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Phylogenetic Analysis of the Spirochete *Borrelia microti*, a Potential Agent of Relapsing Fever in Iran

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We report a role for *Borrelia microti* as a cause of relapsing fever in Iran supported by robust epidemiological evidence. The molecular identity of this spirochete and its relation with other relapsing fever borreliae have, until now, been poorly delineated. We analyzed an isolate of *B. microti*, obtained from *Ornithodoros erraticus* ticks, by sequencing four loci (16S rRNA, *flaB*, *glpQ*, intragenic spacer [IGS]) and comparing these sequences with those of other relapsing fever borreliae. Phylogenetic analysis using concatenated sequences of 16S rRNA, *flaB*, and *glpQ* grouped *B. microti* alongside three members of the African group, *B. duttonii*, *B. recurrentis*, and *B. crocidurae*, which are distinct from *B. persica*, the most prevalent established cause of tick-borne relapsing fever in Iran. The similarity values for 10 concatenated sequences totaling 2,437 nucleotides ranged from 92.11% to 99.84%, with the highest homologies being between *B. duttonii* and *B. microti* and between *B. duttonii* and *B. recurrentis*. Furthermore, the more discriminatory IGS sequence analysis corroborated the close similarity (97.76% to 99.56%) between *B. microti* and *B. duttonii*. These findings raise the possibility that both species may indeed be the same and further dispel the one-species, one-vector theory that has been the basis for classification of relapsing fever *Borrelia* for the last 100 years.

Relapsing fever (RF), as the name implies, is characterized by recurrent febrile episodes. The causative agents of the disease, several *Borrelia* species, are transmitted by soft ticks of the genus *Ornithodoros*. The only exception is the spirochete *Borrelia recurrentis*, the etiological agent of epidemic relapsing fever that is transmitted by the human body louse (*Pediculus humanus humanus*) (4). Tick-borne relapsing fever (TBRF) is a disease endemic to Iran, with 1,415 confirmed cases from the entire country occurring from 1997 to 2006 (16). *Borrelia persica* accounts for most of these cases in locations where its argasid tick vector, *Ornithodoros tholozani*, is commonly encountered. Other *Borrelia* species, including *Borrelia microti*, *Borrelia latyschewii*, and *Borrelia baltazardii*, have also been described in Iran (14, 19). Unlike some TBRF *Borrelia*, Iranian species are not named after their specific argasid tick vectors; *B. persica* is transmitted by *O. tholozani*, *B. microti* is transmitted by *O. erraticus*, and *B. latyschewii* is transmitted by *O. tartakowskyi*. No argasid tick vector has yet been described for *B. baltazardii* (14). There are no recent reports on the occurrence of *B. latyschewii* and *B. baltazardii* relapsing fevers in Iran; in contrast, the epidemiological evidence for *B. microti* causing human infections is strong (12, 16). The spirochete was described in 1947 following isolation from *Microtus* spp. in the Kazeroun area, southern Iran. Later, following reports of sporadic human cases from the same area, the spirochetes were isolated from *O. erraticus* ticks collected from rodent burrows (12). *O. erraticus* tick infection rates may reach 50%, and the occurrence of TBRF in areas without *O. tholozani* ticks suggests a potential role for *B. microti* as the second TBRF causative agent in Iran (16). Relapsing fever cases in Hormozgan Province, south Iran, which comprise 3.7% of total RF cases reported, are mostly from localities in which *O. erraticus* ticks predominate (16). The distribution of *O. erraticus* ticks in Iran covers a vast area from the central to the south of country; these ticks are not inclined to enter domestic dwellings and are commonly found in burrows of wild rodents, including *Tatera* spp., *Meriones* spp., and *Microtus* spp. (12, 13).

Previously, the nomenclature of *Borrelia* species was based on the cospecies concept, taking into account the association of the *Borrelia* spp. with a specific tick, its geographical location, and virulence tests using laboratory animals (1). Virulence testing successfully differentiated most Iranian TBRF-causing borreliae; however, some isolates remained ambiguous (1, 13). Molecular analysis of *B. persica* and its taxonomic position with other *Borrelia* species has recently been assessed (2, 11, 21); however, there are few data on other Iranian *Borrelia* species. An initial study designed to provide species-specific PCR for detection of *B. persica* and *B. microti* using *glpQ* and 16S rRNA revealed the divergence between these two Iranian species (18).

Here, we present a more comprehensive report on the molecular characterization of *B. microti*, a potential agent of TBRF in Iran, using four loci, and demonstrate its close relationship to *B. duttonii* and *B. recurrentis*, two well-characterized RF causative agents in Africa.

MATERIALS AND METHODS

An isolate of *Borrelia* was originated from *O. erraticus* ticks that were collected from rodent burrows by scraping the walls of tunnels and sieving the contents. Tick collections were made in the Hesark region, south of Karaj City in Alborz Province, Iran, during 2000 (M. Assmar, personal communication). The isolate was maintained via continual passage in outbred white laboratory mice aged 8 to 10 weeks for about 9 successive years. Virulence tests confirmed that this isolate was distinct from *B. persica*. Unlike *B. persica*, which causes a high spirochetemia in adult guinea pigs (2, 13), in the present study, inoculation of 200 μl of infected blood

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TABLE 1 Details of 16S rRNA, *glpQ*, and *flaB* gene sequences used in this study

Species	Gene	GenBank accession no.	Reference
B. duttonii	16S rRNA	AF107364	6
	glpQ	DQ346787	8
	flaB	DQ346833	8
B. microti	16S rRNA	JF803950	This study
	glpQ	JF825473	This study
	flaB	JF825472	This study
B. recurrentis	16S rRNA	AF107367	6
	glpQ	DQ346777	8
	flaB	DQ346814	8
B. crocidurae	16S rRNA	GU350713	28
	glpQ	GU357579	28
	flaB	GU357619	28
B. persica	16S rRNA	U42297	21
	glpQ	HM161661	22
	flaB	HM194740	22
B. hispanica	16S rRNA	GU350708	28
	glpQ	GU357574	28
	flaB	GU357614	28
B. hermsii	16S rRNA	EU203150	24
	glpQ	EU194845	24
	flaB	EU194843	24
B. coriaceae	16S rRNA	U42286	21
	glpQ	AF247158	20
	flaB	D82864	9
B. parkeri	16S rRNA	AY934598	23
	glpQ	AY934635	23
	flab	AY934623	23
B. turicatae	16S rRNA	AY934610	23
	glpQ	AY934641	23
	flaB	AY934629	23

TABLE 2 Details of IGS sequences used in this study

Species	Strain designation	GenBank accession no.	Reference
B. duttonii	TzBd6	GQ401250	7
B. duttonii	TzBd19	GQ401251	7
B. recurrentis	A11	DQ000278	25
B. recurrentis	Br12	GQ401266	7
B. recurrentis	SJC13	GQ401252	7
B. duttonii	Bd4	GQ401270	7
B. duttonii	MA/15	DQ000280	25
B. duttonii	MA/18	DQ000281	25
B. recurrentis	A1	DQ000277	25
B. recurrentis	SJC4	GQ401246	7
B. recurrentis	Br5	GQ401247	7
B. recurrentis	Br7	GQ401278	7
B. duttonii	Ly	DQ000279	25
B. duttonii	TzBd4	GQ401257	7
B .duttonii	Bd6	GQ401268	7
B. duttonii	Bd10	GQ401261	7
B. duttonii	TzBd14	GQ401262	7
B. microti	IR-1	JQ436580	This study
B.duttonii	1120K3	GU350721	28
B. duttonii	CR2A	GU350722	28
B. duttonii	WM	DQ000282	25
B. duttonii	Tzbd1	GQ401245	7
B. duttonii	Bd9	GQ401244	7
B. duttonii	Bd11	GQ401243	7
B. crocidurae	Achema	GU350723	28

samples with spirochetes at concentrations ranging from 250 to 2,750 spirochetes per microliter caused occult infections (not detectable in fresh blood by dark-field microscopy or by examination of Giemsa-stained slides) in adult guinea pigs, with concentrations reaching as high as 2.1×10^7 spirochetes per ml in adult mice 2 to 4 days postinoculation. These infections were followed by 2 to 3 relapses.

Between 200 and 300 μl of blood was collected from adult mice 3 to 4 days following inoculation, when spirochete concentrations reached 1.2×10^7 to 2.1×10^7 per ml of blood. DNA extraction from blood samples was performed using a Miniprep DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions, and partial sequences of the 16S rRNA, flagellin (*flaB*), and glycerophosphodiester phosphodiesterase (*glpQ*) genes as well as an intragenic spacer (IGS) region were amplified using the primers and conditions detailed by others (2, 7, 11, 21). Final reaction volumes of 25 μl contained 20 pmol of each primer, 2 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 200 μM deoxynucleoside triphosphates, 1 U of *Taq* (Roche, Mannheim, Germany), and 3 μl of DNA. A negative control containing all reagents except DNA was included in all amplifications. PCR products were resolved on 1% agarose gels in 1× TAE (Tris-acetate-EDTA) buffer, and amplicons of the expected size were purified using a gel band purification kit (Pharmacia, Piscataway, NJ) according to the manufacturer's recommendations and later sequenced in both directions using the same primers at concentrations of 10 pmol at the SeqLAb laboratory in Germany. The 16S rRNA (1,052-bp), *glpQ* (667-bp), and *flaB* (718-bp) sequences of our isolate and those of nine other relapsing fever *Borrelia* species from GenBank (Table 1) were concatenated using the BioEdit sequence alignment program (version 7.1.3.0) (10) and aligned by using Clustal X software (27). The distances between concatenated sequences were calculated, and phylogenetic trees were constructed by using the Jukes-Cantor option of the neighbor-joining method in a complete deletion procedure using MEGA 4 software (26). The robustness of the topologies was estimated through 1,000 bootstrap replications. The IGS region (526 bp) of *B. microti* obtained in this study and the IGS regions from three Old World *Borrelia* species, including *B. duttonii*, *B. recurrentis*, and *B. crocidurae* (Table 2), were compared, and distances and a phylogenetic tree were obtained as described above.

Nucleotide sequence accession numbers. The sequence data for the 16S rRNA, *glpQ*, *flaB*, and IGS loci were submitted to GenBank with accession numbers JF803950, JF825472, JF825473, and JQ436580, respectively.

RESULTS

A total of 2,437 nucleotides were concatenated over three loci for 10 relapsing fever borreliae, revealing sequence homologies of from 92.11% to 99.84% (Table 3). *B. microti* showed the greatest homology (99.84%) with *B. duttonii*. The *glpQ* sequences of these two species were 100% identical over the entire 667 bp determined. Comparison with other published *glpQ* nucleotide sequences showed 99.85% homology with *B. recurrentis*, 97.72% with *B. crocidurae*, 96.15% with *B. hispanica*, 87.82% with *B. persica*, 85.49% with *B. hermsii*, 84.39% with *B. turicatae*, and 84.20% with *B. parkeri* and *B. coriaceae*. Over 718-bp *flaB* gene sequences,

TABLE 3 Levels of similarity for three concatenated loci (16S rRNA, *flaB*, and *glpQ*) totaling 2,437 bp

Species	% similarity									
	B. microti	*B. duttonii*	*B. recurrentis*	*B. crocidurae*	*B. hispanica*	*B. persica*	*B. hermsii*	*B. turicatae*	*B. parkeri*	*B. coriaceae*
B. microti	100									
B. duttonii	99.84	100								
B. recurrentis	99.67	99.84	100							
B. crocidurae	98.97	99.05	98.97	100						
B. hispanica	98.21	98.21	98.13	98.17	100					
B. persica	93.46	93.51	93.42	93.60	94.11	100				
B. hermsii	92.67	92.67	92.58	92.67	92.66	92.31	100			
B. turicatae	92.62	92.71	92.62	92.80	92.84	92.86	95.56	100		
B. parkeri	92.58	92.67	92.58	92.67	92.84	92.77	95.34	99.05	100	
B. coriaceae	92.20	92.20	92.11	92.11	92.51	92.21	93.96	95.33	95.20	100

B. microti differed at three nucleotides (99.56% homology) from both *B. duttonii* and *B. recurrentis*; with this sequence, the highest homology (100%) was between *B. duttonii* and *B. recurrentis*. Our *flaB* sequence exhibited 100% identity with another sequence from Iran previously deposited in GenBank (accession no. JF70895; unpublished data). Sequencing of the 16S rRNA over 1,052 bp gave a poor resolution for separating these *Borrelia* species, with similarity values ranging from 98.17% to 100% and with only a single nucleotide difference separating *B. microti* from *B. duttonii* (99.9% homology). It is noteworthy that *B. microti* shared the same nucleotide difference with all of the eight other species assessed and showed 100% identity with another *B. microti* 16S rRNA sequence previously submitted from Iran (GenBank accession no. JF681792; unpublished data). High DNA similarities were also observed between *B. microti* and *B. recurrentis* at three loci (16S rRNA, 99.62%; *glpQ*, 99.85%; and *flaB*, 99.56%). All three genes, the 16S rRNA, *flaB*, and *glpQ* genes, clustered *B. microti* with the Old World group (*B. duttonii*, *B. recurrentis*, *B. crocidurae*, and *B. hispanica*) yet separate from *B. persica*; thus, we show only the phylogenetic tree based on concatenated sequences for three loci in *B. microti* and nine other relapsing fever agents (Fig. 1). The IGS region of *B. microti* displayed 97.76% to 99.56% identity with *B. duttonii*, 98.01% identity with *B. recurrentis*, and 94.58% identity with *B. crocidurae*. The greatest IGS homologies were seen with *B. duttonii* strain CR2A, giving 99.56% identity; strain 1120k, giving 99.34% identity; and strains MW, Tzbd1, Bd9, and Bd11, demonstrating 99.12% identity. Strain CR2A was obtained from *O. erraticus* ticks in West Africa, and strain 1120K was obtained from *O. moubata* ticks in Congo. The phylogenetic position of *B. microti* based on its IGS sequence is shown in the supplemental material.

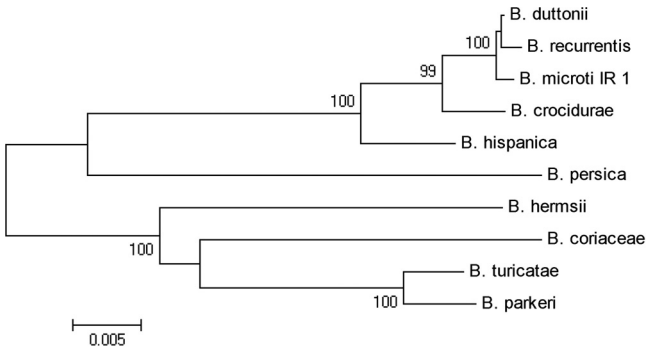

FIG 1 Phylogenetic tree based on concatenation of 16S rRNA, *glpQ*, and *flaB* DNA sequences. The scale bar corresponds to a distance of 0.005. The accession numbers of gene sequences used for construction of the tree are shown in Table 1.

DISCUSSION

The aim of this study was to establish the taxonomy of *B. microti*. Preliminary molecular analysis of *B. microti* based on partial sequences of the *glpQ* and 16S rRNA genes (GenBank accession numbers EU914142 and EU914144, respectively) suggested a close association of this species with the African TBRF group rather than *B. persica* (18). The four targets of *B. microti* sequenced herein all corroborated homology between our isolate and African species, particularly *B. duttonii* and *B. recurrentis*. Concatenated coding genes can be used to draw phylogenetic inference, while IGS, being noncoding, provides a more discriminatory typing tool. Our findings revealed a high degree of homology between *B. microti* and isolates of *B. duttonii* and *B. recurrentis*, while typing of IGS proved to be more discriminatory than typing of other loci but could show overlap between subgroups of *B. duttonii* and *B. recurrentis* (7, 25, 28). The greatest nucleotide homology between *B. microti* and *B. duttonii* over the loci analyzed (Fig. 1; see Fig. S1 in the supplemental material) raises the possibility that *B. microti* and *B. duttonii* may belong to a single species. As full genomic sequences are now becoming available, it might be better to consider this cluster of strains to be variants within a complex rather than individual species (15). This would embrace both the within-species variation disclosed by IGS typing and the between-species diversity reported herein.

Transmission of *B. duttonii* is associated with living in traditional mud-built dwellings that are commonly infested with *O. moubata* ticks, while exposure to *B. microti*, in common with *B. crocidurae*, appears to correlate with sleeping in the open and proximity to tick-infested burrows; these conditions are more likely to be provided in southern Iran, owing to the long summers and very mild winters. Intriguingly, humans are the only known vertebrate hosts for *B. duttonii* (and its derived louse-borne variant, *B. recurrentis*). This was recently challenged through the finding of *B. duttonii* DNA in blood samples of chickens and swine living close to humans, suggesting potential alternative reservoirs for infection (17); however, the competence of transmission from these hosts remains to be substantiated. In contrast, isolation of *B. microti* from *Microtus* spp. suggests that it may be maintained by enzootic cycles that involve rodents and other small mammals

(12, 13) and that thus resemble the life cycle of *B. crocidurae* (3, 5, 29). Interestingly, the isolate of *B. duttonii*, which showed the greatest homology with our *B. microti* isolate, was derived from *O. erraticus* ticks in West Africa (strain CR2A), initially being classified as *B. crocidurae* rather than *B. duttonii* (28). It is tempting to speculate whether such as yet undisclosed links hold the key to the evolutionary origins of these isolates.

Differential pathogenicity has historically been used to differentiate species of TBRF. *B. duttonii* is known to cause severe infection and significant perinatal mortality in East Africa (5), while infection with *B. microti* presents as a milder form of the disease in Iran (13). Again, this might parallel the milder TBRF seen to be associated with *B. crocidurae* infection in West Africa (5). *Borrelia duttonii* isolates Bd9, Bd11, and WM were from spirochetemic febrile patients, and strain TzBd1was from an asymptomatic blood donor in Tanzania (7, 25). Whether the difference in virulence is due to adaptation to different tick vectors is open to question; this can be assessed following cross-vector exchanges of spirochetes derived from *O. erraticus* and *O. moubata*. Molecular analysis of isolates of *B. microti* from various geographical regions together with *in situ* analysis from either clinical or vector samples will help to provide more accurate insights into the evolutionary origins of *B. microti*.

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