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1 Ticks and Tick-borne Diseases

2 Short Communication

3 Tick-borne encephalitis virus in *Ixodes ricinus* (Acari: Ixodidae) ticks, Tunisia

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## 20 ABSTRACT

21 Tick-borne encephalitis virus (TBEV) is the etiologic agent of tick-borne encephalitis (TBE)  
22 and transmitted by *Ixodes ricinus* (Linnaeus, 1758) in Europe. The geographical distribution  
23 of *I. ricinus* in the Palearctic region covers also northern Africa, including northwestern  
24 Tunisia. While the eco-epidemiology of TBE in Europe is well documented, no data  
25 concerning TBEV from northern Africa are available. We investigated whether TBEV is  
26 circulating in Tunisia. A total of 877 adult *I. ricinus* collected from northwestern Tunisia were  
27 examined in pools for the presence of TBEV by nRT-PCR. Viral RNA was detected in one  
28 pool of three engorged ticks, yielding a minimum infection rate of 0.11% (1/877).  
29 Phylogenetic analysis showed that Tunisian TBEV strain belongs to the European lineage.  
30 We report for the first time the presence of TBEV in *I. ricinus* from northern Africa. These  
31 preliminary data led us to suspect the presence of TBE in Tunisia. Therefore, more studies are  
32 needed to assess the public health importance of TBEV in northern Africa.

33

34 *Key words:* Tick-borne encephalitis virus; Ticks; Tunisia

35

## 36 **1. Introduction**

37 Tick-borne encephalitis virus (TBEV) is the etiologic agent of tick-borne encephalitis (TBE)  
38 which is the most important viral tick-borne disease in Europe (Süss, 2010). Five main  
39 lineages of TBEV are recognized: European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern  
40 (TBEV-FE), Baikalian (TBEV-Bkl) and Himalayan (Ecker et al., 1999; Zlobin et al., 2001;  
41 Demina et al., 2012; Kovalev et al., 2017). Case-fatality rate for TBEV-Eu and TBEV Sib is  
42 low (1 to 2%) while for TBEV- FE is up to 30% (Gritsun et al., 2003). While the TBEV-Eu  
43 is transmitted mainly by *Ixodes ricinus* (Linnaeus, 1758) (Lommano et al., 2012), TBEV-Sib  
44 and TBEV-FE are transmitted by *Ixodes persulcatus* Schulze (Kholodilov et al., 2019).

45 *Ixodes ricinus* has a very wide geographic distribution throughout Europe and in parts  
46 of northwestern Africa (Gern and Humair, 2002; Piesman and Gern, 2008). In Tunisia, *I.*  
47 *ricinus* is principally located in oak forests, in humid to semi-humid microclimatic zones of  
48 the Kroumirie Mountains in the northwest (Zhioua et al., 1989, 1999; Younsi et al., 2005;  
49 Dsouli et al., 2006). In previous studies, we reported for the first time the presence of *Borrelia*  
50 *lusitaniae* (Zhioua et al., 1999) and *Borrelia garinii* (Younsi et al., 2005) in *I. ricinus*  
51 collected from northwestern Tunisia. While *I. ricinus* is considered the main vector of TBEV  
52 in Europe (Lommano et al., 2012; Alfano et al., 2020), this arbovirus has never been studied  
53 before in northern Africa. Therefore, this study was undertaken to assess the presence of  
54 TBEV in *I. ricinus* from northwestern Tunisia by molecular and phylogenetic analysis.

## 55 **2. Materials and Methods**

### 56 *2.1. Study area, tick sampling, and tick identification*

57 Ticks were collected from the oak forest of EL Jouza (36°58'N, 9°05'E), located in  
58 northwestern Tunisia by flagging and from grazing cattle during the period from November  
59 2015 through February, 2016, a period corresponding to the peak activity of only adult *I.*  
60 *ricinus* in Tunisia (Dsouli et al., 2006). Larvae and nymphs are active on vegetation from

61 March to August (Dsouli et al., 2006). Ticks were identified to species level by using  
62 available adult *I. ricinus* morphological identification keys (Perez, 2007; Estrada-Peña et al.,  
63 2004). It is of major importance to point out that a new tick species *Ixodes inopinatus*  
64 (Estrada-Peña et al., 2014), closely related to *I. ricinus* has been described by Estrada-Peña et  
65 al. (2014). *Ixodes inopinatus* is distributed mainly in the dry areas of the Mediterranean region  
66 (Estrada-Peña et al., 2014). Both species are sympatric in northwestern Tunisia (Younsi et al.,  
67 2019), and in Central Europe (Chitimia-Dobler et al., 2018). Identifying adult ticks is difficult  
68 as both species have intermediate morphological characters, and therefore, molecular analysis  
69 of 16SrRNA sequences (Mangold et al., 1998) was used to differentiate between *I. ricinus*  
70 and *I. inopinatus* (Estrada-Peña et al., 2014; Chitimia-Dobler et al., 2018; Younsi et al., 2019;  
71 Hauck et al., 2019). Taking into account that both species cannot be differentiated based  
72 solely on morphological characters (Younsi et al., 2019), and since no molecular analysis was  
73 performed to confirm morphological identification, we are aware that many *I. inopinatus* have  
74 been misclassified as *I. ricinus*. Therefore, bearing in mind the limited scope of this  
75 preliminary work, we considered both species as *I. ricinus*.

## 76 2.2. Extraction of nucleic acid, detection and identifying TBEV infections

77 Ticks were pooled according to the collecting date with a maximum of 10 specimens per pool  
78 for the non-engorged ticks and up to 5 per pool for engorged ticks, and directly stored at -  
79 80°C until use. Ticks were homogenized in 500 µl of Minimum Essential Medium (MEM)  
80 using a PreCellys.24-Bertin instrument. Total RNA was extracted according to the Qiagen  
81 RNA Mini Kit (Qiagen, Hilden, Germany) from 140 µl of tick's supernatant. During the RNA  
82 extraction, no positive control was used to preclude any risk of contamination. Instead, only  
83 negative control consisting of water solution was used. The presence of TBEV RNA was  
84 determined by conventional flavivirus reverse transcription heminested PCR  
85 (Scaramozzino et al., 2001) using the SuperScript One-Step RT-PCR with Platinum Taq

86 (Invitrogen Life Technologies) according to the manufacturer's instructions. West Nile virus  
87 pre-extracted RNA was used as positive control. PCR products were visualized in a 2%  
88 TBE/agarose electrophoresis gel and sequenced in both directions using the Big Dye  
89 Terminators v3.1 kit (Applied Biosystems). Resulting sequence was firstly submitted to the  
90 NCBI BLASTN and BLASTX programs ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) for identification.  
91 A partial NS5 polymerase fragment of 218-nt was used for phylogenetic analysis by MEGA  
92 v.5.22 (Tamura et al., 2001). Phylogenetic tree was obtained according to the maximum  
93 likelihood analysis method and the tamura-3 model with general time-reversible rates among  
94 sites. The tree topology was supported by 500 bootstrap replicates. Available Louping ill  
95 virus (LIV) sequences were added to the TBEV collected database. The Omsk hemorrhagic  
96 fever virus (OHFV) sequence was used as an out-group with TBEV sequences. Genetic  
97 distances were calculated using the neighbor joining and p-distance methods.

### 98 **3. Results and Discussion**

99 Of a total of 882 adult ticks collected during the capture season from November 2015  
100 to February 2016, 877 ticks were identified as *I. ricinus*. Only 5 ticks were identified as  
101 *Hyalomma marginatum*. Of a total of 120 pools, including 92 pools from non-engorged and  
102 28 pools from engorged ticks, only one pool of three engorged females *I. ricinus* was positive  
103 by reverse transcription heminested PCR. Subsequently, the minimum infection rate (MIR) is  
104 0.11% (1/877). The sequence of the heminested positive PCR product was identified as  
105 TBEV by BLAST analysis (GenBank accession number: KY412768).

106 Phylogenetic analysis showed that five main lineages of TBEV are described (Figure  
107 1). While the Baikalian and Himalayan lineages were represented by one sequence each, the  
108 European, the Far Eastern and the Siberian subtypes are more represented (Figure 1), and they  
109 seem to share the same properties in their genetic diversity with 2.8%, 4.4% and 3.7%,  
110 respectively. The newly detected Tunisian TBEV sequence is included in the TBEV European

111 subtype, supported by a 99% bootstrap value. The European sequences within the European  
112 subtype were genetically very close to each other (0–3.7% divergence) and clearly different  
113 from the Tunisian TBEV sequence (6–8.3% divergence).

114 While there are substantial data of TBEV in *I. ricinus* from Europe, there are no data  
115 concerning this arbovirus from northern Africa. To our knowledge, this is the first report of  
116 TBEV in *I. ricinus* from northern Africa. The 0.11% MIR observed in Tunisia is in line with  
117 TBEV prevalence rates in ticks reported from European countries. The overall TBEV  
118 prevalence rates in unfed nymphal and adult *I. ricinus* from endemic areas in northern Italy  
119 varied from 0.17% (Alfano et al., 2020) to 0.21% (D'Agaro et al., 2009). The minimal  
120 infection rates of questing nymphal and adult *I. ricinus* with TBEV collected from a new  
121 endemic area in western Switzerland ranged from 0.95% to 0.21% (Lommano et al., 2012).

122 It is important to note that the detection of RNA corresponding to a region of the  
123 TBEV polymerase in a pool of three engorged female ticks may indicate that the virus could  
124 have been present in the blood meal and not actually infecting the ticks. However, the  
125 detection TBEV provide strong evidence of the circulation of the virus in this natural biotope  
126 of *I. ricinus* where *B. burgdorferi* sensu lato have been detected previously (Zhioua et al.,  
127 1989, 1999; Younsi et al., 2005; Dsouli et al., 2006).

128 Taking into account that Tunisia is positioned on the flight path of the Palearctic-  
129 African bird migration route, and migratory birds play an important role in spreading TBEV  
130 infected ticks (Waldenström et al., 2007; Lommano et al., 2014), it is not excluded that TBEV  
131 might be introduced in northwestern Tunisia, a hotspot of biodiversity where oak forests offer  
132 suitable habitat for *I. ricinus* and favorable conditions for the maintenance of this arbovirus in  
133 ticks and vertebrate hosts. This is supported by the fact that both immature stages of *I. ricinus*  
134 are active in synchrony in Tunisia (Dsouli et al., 2006), a prerequisite for efficient co-feeding

135 transmission of TBEV (Randolph et al., 1999), which is a mechanism of maintenance of the  
136 virus in nature that could not be excluded (Labuda et al., 1993).

137         The Tunisian strain of TBEV belongs to the European subtype of TBEV but distant  
138 phylogenetically from European strains. This divergence could be explained by the strong  
139 genetic differentiation between Eurasian and North-African populations of *I. ricinus* (De  
140 Meeûs et al., 2002; Noureddine et al., 2011), which in turn may modify the epidemiological  
141 landscape of the pathogens vectored by this tick species in northern Africa. Further studies are  
142 needed to clarify this hypothesis.

143         In North Africa, including Tunisia, the problem of arboviral infections has been  
144 neglected in the past except for West Nile virus infection (Fares et al., 2016). With the recent  
145 report of the first autochthonous fatal case of TBE from southern Italy (Spagnolo et al., 2018),  
146 a region acting as natural bridge connecting continental Europe to northern Africa and a  
147 common pathway of migratory birds infested mainly with *I. ricinus* (Falchi et al., 2012), the  
148 detection of TBEV in *I. ricinus* support the suspicion of the presence of TBE in northwestern  
149 Tunisia. Thus, more studies are needed to attempt isolating the virus from field-collected *I.*  
150 *ricinus* and to ascertain the circulation of TBEV in animals and human populations at risk  
151 through serosurveys.

152

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159 **Conflict of interests**

160 The authors declare that they have no competing interests

161

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- 271

272 Figure Caption

273 Figure 1. Phylogenetic analysis was performed on the partial polymerase 218-nt aligned  
274 sequences of 24 LIV strains, 74 TBEV strains retrieved from the GenBank database and the  
275 Tunisian TBEV sequence (KY412768, in bold) obtained during this study. The Omsk  
276 hemorrhagic fever virus (OHFV) sequence was used as an out-group with TBEV sequences.  
277 Phylogenetic tree was obtained according to the maximum likelihood analysis method and the  
278 tamura-3 model with general time-reversible rates among sites. The tree topology was  
279 supported by 500 bootstrap replicates.

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