

Original Articles

Study on Presence of *Borrelia persica* in Soft Ticks in Western Iran

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Abstract

Background: A molecular survey was conducted to investigate the presence of pathogenic *Borrelia persica* species causing the tick borne relapsing fever (TBRF) in Takistan district Qazvin Province, western Iran.

Methods: A number of 1021 soft ticks were collected from 31 villages including previously reported infected and none-infected TBRF cases and individually examined for the presence of *B. persica* DNA by conventional PCR targeting the 16S rRNA.

Results: A total of 1021 soft ticks of three species of *Ornithodouros tholozani* (120: 11.75%), *O. lahorensis* (461: 45.15%) and *Argas persicus* (440: 43.1%) were collected and tested against *Borrelia* infection. Soft ticks were more prevalent (67%) in infected areas than none infected areas. The rate *O. tholozani* in infected areas was much greater (29 times) than none infected areas. Ninety seven percent of soft ticks in none infected areas were of *O. tholozani*. Sixteen (16.7%) ticks of tested (n=95) *O. tholozani* were infected with *B. persica*. Three (1.3%) out of 205 soft ticks of *O. lahorensis* were positive for *Borrelia* sp., and no infection was observed in *A. persicus*. *TaqI* RFLP analysis and sequence analysis of the positive PCR products showed the presence of *B. persica*. The RFLP analysis showed that the positive ticks of *O. lahorensis* were infected with unknown *Borrelia* species.

Conclusion: This study showed that although there were no TBRF cases in Takisan, but still infected *O. tholozani*, the known vector of TBRF, presented in the region. Control measures needs to be fulfilled in Thakistan.

Keywords: *Borrelia persica*, tick borne relapsing fever (TBRF), PCR-RFLP, Takistan, Iran

Introduction

The tick borne relapsing fever (TBRF) is an acute infectious disease and one of its basic properties is the recurring attacks of fever and chill. This disease is one of the important hygiene problems in Middle East and Central Asia (Karimi et al. 1979, Karimi 1980, Barbour and Hayes 1986, Arshi et al. 2002). The disease is reported from North and South America, Africa, Asia and Europe. The epidemiology of the disease in each region depends on the relationships between the tick and *Borrelia* species as well as environmental condition in its distribution area. Reservoir hosts

are usually wild rodents (Rebaudet and Parola 2006). Feeding of a tick on an infected vertebrate is the natural route of tick infection. The pathogen is transmitted to another vertebrate host during tick feeding in the next life stage.

The development of the PCR has offered a new dimension in the diagnosis of infectious diseases. Capable of amplifying minute amounts of DNA into billions of copies in just a few hours, PCR facilitates the sensitive and specific detection of DNA or RNA of pathogenic organisms. PCR for the diagnosis of infectious diseases has been directed pri-

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marily toward the detection of pathogens for which conventional diagnostic techniques are either too insensitive or too slow such as *Borrelia* spp. There are many research studies involving PCR in the diagnosis of *Borrelia* spp. particularly *B. burgdorferi* infections in clinical samples (Schwartz et al. 1997, Cerar et al. 2008). The PCR method have been used successfully for identification of *B. burgdorferi* in hard ticks of *Ixodes ricinus* (Horka et al. 2008) and *B. persica* in soft ticks of *O. tholozani* (Oshaghi et al. 2010a, b).

Tick-borne relapsing fever is one of the endemic diseases in Iran and its prevalence was considerable in the past, but the incidence rate of it decreased to 0.06/100,000 in 2006 (Asl et al. 2009). The cause of this disease in Iran is mainly *Borrelia persica* Dschunkowski 1912, that conveyed by the bite of the *Ornithodoros tholozani* soft tick (Karimi 1980, Masoumi Asl et al. 2009). However, other species such as *B. latshywi*, *B. microtti* and *B. balthazardi* are responsible for the spread of the disease in certain areas of the country (Karimi 1980).

Qazvin Province is amongst the most important foci of TBRF in Iran (Asl et al. 2009). The province covers 15821 km² between 45-48 to 50-50 east of Greenwich Meridian of longitude and 35-37 to 36-45 north latitude of the equator (Aghighi et al. 2007). Takistan district with almost 180,000 populations, is located in west of the province. In Takistan district there are no data regarding *B. persica* circulation in enzootic sites, nor the presence of these bacteria in ticks, even if there were human case records (MHME). On average, five cases of TBRF had been reported from Takistan, however, since 2007, no report of TBRF has been evidenced in the district. Since the risk of infection to humans with *Borrelia* depends on outdoor recreational activity, on the density of tick populations, and on the infection of the ticks with *Borrelia*. Therefore, data describing the prevalence of *Borrelia* in ticks can be used to assess the

risk of TBRF for public health (Rauter and Hartung 2005). In this study we tried to assess the potential risk of TBRF by testing the presence of *B. persica* in soft ticks, particularly *O. tholozani*, from Takistan that had previously witnessed some outbreaks of TBRF transmitted by this tick species.

Materials and Methods

Study area

Surveys were performed in Takistan district of Qazvin Province in north-west Iran. It covers 2430 km² and the terrain of the survey areas consists of 4 zones with 133 villages (Vs) distributed in different mountainous, hilly, and plain regions. It has a temperate to dry climate with annual precipitation of about 232 mm. The temperature ranges from -20 to 40° C, with an average of 14° C. The study was performed in all previously infected (ten) and 21 none infected villages. These 21 villages were randomly selected from mountainous (8), hilly (6), and plain (5) areas.

Specimen collection

This survey was conducted from August 2007 to July 2008. Ticks were actively searched directly by eye and help of torch light and collected from cracks, crevices, ceiling, and floor of human dwelling, poultry and animal shelters or rodent burrows. Process of collection took 30 min each time and the specimens transferred into the holding tubes. All ticks were handled with forceps. All the specimens were identified on the basis of their morphological characteristics (Zaim and Shayeghi 1989, Estrada-Pen˜a et al. 2004).

DNA extraction

DNA extraction from ticks was performed as previously described (Cao et al. 2000). Briefly, each tick was placed into a microtube and mechanically disrupted with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.1 % SDS, and

500 µg proteinase K ml⁻¹). The sample was incubated for 2 h at 56 °C, and then boiled at 100 °C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to a fresh sterile microtube and purified by extracting twice with an equal volume of phenol/chloroform before use in PCR.

16S rRNA gene PCR

A conventional PCR was performed with primers designed to amplify the 16S ribosomal RNA (16S rRNA) gene of the *Borrelia* spp. Primers rec4 (5'-ATG CTA GAA ACT GCA TGA-3') and rec9 (5'-TCGTCTGAGTCCCC-ATCT-3') were used as previously described (Ras et al. 1996). These primers are expected to yield a 523-bp PCR products regardless of *Borrelia* species. PCR amplification were performed in a volume of 30 µl containing 3 µl 10xPCR buffer (containing 100 mM Tris/HCl, 500 mM KCl, 15 mM MgCl₂), 0.3 µl Taq DNA polymerase (5 U µl⁻¹), 0.3 µl dNTP mix (10 mM) (all from Bioneer, S. Korea), 17.4 µl deionized water, 5 µl DNA template and 2 µl of each primer (10 µM). The cycling conditions for amplification involved 5 min denaturation at 95 °C, followed by 35 cycles of 94 °C for 60 s, 45 °C for 60 s and 72 °C for 120 s, and a final extension at 72 °C for 7 min. In parallel with each amplification, a positive control (previously extracted DNA of standard *B. persica* from Pasteur Institute of Iran) and two negative controls including previously extracted DNA of standard *B. microtti* from Pasteur Institute of Iran and distilled water were included.

All the PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. To minimize contamination, DNA extraction, the reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms.

RFLP analysis

In order to identify the species of *Borrelia* spp. infecting the ticks, the positive PCR

products were analyzed by RFLP as described by Oshaghi et al. (2010). Previously extracted DNA of standard *B. persica* was used as positive controls. For each positive sample, 10-15 µl amplified DNA was digested at 65 °C overnight with endonuclease *TaqI* (Fermentas) according to the manufacturer's recommendations. Electrophoresis was conducted in 2% agarose gel at 100 V for 5 min and then 80 V for 1 h. The gels were stained with ethidium bromide, and visualized under UV light. A 100 bp DNA Ladder Marker (Cinagen, Fermentas) was used as a molecular size marker. RFLP profile of *B. persica* was identified by digestion of 523-bp into 324- and 199-bp according to Oshaghi et al. (2010). 16S rDNA PCR products of *B. microtti* was used in parallel to compare the RFLP profiles of both species.

DNA sequencing of PCR product

In order to confirm the species of *Borrelia* spp. infecting the ticks, a sample from those PCR products that showed unique RFLP profile was selected for PCR-direct sequencing. The nucleotide sequences were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (Seqlab, Germany). The sequence obtained in the present study was compared with the previously published sequences deposited in GenBank using the BLAST program from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>). The accession number of the 16S rRNA gene sequence obtained in this study is EU914141.

Results

Tick prevalence and distribution

A total of 1021 soft ticks of three species of *Ornithodoros tholozani* (120: 11.75%), *O. lahorensis* (461: 45.15%) and *Argas persicus* (440: 43.1%) were collected and tested against *Borrelia* infection. Soft ticks were more prevalent (67%) in infected areas than

none infected areas. The rate *O. tholozani* in infected areas was 29 times more than none infected areas. 97% of soft ticks in none infected areas were of *O. tholozani*. On average, number of females was twice as males. *Ornitorodorus lahorensis* with 45.15% was the most common species in the study area. Among ten previously infected villages, *O. tholozani* specimens were found only in four villages of Mehin, Meshkin, Andagh, and Ghalat. In none previously infected villages, different species of *Ornitorodorus* spp. were present in ten out of 21 villages, though *O. lahorensis* with 97.9% was the most prevalent soft ticks.

***Borrelia persica* prevalence in ticks**

DNA of *B. persica* was detected by a conventional PCR specifically targeting the 16S rRNA gene. A total of 344 soft ticks including 95 *O. tholozani*, 205 *O. lahorensis*, and 44 *A. persicus* were selected based on the collection sites and individually examined by PCR against *Borrelia* genome. The target region (16S rDNA) was successfully amplified

for 5.5% (19/344) of the investigated ticks. Sixteen (16.7%) ticks of *O. tholozani* were infected with *B. persica*. Three (1.3%) soft ticks of *O. lahorensis* were also positive for *Borrelia* sp., but no infection was found in *A. persicus*. So, two *Borrelia* species, *B. persica* and *Borrelia* sp. (unknown) were identified by PCR-RFLP: *B. persica* was the most frequently detected species (16/19, 84.2%). All of the infected ticks with *B. persica* collected in the sites previously TBRF was reported whereas 100% of ticks infected with *Borrelia* sp. were found in none previously TBRF reported areas. Infection rates of ticks with respect to the area of sample collection are shown in Table 1.

TaqI RFLP and sequence analysis of the amplified products from *O. tholozani* ticks specimens resulted in unique profile. On the basis of *TaqI* RFLP patterns of positive controls of *B. persica* the profiles obtained in this study consist of restriction pattern for *B. persica* species (Fig. 1).

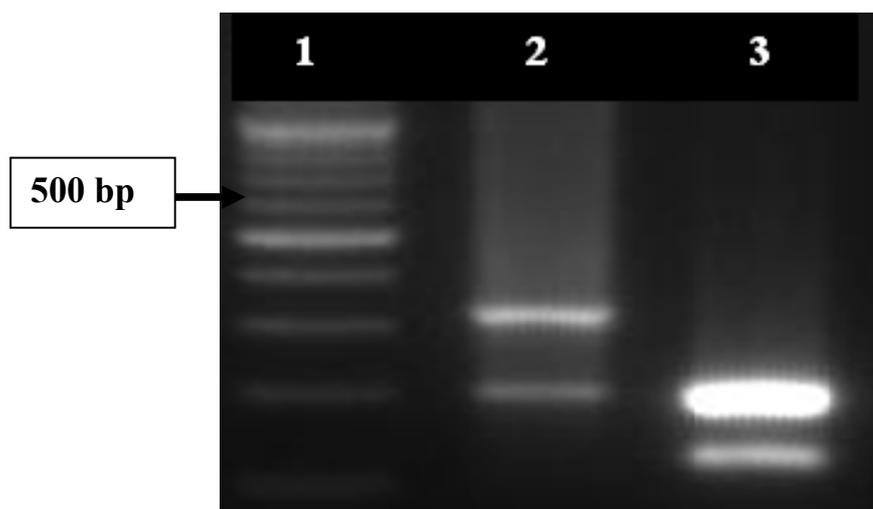


Fig. 1. PCR-RFLP profile of 523bp of 16SrDNA gene of *Borrelia persica* (no. 2) and *B. microtti* (no. 3) digested by *TaqI* restriction enzyme (Cinnagen). No. 1: Molecular size marker (100 bp Cinnagen), *B. persica* PCR was broken down into two fragments of 324 bp and 199 bp and *B. microtti* into three fragments of 205, 199, and 119 bp.

Table 1. PCR-based *Borrelia* spp infection rate in *O. tholozani* and *O. lahoriensis* in Takistan

Area studies	<i>O. tholozani</i>			<i>O. lahoriensis</i>		
	No. tested	Infected (%)		No. tested	Infected (%)	
		<i>B. persica</i>	<i>B. sp</i>		<i>B. persica</i>	<i>B. sp</i>
Infected	96	16 (16.6)	0.0 (0)	95	0.0 (0)	0.0 (0)
Clean	4	0.0 (0)	0.0 (0)	130	0.0 (0)	3(2.3)
Total	100	16 (16)	0.0 (0)	225	0.0 (0)	3(1.3)

Discussion

In this study we confirmed the presence of *B. persica* in 16.7% of the soft tick *O. tholozani* in the previously reported TBRF areas of Takistan district. This infection rate is higher than the prevalence of 3.7% in the same tick species that were previously collected in Qazvin, neighbor district of Takistan (Aghighi et al. 2007). This is mainly attributable to sensitivity of PCR methods in comparison with microscopic following xenodiagnosis (Rafinejad et al. 2010). In addition, in this study we could use the PCR technique for either dead or alive, and 4 different development stage of ticks. However, this prevalence is comparable to the overall prevalence average in Iran and other countries. This rate was 3.7% in Hamadan (Ghaderi 2001), 8.8% in Qazvin (Aghighi et al. 2007), 20% in different areas of Iran (Rafiei and Rak 1986), 36.6% in Semnan (Nekoui et al. 1999), and 2-40% in other parts of the world (Assous and Wilamowski 2009). These differences in infection rates among localities could be attributable to geographical and seasonal variations of infected ticks or to different sampling approaches and examination methods. Among these factors, the examination method is more critical since the classical method, which is mainly based on xenodiagnosis, is extremely laborious, slow, expensive, and requires preparation of the samples. Also we should consider the fact that the density of *B. persica* in field ticks is very low and hard to be detected by the classical methods.

In Takistan, people are involved with poultry and animal husbandry and there is close relationship between people and animals. Also there is an abundant small wild animals such rodent and canine fauna, thus it can be suggested that *B. persica* or other *Borrelia* spp. are maintained particularly in enzootic cycles involving the wild and domestic mammals in the region.

The two soft ticks of *O. lahorensis* and *A. persicus*, which are mainly distributed in Takistan, lack any *B. persica* spirochetes. In spite of high prevalence (41.15%) of *O. lahorensis* in the area, it was not infected with *B. persica*. Instead, it was found infected with an unknown *Borrelia* species. Similarly, other investigators have already revealed infection of *O. lahorensis* with unknown *Borrelia* in Iran (Arshi et al. 2002) and Nablos in Lebanon. The results of our research and previous studies on infection of *O. lahorensis* to *Borrelia* sp needs further investigations to identify the spirochetes presence in this particular soft tick.

Argas persicus, with 43% prevalence, which are mainly distributed in Takistan (43%), was not found infected with any kind of *Borrelia*. These diversities in vectorial capacity of different tick species is attributable to the highly specific *Borrelia*-tick interactions as each species of *Borrelia* is only transmitted by one or a few closely related species of ticks. For example, *O. tholozani* is the main vector of *B. persica*, and *O. erraticus* is vector of *B.*

microtti (Assmar et al. 2002, Masoumi Asl et al. 2009). In Azerbaijan, *O. verrucosus* is a vector of *B. caucasica*, which causes a very severe TBRF. In Iran and the countries of Central Asia of the former USSR, *O. tartakowskyi* is the vector of *B. latyshewii*, which causes a very mild RF without apparent clinical relapses (Parola and Raoult 2001). *Ornithodoros sonrai* is recognized as the only vector of *B. crocidurae* causing human relapsing fever in West Africa (Vial et al. 2006). The mechanisms responsible for this strict species specificity in transmission of one species of spirochete by only one species of tick are not known (Schwan and Piesman 2002).

In conclusion, pathogenic *B. persica* infection in *O. tholozani* ticks poses a potential health threat both to local residents and to tourists and animals in this area. It will be necessary to alert public health officials and clinicians about the existence of *B. persica*-positive in ticks in previously infected areas of Takistan district to continue vector control measures.

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