A novel, fast-growing *Borrelia* sp. isolated from the hard tick *Hyalomma aegyptium* in Turkey

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A novel, fast-growing spirochaete was isolated from the hard tick *Hyalomma aegyptium* (family *Ixodidae*, subfamily *Metastriata*) using Barbour–Stoenner–Kelly (BSK) II medium. Tick samples were taken during the summer of 2000 from the Istanbul area in northwestern Turkey. Sixty-seven of 153 adults (44 %) and 72 of 185 nymphs (39 %) were infected with the novel spirochaete, whereas none of the 20 larvae examined were infected. The optimal growth temperature of the spirochaete in BSK II medium was 34–37 °C, and it could grow at 39 °C. Doubling times at 34 and 37 °C were 5-3 and 5-1 h, respectively. Six pure cultures of the spirochaete were obtained and characterized by microscopic observation, sequence analysis of the flagellin gene (*flaB*), SDS-PAGE and Western blotting. The spirochaete was morphologically similar to those of the genus *Borrelia* and contained a 41 kDa protein reactive with mAb H9724 specific to the flagellin of a *Borrelia* species. Polyclonal antibody raised to this spirochaete reacted with several antigen bands, whereas no bands were detected with *Borrelia burgdorferi*, *Borrelia hermsii*, *Borrelia turicatae* and *Borrelia parkeri*. The *flaB* sequences of the six isolates showed high similarity, with sequence similarity values ranging from 99-2 to 100 %; however, the similarity of the isolates’ *flaB* sequences to those of the Lyme-disease-related *Borrelia* and relapsing-fever-associated *Borrelia* species was less than 90 %. These findings suggest that the unique spirochaete is a member of the genus *Borrelia*, and differs from previously described *Borrelia* species.

INTRODUCTION

Lyme borreliosis and relapsing fever are caused by *Borrelia* species (Barbour & Hayes, 1986). Lyme-disease-related *Borrelia* species (Barbour et al., 1995; Miyamoto & Masuzawa, 2002), and these are transmitted by the *Ixodes* species (family *Ixodidae*, subfamily *Prostriata*) of hard tick. Relapsing-fever-associated *Borrelia* species, including *Borrelia coriaceae*, which is suspected of involvement in the aetiology of epizootic bovine abortion, are transmitted by soft ticks of the genus *Ornithodoros* (family *Argasidae*, subfamily *Ornithodorinae*). An avian borreliosis agent, *Borrelia anserina*, is carried by the soft tick *Argas persicus* (family *Argasidae*). Other relapsing-fever-associated *Borrelia* species are *Borrelia recurrentis*, transmitted by the human body louse *Pediculus humanus*, and a bovine borreliosis agent, *Borrelia theileri*, transmitted by *Rhipicephalus evertsi* and *Boophilus* spp. (family *Ixodidae*, subfamily *Metastriata*) of hard ticks (Parola & Raoult, 2001). Furthermore, *Borrelia miyamotoi* (Fukunaga et al., 1995) and ‘*Borrelia lonestari’* (Barbour et al., 1996), carried by hard ticks, namely *Ixodes persulcatus* in Japan and *Amblyomma americanum* (family *Ixodidae*, subfamily *Metastriata*) in the USA, respectively, are classified with the relapsing-fever-associated *Borrelia* species, but their pathogenicity is still unknown. We have isolated a novel, fast-growing spirochaete from the hard tick *Hyalomma aegyptium* (family *Ixodidae*, subfamily *Metastriata*), from samples collected during the summer of 2000 from the Istanbul area in northwestern Turkey. The aim of this study was to characterize this spirochaete.

METHODS

Survey sites. Ticks were sampled from a seashore resort located on the European side of the Istanbul metropolitan area, 80 km west of
the city centre (coordinates: 41° 23′ N, 28° 21′ E). The sampling area was 20 m above sea level and 100–150 m from the coast line, and included four main zones of vegetation: (1) woods (poplar, acacia, oleaster, elm, willow), (2) high shrubs (1 m or taller), (3) low shrubs and (4) leaves and/or grass litter cover. *H. aegyptium* ticks were sampled from captured tortoises (*Testudo graeca*). Ticks were collected with forceps, placed into screw-capped tubes containing small pieces of cotton moistened with 1% mycostatin solution, to prevent desiccation and mould growth, and kept at 5°C until dissection.

**Isolation of spirochaetes.** Ticks were dipped into 70% isopropyl alcohol for 2 min, air-dried in a biological safety cabinet, and dissected using sterile jeweller’s forceps and Iris scissors. Midgut tissues were dissected, inoculated into 5 ml of Barbour–Stoenner–Kelly (BSK) II medium (Barbour, 1984) containing 7% heat-inactivated (56°C, 45 min) rabbit serum in 5 ml screw-capped tubes, and incubated at 33°C for 3 months. These cultures were examined under a dark-field microscope at ×200 magnification.

**Determination of spirochaete prevalence in ticks.** Ticks were dissected with forceps and scissors, and examined under a dark-field objective. One hundred fields of view were scanned under a dark-field microscope at ×100 magnification before determining whether a tick was infected or not.

**Cultivation of spirochaetes.** Six strains (ISTF1, IST2, ISTF2, IST4, IST6 and IST7) isolated from *H. aegyptium* and five species of *Borrelia*, namely *Borrelia burgdorferi* strains B31 and 297, *Borrelia parkeri* (undesignated), *Borrelia turicatae* (undesignated) and *Borrelia hermsii* strain H51, were grown in BSK II medium at 34°C. Growth of the spirochaetes was examined in BSK II medium by inoculation of exponential-phase cells (7 to 8 × 10⁶ cells ml⁻¹) at 25, 34, 37 and 39°C. Enumeration of the spirochaetes was done using a cell counting chamber (10μm thickness; Nippon Rinsyou Co.) under the dark-field microscope.

**Preparation of antiserum.** Isolate IST7 was inactivated at 56°C for 1 h and intraperitoneally inoculated (1 × 10⁷ cells) three times at 1-week intervals into 7-week-old ddY mice (Japan SLC, Hamamatsu). Serum was collected by cardiac puncture after the mice were killed by CO₂ inhalation.

**SDS-PAGE and Western blotting.** SDS-PAGE and Western blotting were carried out according to the method described previously (Masuzawa et al., 1991). After electrophoresis, the gels were stained with Coomassie brilliant blue. Antigens were transferred onto PVDF membranes (Bio-Rad), and specific antigen bands were subsequently detected by immunostaining with mAbs and antiserum raised against strain IST7. The mAbs used were H9724 to flagellin (Barbour et al., 1986), H5352 specific to OspA of Lyme-disease-related *Borrelia* (Barbour et al., 1983) and G7 to OspC (Masuzawa et al., 1996a).

**Microscopy.** The cellular morphology of isolate IST7 was examined by dark-field microscopy, transmission electron microscopy and scanning electron microscopy. To prepare negative staining specimens, one drop of the cell suspension concentrated by centrifugation was allowed to adsorb onto carbon-coated Formvar film supported on copper grids. The cells were stained with 2% sodium phosphotungstate for 30 s. The specimens were examined with an H-7000 transmission electron microscope (Hitachi) at an acceleration voltage of 100 kV. Samples for scanning electron microscopy were fixed with 2.5% glutaraldehyde in sodium phosphate buffer for 5 h. After washing, the cells were seeded on glass coverslips precoated with poly-L-lysine (Sigma) overnight at 4°C. The coverslips were transferred into 2% osmium tetroxide solution for 4 h. The coverslips were then treated sequentially (15 min each) with 50, 70, 80, 90 and 95% ethanol. After treatment twice with 100% ethanol, the coverslips were dried using a critical point dryer. The samples were coated with osmium by a plasma coating method (Osmium Plasma Coater; Nippon Laser & Electronics Lab.) and examined with a Hitachi S-400 and an S-2500 scanning electron microscope.

**Infectivity test.** Isolate IST7 (1 × 10⁷ cells) of low passage in BSK II medium was inoculated intraperitoneally or subcutaneously into ddY mice (5-week-old males; Japan SLC). Some mice were treated with 160 mg cyclophosphamide (kg body weight)⁻¹ at 1 day before and 1 and 3 days after the inoculation. On day 14 after inoculation, the mice were killed by CO₂ inhalation and the heart, spleen, bladder, ear and blood were inoculated into BSK II medium. The cultures were incubated at 34°C for 2 months.

**PCR of 5S–23S rDNA intergenic spacer.** Primers corresponding to the 3′ end of the 5S rDNA (rfJ) (5′-CTG CGA GTT CGC GGG AGA-3′) and the 5′ end of the 23S rDNA (rfB) (5′-TCC TAG GCA TTC ACC ATA-3′) (Masuzawa et al., 1996b; Postic et al., 1994) were synthesized. PCR was performed by the method of Postic et al. (1994).

**PCR and sequencing of the flagellin gene.** The flagellin gene (flaB) was amplified for 30 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, with primer A corresponding to the 5′ end of fla (5′-TCT GAT GAT GCT GCT GGT ATG G-3′) and primer D corresponding to the 3′ end of fla (5′-AGG TTT TCA ATA GCA TAC TC-3′). For DNA sequencing analysis, primers A and D labelled with Texas red (Fasmac) were used. The amplicons of flaB (about 740 bp) obtained after PCR were purified with a DNA purification column (Microcon-PCR; Millipore). The DNA cycle sequencing reaction was performed using the Thermo Sequenase pre-mixed cycle sequencing kit (Amersham Biosciences) according to the manufacturer’s recommendations. The partial DNA sequences were determined with a model SQ5500EL DNA-sequencer (Hitachi).

**Phylogenetic analysis.** The CLUSTAL_X software originally described by Thompson et al. (1997) was used to align the sequences, and the phylogenetic distances were calculated using the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree was drawn using NJPLOT software. Bootstrap values were obtained with 1000 resamplings.

**RESULTS**

**Prevalence**

Larval, nymphal and adult *H. aegyptium* ticks were collected from tortoises between May and September of 2000. A total of 150 tortoises were captured in 30 days in a sampling area of 500 m². On average, five ticks per day were caught depending on the weather conditions; heavy rain, warm weather (above 30°C) and cold temperatures (below 15°C) affected the activities of the tortoises. Adult ticks were found on almost every tortoise host (3-4 ticks per tortoise). Nymphal *H. aegyptium* predominated in early summer, and larvae were abundant in mid-summer. The densities of nymphs on tortoises peaked earlier than larvae in the summer, the mean number of nymphs per tortoise was 5-75 in early June. During the larval activity peak, the tortoises averaged 15-3 larvae per host. Only adult ticks were found by the end of August and September. The number of ticks per tortoise did not differ significantly among the different types of vegetation.
Spirochaete prevalence in the tick midgut was determined under the dark-field microscope. Sixty-seven of 153 adults (43.8%) and 72 of 185 (38.9%) nymphs were infected with the spirochaete, whereas none of the 20 larvae examined were infected. We did not find any free-living *H. aegyptium* by flagging the vegetation and leaf/grass litter cover. We isolated a total of 26 isolates of the spirochaete as pure cultures. Since most of the isolates did not re-grow from liquid nitrogen stocks, six strains, ISTF1, IST2, ISTF2, IST4, IST6 and IST7, were used for subsequent study.

**Morphology**

Spirochaetes isolated from *H. aegyptium* were slender and helically shaped and were highly motile in the BSK II medium under the dark-field microscope (Fig. 1a). On transmission and scanning electron microscopy observation of the specimens (Fig. 1b, c, d), the cells showed the characteristic borrelia-like shape, i.e. a helical shape with tapered ends. The length of the cells was variable, about 10–25 μm, and the diameter was 0.2–0.28 μm. Five insertion points of flagella were observed in the tapered ends (Fig. 1b). Numbers of flagella found were more than 10 in each end of the cell. The spirochaete divided by binary fission (Fig. 1d). These ultrastructural features were similar to those of members of the genus *Borrelia*.

**Growth characteristics of the spirochaete isolates**

The growth rate of isolate IST7 in BSK II medium was higher than that of *B. burgdorferi* at 34, 37 and 39 °C (Fig. 2). The optimal growth temperature was 34–37 °C, and the isolate could grow at 39 °C (*B. burgdorferi sensu stricto* could not). The doubling times of isolate IST7 at 34 and 37 °C were 5.3 and 5.1 h, respectively, and its growth rate was twice as fast as that of *B. burgdorferi sensu stricto*. All six isolates started to grow at the early-exponential phase and reached the stationary phase after 48 h. The maximum cell numbers obtained were 5 × 10⁸ cells ml⁻¹. Therefore, this spirochaete from *H. aegyptium* was considered to be a fast-growing *Borrelia* sp. in comparison with previously known *Borrelia* species (Kelly, 1984).

**Infectivity**

Normal ddY mice and cyclophosphamide-treated mice did not show any symptoms of disease after inoculation with IST7, and spirochaetes could not be re-isolated from the spleen, kidney, ear, blood, bladder and heart from these animals.
Protein profiles and reactivity with mAbs and antiserum

The protein profiles of the six isolates from *H. aegyptium* were similar to each other. These isolates contained a 41 kDa protein reactive with mAb H9724 specific to the flagellin of *B. burgdorferi* (Fig. 3a). The 38 kDa flagellin was specifically found in relapsing-fever-associated *Borrelia* species, whereas the flagellin of Lyme borreliosis agents was situated at 41 kDa. However, no reactivity of our isolates was observed with mAbs H5332 and G7 reactive to OspA and OspC of Lyme disease borrelia, respectively (data not shown). Polyclonal antibody raised against IST7 reacted with 15, 31, 33, 35, 40–45 and 49 kDa bands and also several bands of more than 70 kDa (Fig. 3b). However, no bands were detected in *B. burgdorferi*, *B. hermsii*, *B. turicatae* and *B. parkeri* because the polyclonal antiserum did not show reactivity with flagellin, which was the cross-reactive antigen of *Borrelia* cells.

PCR analysis of the 5S–23S rDNA intergenic spacer

A specific amplicon was not obtained from cultures of the six isolates by PCR of the 5S–23S rDNA intergenic spacer (data not shown).

Phylogenetic analysis based on *flaB* sequences

A phylogenetic tree based on partial *flaB* sequences (596 bp) was constructed on the basis of a sequence similarity matrix (Fig. 4). The *flaB* sequences of the six isolates showed high similarity, with sequence similarity values ranging from 99.2 to 100%; however, the similarity of the isolates’ *flaB* sequences to those of the Lyme-disease-related *Borrelia* and relapsing-fever-associated *Borrelia* species was less than 90%. On the phylogenetic tree, the sequences of the novel isolates clustered together and were situated between the clusters of Lyme-disease-related *Borrelia* and relapsing-fever-associated *Borrelia* species. These observations suggested that these isolates were unique and differed from previously described *Borrelia* species.

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**Fig. 3.** SDS-PAGE profiles and Western blot analysis of spirochaetes isolated from *Hyalomma aegyptium*. Ten micrograms of protein were applied to each lane of the 12.5% polyacrylamide gel. (a) Coomassie-brilliant-blue-stained proteins in whole-cell lysates; mAb H9724 reactive bands are indicated with arrow heads. Lanes: 1, *B. hermsii*; 2, *B. turicatae*; 3, *B. parkeri*; 4, ISTF1; 5, IST2; 6, ISTF2; 7, IST4; 8, IST6; 9, IST7; 10, *B. burgdorferi* B31. Molecular mass standard proteins (M, in kDa) are indicated on the left. (b) Western blot probed with antiserum raised against IST7. Lanes: 11, IST7; 12, *B. parkeri*; 13, *B. turicatae*; 14, *B. hermsii*; 15, *B. burgdorferi*.

**Fig. 4.** Phylogenetic tree based on *flaB* sequences. Sequences of strains ISTF1, ISTF2, IST2, IST4, IST6 and IST7 were determined in this study. Accession numbers are shown in parentheses. Bar, 5.0% sequence divergence.
**DISCUSSION**

Relapsing fever in Turkey was described in 1901 by Dr Numan (Ozsan, 1954). The *Borrelia* species isolated from *Ornithodoros erraticus* collected in Southern Turkey, which caused death in laboratory rabbits, was named *Borrelia crocidurae* (Baltazard, 1953). Other *Borrelia* species found in Turkey were the Lyme borreliosis agent *B. burgdorferi sensu lato*, which we recently isolated from *Ixodes ricinus* collected from the European side of the Black Sea coast area and identified as *B. burgdorferi sensu stricto* (Güner et al., 2003), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia valaisiana* and *Borrelia lusitaniae*.

During our survey of the Lyme borreliosis agent, we isolated six strains of a unique *Borrelia* sp. from *H. aegyptium* on tortoises (*T. graeca*) during the summer of 2000 near the Istanbul area in northwestern Turkey. We compared the biological and genetic characteristics of this spirochaete with those of Lyme-disease-related *Borrelia*, *B. burgdorferi sensu lato* strains and relapsing-fever-associated *Borrelia* species. During the course of our study, the unique characteristics of this spirochaete were revealed. The spirochaete was morphologically similar to members of the genus *Borrelia*. However, its growth rate in BSK II medium was high compared to that of *B. burgdorferi sensu stricto*. The doubling time of the novel spirochaete at optimal temperature, 34–37 °C, was less than 6 h, although the doubling time of other cultivable *Borrelia* spp. is 12–18 h (Kelly, 1984). The spirochaete reached high numbers in 2 days, which can be considered astonishing when compared to well-growing *Borrelia* spp., such as *B. burgdorferi sensu stricto*, and fastidious spirochaetes, such as *Borrelia duttonii*, which has recently been cultured in vitro (Cutler et al., 1999).

The spirochaete isolates from *H. aegyptium* showed an antigen band that reacted with mAb H9724 specific to borrelial flagellin (Barbour et al., 1986), in contrast to mAb G7, which recognizes the broad reactive epitope of OspC of Lyme-disease-related *Borrelia*. The 5S–23S rDNA intergenic spacer, which is commonly found in Lyme-disease-related *Borrelia* species, but not in relapsing-fever-associated *Borrelia* species, was also not found in this novel spirochaete, suggesting that this novel *Borrelia* species might not be a member of the Lyme-disease-related *Borrelia* complex.

Ras et al. (1996) determined the phylogenetic relationships of relapsing-fever-associated *Borrelia* species on the basis of 16S rDNA sequences. According to the results of their study, *B. duttonii* and *B. recurrentis* belong to the same group and *B. hermsii* is included in another group shared by other *Borrelia* species such as *B. parkeri* and *B. turicatae*. Interestingly, the phylogenetic tree constructed using flaB sequences (Fig. 4) showed a similar topology to that constructed using 16S rDNA sequences. The spirochaete isolated from *H. aegyptium* clustered between relapsing-fever-associated *Borrelia* species and Lyme borreliosis agents and showed a relationship with *Borrelia* species from both these groups. These biological, genetic and immunological features of this spirochaete were unique, which suggested that this spirochaete represented a new member of the genus *Borrelia*.

Regarding the vector and reservoir animals, the spirochaete described here was found in the nymphal and adult stages of *H. aegyptium* feeding on tortoises, but not in the larval ticks, which may indicate that this *Borrelia* sp. originated from the tortoises and is transmitted from the tortoises to the ticks via blood-feeding. In experimental infection of the low-passaged isolate in laboratory mice, these mice were not infected with the *Borrelia* sp., which may suggest that mice were not a preferred host for this spirochaete. However, the infectivity and pathogenicity of the *Borrelia* sp. to laboratory mice and tortoises have not been elucidated. Further investigations are needed.

The *H. aegyptium* tick is found in southwest and mid Asia, northern and southern Africa, southern Europe and the Middle East. Hoogstraal reported in 1956 (Merdivenci, 1969) that *H. aegyptium* may infest the tortoise, lizard, partridge (*Perdix* sp.), hare (*Lepus europaeus*), hedgehog (*Erinaceus* sp.), some rodents and also humans in southeast Anatolia. Merdivenci (1969) reported that they could not succeed in feeding *H. aegyptium* on guinea pigs, mice and rabbits in the laboratory. However, investigations by Hoogstraal (Merdivenci, 1969) suggested that this tick may feed on humans, even though it is usually found on tortoises, indicating that humans may be alternative hosts for *H. aegyptium*, leading to the possibility that this tick species may transmit the *Borrelia* sp. to humans if there is no other available host.

Most relapsing-fever-associated *Borrelia* species are transmitted by soft ticks, although two relapsing-fever-associated *Borrelia* species, *B. miyamotoi* (Fukunaga et al., 1995b) and ‘*B. lonestari*’ (Barbour et al., 1996), are carried by hard ticks, namely *I. persulcatus* in Japan and *A. americanum* in the USA, respectively. Lyme-disease-related *Borrelia* species are transmitted by the *Ixodes* species of hard ticks. However, there has been one report concerning the detection of *Borrelia* by PCR from *Hyalomma marginatum* hard ticks collected in Portugal, which were identified as Lyme-borreliosis-related *B. lusitaniae* (De Micheli et al., 2000). Other pathogens carried by members of the genus *Hyalomma* are Crimean-Congo haemorrhagic fever virus and ‘*Rickettsia mongoliotimonae*’ (Parola & Raoult, 2001). Some species of the genus *Hyalomma* transmit *Theileria*, the causative agent of piroplasmosis in the cow, horse, deer and water buffalo. To our knowledge, this is the first report of the isolation of a *Borrelia* sp. from the hard tick *H. aegyptium*. The pathogenicity of this *Borrelia* species in humans, domestic animals, laboratory mice and tortoises, and a detailed classification, remain to be determined. The great interest is the molecular mechanism involved in the astonishing growth of this *Borrelia* sp.
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