the co-feeding process [45]. These findings provide a foundation for future investigations into the factors influencing the genetic diversity and population structure of *I. inopinatus* and the associated *B. lusitaniae* isolates. Subsequent studies could explore ecological and environmental variables contributing to the observed patterns and seek to elucidate the mechanisms underlying the transmission of tick-borne diseases in different geographic regions.

5. Conclusion

This present study provides new evidence for the presence of Lyme borreliosis in North Africa. It is crucial to enhance our understanding of the factors that contribute to the maintenance of this disease in nature in order to minimize the risk of infection and disease. Further investigations are needed to assess the transmission risk of borreliosis by hard ticks in the study area. The detection of *B. lusitaniae* DNA in *I. inopinatus* ticks suggests the potential role of this tick species as a vector for these pathogens, but confirmation through transmission experiments is necessary. These future studies will help improve our knowledge of the epidemiology and dynamics of Lyme borreliosis in the region and inform effective prevention and control strategies.

Author contributions

R.S. conceived the idea. R.S. and M.B.A. carried out the ticks sampling. K.A., H.B., A.M., R.S. performed the experiments. M.B.S. and M.K. performed risk factor analysis, and bioinformatic study. R.S., H.B. and M.B.S. wrote the manuscript. M.B.S. and L.M. edited and finalized it. All authors read and approved the final version.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data available statement

All data supporting this study are included in the manuscript.

Ethics statement

Not applicable.

Informed consent

Not applicable.

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