First isolation and characterization of *Borrelia burgdorferi sensu lato* strains from *Ixodes ricinus* ticks in Turkey

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In order to investigate the presence and prevalence of Lyme borreliosis (Lyme disease) *Borrelia* species, 312 unfed ticks were collected by flagging at a woodland area in Trakya, in the European side of Turkey, in May 2002. Twelve of 299 *Ixodes ricinus* ticks were infected with *Borrelia* spp., as determined by cultivation in BSK medium (prevalence rate 4.0%). Ten pure cultures were subjected to further characterization by sequencing analysis of the 5S–23S rDNA intergenic spacer, 16S rDNA and flagellin gene. One isolate of *Borrelia burgdorferi sensu stricto*, two of *Borrelia garinii* (Eurasian type), two of *Borrelia afzelii*, four of *Borrelia lusitaniae* and one of *Borrelia valaisiana* were identified. However, no Asian-type *B. garinii* was found. Interestingly, all *Borrelia* species that are known to be carried by *I. ricinus* were discovered among the 10 isolates. These results provide the first evidence for the existence of the Lyme borreliosis agent in Turkey.

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INTRODUCTION

Lyme borreliosis is the most prevalent tick-borne zoonosis in Europe, North America and the Far East (Burgdorfer *et al.*, 1982; Gern *et al.*, 1998; Steere, 2001; Yanagihara & Masuzawa, 1997). *Borrelia burgdorferi* (Johnson *et al.*, 1984) *sensu lato* is classified into 11 species (Masuzawa *et al.*, 2001). In Europe, *Ixodes ricinus* is the prominent vector tick and transmits five species, *B. burgdorferi sensu stricto*, *Borrelia garinii* (Baranton *et al.*, 1992), *Borrelia afzelii* (Canica *et al.*, 1993), *Borrelia lusitaniae* (Le Fleche *et al.*, 1997) and *Borrelia valaisiana* (Wang *et al.*, 1997). Of these five species, *B. burgdorferi*, *B. afzelii* and *B. garinii* are pathogenic to humans. On the other hand, the pathogenicity of *B. lusitaniae* and *B. valaisiana* remains to be elucidated (Escudero *et al.*, 2000; Wang *et al.*, 1999). *B. burgdorferi* has been isolated and identified in Europe from the vector tick, *I. ricinus*, wild mammals and patients, in contrast to Turkey, a country that constitutes a bridge between Europe and Asia. Based on RFLP analysis of the 5S–23S rRNA gene spacer, *B. garinii* were further classified into two subtypes, Eurasian type and

Asian type. Eurasian-type *B. garinii* is found in both Europe and Asia; on the other hand, the Asian type is distributed only in east-Asian countries. *Ixodes persulcatus* is the vector of the Lyme disease borreliae *B. garinii* (Eurasian and Asian types) and *B. afzelii* in Asian countries such as Japan (Masuzawa *et al.*, 1991, 1996a), far-eastern Russia (Masuzawa *et al.*, 1997; Postic *et al.*, 1997; Sato *et al.*, 1996), north-eastern China (Li *et al.*, 1998; Takada *et al.*, 1998), western China (Uighur; Takada *et al.*, 2001b) and Korea (T. Masuzawa, K. H. Park and M. K. Cho, unpublished). *Borrelia* strains genetically related to *B. valaisiana* found in Europe are distributed in the southern part of Asia, on Okinawa island, the southernmost islands of Japan (Takada *et al.*, 2001a), Taiwan (Masuzawa *et al.*, 2000), the southern part of China (Masuzawa *et al.*, 2001) and Korea (Masuzawa *et al.*, 1999).

The aims of this study were to isolate *B. burgdorferi sensu lato* from ticks and to identify the vector tick species on the Black Sea coast of the European side of Turkey, in the Trakya region, and to verify the transmission of *Borrelia* species, especially *B. valaisiana* and *B. garinii* (Asian type), from Asia to Europe.

METHODS

Survey area and tick collection. Unfed adult ticks were collected during May 2002 from vegetation with 1 m² flannel flags at areas in

The GenBank/EMBL/DDBJ accession numbers for sequences of rRNA intergenic spacers, flagellin genes and 16S rDNA from *Borrelia* strains isolated in Turkey are AB091795–AB091804, AB091805–AB091814 and AB091815–AB091824.

Trakya, including a recreational park (Zekeriyaşoy, Belgrad Forest) in the Istanbul metropolitan area (Fig. 1). The habitat in this area contained four main zones of vegetation: (i) woods (such as poplar, oak, acacia, oleander bushes, elm and willow), (ii) high shrub (1 m or taller), (iii) low shrub and (iv) leaf and/or grass litter cover. In Trakya, some of the river banks and delta regions on the Black Sea coast are filled with sand and pebbles that lead to the formation of small lagoons and swamps covered with very dense 'langoz forests'. In some parts, thick forests (such as beech and hornbeam) start at the shore and oak forests cover the mountain valleys (such as Yıldız mountain, with an altitude of 1031 m) away from the marine-type climatic conditions. Edirne and inland Trakya have continental-type climates with less precipitation and vegetation cover. Therefore, tick populations are sparse away from the shore. Temperatures average a maximum of 26–29 °C (Istanbul area and Black Sea coast) and 29–32 °C (Edirne and Kırklareli area) and a minimum of 5–10 °C. Temperatures measured during the expedition were 1.5 °C above the mean value of the last 50 years. Precipitation was within normal values in the expedition area during our study (800–1000 mm m⁻² year⁻¹ in Istanbul and the Black Sea coast and 550–680 mm m⁻² year⁻¹ in Edirne and Kırklareli).

Isolation of *Borrelia* strains. A total of 312 unfed ticks collected from vegetation were dissected under the stereoscopic microscope and internal organs were transferred into BSK II medium (Barbour *et al.*, 1984) and incubated at 33 °C for 3 months as described by Takada *et al.* (1998).

Genotyping: *rrf-rrl* rDNA intergenic spacer, 16S rDNA and flagellin gene sequences. Aliquots (0.5 ml) of cultures were washed and cells were resuspended in 100 µl water. The resultant cell suspensions were boiled at 100 °C for 10 min. PCR was performed by the method of Masuzawa *et al.* (1996b). Primers corresponding to the 3' end of the 5S rDNA (*rrf*) (5'-CTGCGAGTTCGCGGGAGA-3') and the 5' end of the 23S rDNA (*rrl*) (5'-TCCTAGGCATTACCATA-3') (Postic *et al.*, 1994) were synthesized by a custom oligonucleotide-synthesis service (Lifetec Oriental Co.). The 16S rDNA and the flagellin gene of isolates were amplified with the primers 16SMF, corresponding to the 5' end of the 16S rDNA (5'-GCGAACGGGTGAGTAACG-3'), and 16SMR, corresponding to the 3' end of 16S rDNA (5'-CCTCCCTTACGGGTTAGAA-3'), and primers A, corresponding to

the 5' end of *fla* (5'-TCTGATGATGCTGCTGCTGGTATGG-3'), and D, corresponding to the 3' end of *fla* (5'-TCCAAAAGTTATCGTATGAG-3'). Amplicons from the intergenic spacer sequence (about 250 bp), the flagellin gene (about 750 bp) and the 16S rDNA (about 1360 bp) were purified with DNA purification columns (Microcon-PCR; Millipore). DNA cycle-sequencing was performed by using a Thermo Sequenase pre-mixed cycle-sequencing kit (Amersham) according to the manufacturer's recommendation. DNA sequences were determined by using a model SQ5500EL DNA sequencer (Hitachi). Based on the determined DNA sequence of the amplicon, RFLP analysis of the 5S–23S rDNA intergenic spacer was determined by using Genetyx-Mac software (Hitachi).

Phylogenetic analysis. CLUSTALX (Thompson *et al.*, 1997) was used to align the sequences and phylogenetic distances were calculated using the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree was drawn using N-J plot software. Bootstrap values were obtained from 1000 resamplings.

RESULTS AND DISCUSSION

Isolation of *Borrelia* strains

We examined 312 unfed ticks from four different species (*I. ricinus*, *Rhipicephalus haemaphysaloides*, *Haemaphysalis aponommoides* and *Ixodes* sp. near *turdus*), and 12 (three from nymphs, four from adult males and five from adult females) of 299 *I. ricinus* ticks were positive for spirochaetes by dark-field microscopy (Table 1). All spirochaetes were isolated from *I. ricinus*, rather than from other tick species. Overall, the prevalence rate was 4.0 %. The infection rate among *I. ricinus* was highest in Langoz (16.6 %). The prevalence rate in our survey area is relatively lower than rates found in Bulgaria (17 % by culture and more than 30 % by PCR; Christova *et al.*, 1998, 2001). Incidence of Lyme borreliosis in Central European countries such as Slovenia and Austria is relatively higher and it gradually decreases

Fig. 1. Collection site of vector ticks in Trakya, in the European part of Turkey. Numbers denote expedition areas: 1, Zekeriyaşoy; 2, Edirne; 3, Demirköy; 4, Yenice; 5, Langoz; 6, Sivriyer; 7, Hamidiye; 8, Kiyiköy; 9, Danamandira; 10, Karamandere; 11, Ormanli; 12, Kemerburgaz. Original scale: 1 : 2 000 000. Reproduced by kind permission of İki Nokta, Istanbul.

Table 1. Isolation and identification of Lyme disease-related *Borrelia* strains

Site	Location	Ticks infected/examined (prevalence, %)		Isolates (tick developmental stage)†
		<i>I. ricinus</i>	Other species*	
1	Zekeriyaoy	2/41 (4.9)	–	Tr29 (M), Tr38 (M)
2	Edirne	–	0/1 (0) ^a	
3	Demirkoy	2/102 (2.0)	–	Tr77 (N), Tr96 (N)
4	Yenice	0/9 (0)	0/1 (0) ^a	
5	Langoz	2/12 (16.6)	0/7 (0) ^b	Tr161 (F), Tr170 (F)
6	Sivriiler	3/41 (7.3)	0/3 (0) ^b	Tr210 (F), Tr213 (M), ND (F)
7	Hamidiye	1/37 (2.7)	–	ND (M)
8	Kiyikoy	0/20 (0)	–	
9	Danamandira	0/7 (0)	–	
10	Karamandere	1/20 (5.0)	0/1 (0) ^c	Tr293 (N)
11	Ormanli	1/8 (12.5)	–	Tr309 (F)
12	Kemerburgaz	0/2 (0)	–	
	Total	12/299 (4.0)	0/13 (0)	

*Other tick species are indicated as: *a*, *Rhipicephalus haemaphysaloides*; *b*, *Haemaphysalis aponommoides*; *c*, *Ixodes* sp. near *turdus*.

†Tick developmental stages from which isolates were obtained are abbreviated as: N, nymph; M, adult male; F, adult female. ND, Culture positive, but did not grow well in BSK II medium.

from north to south. The annual incidence of Lyme borreliosis in Bulgaria (55.0/100 000) is relatively high among European countries (O'Connell *et al.*, 1998). On the other hand, a seroepidemiological survey done in Greece among 1100 male navy recruits showed that only three of the serum samples were positive for *B. burgdorferi* antibodies (0.27 %) (Stamouli *et al.*, 2000). The prevalence in Tunisia was 34 % by PCR and 5 % by BSK culture in adult *I. ricinus* ticks (Younsi *et al.*, 2001). These reports support our observation that the estimated prevalence rates in Turkey, as determined by cultivation in BSK medium, are within the range of values expected.

Species determination based on 5S–23S rDNA intergenic spacer sequence

Ten of the 12 pure cultures were subjected to further genotyping analyses. All of them amplified about a 250 bp DNA amplicon with the intergenic spacer PCR system. Based on the DNA sequence of the amplicon, the RFLP pattern of the 5S–23S rDNA intergenic spacer was determined (Table 2). Strains Tr161, Tr210 and Tr213 showed RFLP patterns similar to that of *B. lusitaniae* PotiB2^T from *I. ricinus* (Postic *et al.*, 1994). Strain Tr170, identified as *B. lusitaniae*, generated a different RFLP pattern from that of strain PotiB2^T because of the presence of an extra restriction site. Strain Tr293 generated restriction fragments similar to those of *B. burgdorferi* B31^T. The *DraI* RFLP pattern of strain Tr29 was identical to strain VS116^T, the type strain of *B. valaisiana*, but the *MseI* pattern was different because of the absence of two sites. Strains Tr38 and Tr96 and strains Tr77 and Tr309

respectively had identical RFLP patterns to *B. afzelii* VS461^T and *B. garinii* 20047^T. However, none of the strains showed RFLP patterns similar to those of Asian-type *B. garinii* ASF (NT29 type) (Li *et al.*, 1998; Masuzawa *et al.*, 1997; Miyamoto & Masuzawa, 2002; Postic *et al.*, 1994).

These strains clustered with representative strains of each species in the phylogenetic tree (Fig. 2). Strains from Turkey showed the following sequence similarity for the 5S–23S rDNA intergenic spacer sequences: 97.3 % for Tr29 and *B. valaisiana* VS116^T, 99.6 and 97.6 % respectively for Tr38 and Tr96 with *B. afzelii* VS461^T, 100 and 98.8 % respectively for Tr77 and Tr309 with *B. garinii* 20047^T and 97.2 % for Tr293 and *B. burgdorferi* B31^T. Strains Tr161, Tr170, Tr210 and Tr213 showed more similarity to *B. lusitaniae* PotiB2^T, ranging from 94.6 to 97.3 %. From these results, one isolate of *B. burgdorferi sensu stricto*, two of *B. garinii* (Eurasian type), two of *B. afzelii*, four of *B. lusitaniae* and one of *B. valaisiana* were identified; however, no Asian-type *B. garinii* was found.

Sequence similarity of flagellin genes and 16S rDNA

Figs 3 and 4 show phylogenetic trees constructed on the basis of flagellin gene and 16S rDNA sequences. Strains Tr161, Tr170, Tr210 and Tr213 showed more similarity for partial sequences of the flagellin gene to *B. lusitaniae* strain PotiB2^T (98.5–99.3 %). Strain Tr38 and Tr96, identified as *B. afzelii*, showed sequences identical to that of *B. afzelii* VS461^T. Strain Tr77 and Tr309 showed higher similarity to *B. garinii* 20047^T (European type), isolated from *I. ricinus* in France, than to *B.*

Table 2. RFLP analysis of the 5S–23S rDNA intergenic spacer

Species assignments of Turkish isolates were determined in this study. Exact restriction fragment sizes were determined from sequences.

Strain	Amplicon (bp)	Restriction fragments (bp)		Species
		<i>DraI</i>	<i>MseI</i>	
Turkish isolates				
Tr29	255	203, 52	204, 51	<i>B. valaisiana</i>
Tr38	246	174, 52, 20	107, 68, 51, 20	<i>B. afzelii</i>
Tr77	253	201, 52	107, 95, 51	<i>B. garinii</i>
Tr96	246	174, 52, 20	107, 68, 51, 20	<i>B. afzelii</i>
Tr161	256	145, 82, 29	107, 81, 39, 29	<i>B. lusitaniae</i>
Tr170	257	146, 53, 29, 29	107, 52, 40, 29, 29	<i>B. lusitaniae</i>
Tr210	256	145, 82, 29	107, 81, 39, 29	<i>B. lusitaniae</i>
Tr213	256	145, 82, 29	107, 81, 39, 29	<i>B. lusitaniae</i>
Tr293	256	146, 53, 29, 28	107, 52, 40, 29, 28	<i>B. burgdorferi</i>
Tr309	253	201, 52	107, 95, 51	<i>B. garinii</i>
Reference strains				
<i>B. burgdorferi</i> B31 ^T	254	144, 53, 29, 28	108, 51, 38, 29, 28	
<i>B. garinii</i> 20047 ^T (Eurasia)	253	201, 52	107, 95, 51	
<i>B. garinii</i> ASF (Asia)	253	144, 57, 52	107, 57, 51, 38	
<i>B. afzelii</i> VS461 ^T	246	174, 52, 20	107, 68, 51, 20	
<i>B. lusitaniae</i> PotiB2 ^T	257	145, 83, 29	107, 82, 39, 29	
<i>B. lusitaniae</i> PotiB3	255	158, 81, 16	108, 79, 52, 16	
<i>B. valaisiana</i> VS116 ^T	255	203, 52	174, 51, 23, 7

Fig. 2. Phylogenetic tree based on the 5S–23S rRNA intergenic spacer sequence. Accession numbers are shown in parentheses. Bar, 1.0% sequence divergence. Isolates from Turkey are in bold. **Fig. 3.** Phylogenetic tree based on the flagellin gene sequence. Accession numbers are shown in parentheses. Bar, 1.0% sequence divergence. Isolates from Turkey are in bold.

garinii HT22 (Asian type), isolated from *I. persulcatus* in Japan. On the phylogenetic tree constructed from 16S rDNA sequences, the sequences of all isolates in Turkey clustered with those of strains of each species, indicating the accuracy of species determination. Five *Borrelia* species, *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae*, that are

known to be transmitted by *I. ricinus* (Wang *et al.*, 1999) were found among 10 Turkish isolates. The genetic heterogeneity and differences found between other European isolates were not detected in the Turkish isolates. Interestingly, all five *Borrelia* species that can be transmitted by *I. ricinus* have

Fig. 4. Phylogenetic tree based on the 16S rRNA gene sequence. Accession numbers are shown in parentheses. Bar, 0.2% sequence divergence. Isolates from Turkey are in bold. The sequence of *B. garinii* ASF was also determined in this study.

never been isolated from such a limited area, which makes this distribution unique. Regarding the distribution of *B. lusitaniae*, a few strains have been found in Spain (Barral *et al.*, 2002; Escudero *et al.*, 2000), Portugal (De Michelis *et al.*, 2000), Slovakia (Gern *et al.*, 1999), the Czech Republic, Moldova and Ukraine (Postic *et al.*, 1997), Tunisia (Younsi *et al.*, 2001; Zhioua *et al.*, 1999), Poland (Mizak & Krol, 2000) and Switzerland (L. Gern, personal communication). Most of the isolates found in Tunisia and Portugal were identified as *B. lusitaniae*. This terrestrial distribution indicates that this species has been maintained by a narrow range of reservoir host species inhabiting the southern part of the Northern hemisphere, around the Mediterranean Basin (De Michelis *et al.*, 2000). This led us to speculate that the transmission and proliferation of this type of borrelia occurred between *I. ricinus* and some animals that inhabit Southern Europe and which serve as reservoir hosts for *Borrelia* species. On the other hand, *B. garinii* and *B. afzelii* are frequently isolated in Germany, France and Russia (Wang *et al.*, 1999). *B. valaisiana* is found in *I. ricinus* and avian reservoirs from the UK (Kurtenbach *et al.*, 1998), Ireland (Gray *et al.*, 2000; Kirstein *et al.*, 1997), The Netherlands (Rijpkema *et al.*, 1996b), Switzerland (Peter & Bretz, 1992), Germany (Liebisch *et al.*, 1998), Italy (Cinco *et al.*, 1998), Croatia (Rijpkema *et al.*, 1996a), the Czech Republic (Postic *et al.*, 1997), Slovakia (Gern *et al.*, 1999) and Bulgaria (Christova *et al.*, 2001). *B. burgdorferi sensu stricto* isolates are mostly found in Western Europe (Wang *et al.*, 1999).

It has been speculated that the genospecies diversity of *B. burgdorferi sensu lato* decreases towards the southern margin of its European range (De Michelis *et al.*, 2000; Wang *et al.*, 1999). There is one report in which DNA of five different *Borrelia* species was found in *I. ricinus* ticks collected

in a wooded area near Sofia, Bulgaria (Christova *et al.*, 2001); however, spirochaetes were not isolated from these ticks. This is the first report of isolation of Lyme borreliae in Turkey and, moreover, the isolation of five different *Borrelia* species from *I. ricinus* ticks collected from a very limited area in Europe. This finding may suggest that there is a substantial ecological niche occupied by *I. ricinus* in the ecosystem of Trakya.

Fewer than 20 Lyme borreliosis cases have been published in Turkey since 1990 (Aksu *et al.*, 1997; Bil, 1998; Cakir *et al.*, 1990; Ergul *et al.*, 1996; Koksall *et al.*, 1990; Leblebicioglu, 1999; Ozturk *et al.*, 1996). These cases were diagnosed as Lyme borreliosis based on clinical symptoms such as arthralgia, myalgia, extreme fatigue, migratory pain, erythema migrans and positive IgG-ELISA results with antigens derived from European isolates. To our knowledge, there are no reports of *Borrelia* isolates from patients in Turkey, so *Borrelia* strains have not been isolated from either the patients addressed or from others. The authors conclude that case-control studies would be necessary in order for physicians to become more interested in Lyme borreliosis. Further risk assessment for Lyme borreliosis is important, especially in the Zekeriyakoy-Belgrad Forest region, since it is one of the most popular recreational parks of the Istanbul metropolitan area.

In conclusion, our results prove, for the first time, the existence of Lyme borreliosis agent in *I. ricinus* ticks collected in the European part of Turkey.

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