



Detection of *Borrelia persica* Infection in *Ornithodoros tholozani* Using PCR Targeting *rrs* Gene and Xenodiagnosis

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Abstract

Background: Relapsing fever caused by *Borrelia persica*, is an acute tick-borne disease which is transmitted by soft ticks of *Ornithodoros tholozani* to human.

Methods: Value of PCR and xenodiagnosis for detection of *B. persica* in *O. tholozani* ticks was compared. Sixty-four *Borrelia*-free ticks were fed on infected guinea pigs and used for the experiments. For xenodiagnosis, a group of 32 ticks in subsequent blood meal were fed on sterile guinea pigs and the indication of *B. persica* in the animal blood was tested 5-14 days later by dark-field microscopy. For PCR, all 64 ticks were subjected to PCR against *B. persica rrs* gene (16S-rDNA). Also sensitivity of PCR in terms of minimum detectable number of spirochetes as well as the effects of tick sex and post digestion was tested.

Results: PCR revealed *B. persica* DNA in 98.4% ticks, in which *B. persica* were found in 25.0% by xenodiagnosis. PCR was enough sensitive to give positive results for DNA of 1 spirochete. PCR success rates were similar for male or female ticks. Course of time did not affect the efficacy of PCR and similar results were observed for ticks of immediately fed, semi- or completely gravid or completely digested blood ones.

Conclusion: Our results indicate that due to very low specificity and time consuming, xenodiagnosis is not a useful method whereas PCR method has advantages for study the *Borrelia* prevalence in ticks.

Keywords: *Borrelia persica*, *Ornithodoros tholozani*, TBRF, Xenodiagnosis, PCR

Introduction

The tick borne relapsing fever (TBRF) is an acute infectious disease and important health problem in Middle East and Central Asia (1-5). The cause of this disease in Iran is *Borrelia persica* Dschunkowski 1912, that conveyed by the bite of the *Ornithodoros tholozani* soft tick. The natural route of tick infection is the feeding of a tick on an infected host during tick feeding in the next life stage (6). The ability of spirochetes to survive in the tick digestive tract, to migrate to hemolymph and then to the tick salivary glands, and to be transmitted with the saliva into the host skin are the crucial steps of pathogen transmission. Practical confirmation

of spirochete infection in ticks in Iran has relied on xenodiagnosis method in which ticks are let to feed on guinea pigs followed by microscopic identification of the bacteria in blood smears of the animals. The bacteria should be searched for in the blood of guinea pigs daily from days 6 to 12 after the infection (7). Testing blood smear limited to a few days after inoculation and requires a highly experienced observer to identify the spirochetes and may necessitate a tedious and often unsuccessful microscopic search.

The development of the PCR has offered a new dimension in the diagnosis of infectious dis-

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eases. This method can amplify small amounts of DNA into millions of copies in less than 2-4 hours, and facilitates the sensitive and specific detection of DNA or RNA of pathogenic organisms (8). Usually the technique has been primarily used for detection of pathogens for which conventional diagnostic techniques are either too insensitive or too slow such as *Borrelia* spp (5). There are many research studies involving PCR in the diagnosis of *Borrelia* spp particularly *B. burgdorferi* infections in clinical samples (8-15).

Regarding the diagnostic performance of PCR with clinical specimens, the data so far available are variable. However, the diagnostic sensitivities of PCR for identification of *B. persica* in soft ticks have been neither systematically evaluated nor compared with other methods. In a recent but single paper, PCR was reported successfully to detect *B. burgdorferi* in hard ticks of *Ixodes ricinus* (6).

Since analysis and diagnostic PCR could be performed for both dead and alive ticks, and is not development stage dependent, it would be very advantageous and allows the study of different tick biological forms.

The aims of the present study were, by the use of laboratory experimentally infected animals, (i) to compare the diagnostic performance of PCR and xenodiagnosis in soft ticks of *O. tholozani* (ii) to investigate the sensitivity of PCR in terms of minimum detectable number of in vitro cultivated spirochetes (iii) to evaluate the diagnostic performance of PCR for male and female ticks and (iv) to assess the effect of post digestion on diagnostic performance of PCR. The results are expected to predict the efficacies of the methods as diagnostic tools for epidemiology of human TBRF.

Materials & Methods

Bacteria

The species of *B. persica*, strain Bijar isolated from *O. tholozani* in northwestern Iran was prepared from the Pasteur Institute of Iran. A

stock of this bacterium is usually kept in the laboratory by culturing them in animal laboratory of guinea pigs.

Ticks

Laboratory strain of soft tick *O. tholozani* of the Argasidae family originated from the colony maintained at the insectarium of School of Public Health, Tehran University of Medical Sciences (SPH-TUMS). They were tested by PCR and microscopic methods for the presence of *Borrelia* spirochetes with negative results. Sixty four F1 adult ticks were selected and divided into two equal groups of 32 including 16 females and 16 males for xenodiagnosis and PCR experiments.

Guinea pigs

Pathogen-free guinea pigs (10 to 12-week-old females) were selected and maintained under standard conditions in the animal house unit at SPH-TUMS, Iran.

Experimental borreliosis in guinea pigs

We followed the protocol previously described by Lebech et al. (16) and experimental borreliosis were caused by intraperitoneally injection of 10^8 *B. persica* organisms to five pathogen-free guinea pigs.

Tick examination by Xenodiagnosis and PCR

On days 7-10 after inoculation, following confirmation of blood infection in microscopic field, the ticks of both groups were allowed to feed simultaneously at least 3 hours (until they fully blood engorged) on the infected guinea pigs to acquire *Borrelia*. The fed ticks were kept in the laboratory condition till full blood digestion. It normally took place at least 30 days in the laboratory condition. Then 32 ticks (first group) were stored in refrigerator for the following DNA isolation and PCR amplification. For xenodiagnosis method, 32 ticks (2nd group) subsequent of completely gravid with empty abdomen, were allowed to feed again on

a pathogen-free guinea pig for next blood meal and on days 5, 7, 9, 11, and 14 post-infection, blood smear samples were prepared from their ears and examined by dark-field microscopy for growth of spirochetes. Moreover, this group of ticks was stored in refrigerator for further PCR analysis. Another 10 soft tick's *O. tholozani* was used as the control group and were fed on a free pathogen guinea pig.

Isolation of DNA and PCR

The soft tissues of both groups of ticks were individually homogenized in PBS and used for DNA extraction. Genomic DNA was extracted with Bioneer Tissue DNA Spin Kit (Bioneer, South Korea), according to the manufacturer's instructions. The *rrs* or 16S ribosomal RNA (16SrRNA) gene of *Borrelia* genome was amplified by PCR using forward primer Rec4 5'-ATGCTAGAACTGCATGA-3' and reverse primer Rec9 5'-TCGTCTGAGTCCCATCT-3' as previously described (13). Amplicons of 523 bp were visualized on agarose 1% (w/v) gels stained with ethidium bromide. In brief, 5 μ L of isolated DNA was amplified with the primers using initial denaturation of 3 min at 94°C followed by 35 cycles of 1 min at 93°C, 1 min at 45°C, and 1 min at 72°C, followed by a 7-min hold at 72°C. PCR were performed in a volume of 25 μ L including 10 pmol of each primer, Gelatin 0.1%, 1.25 units *Taq* DNA polymerase, 1.5mM MgCl₂, 2.5 μ L 10X buffer, 200 μ M dNTPs, and 5 μ L of DNA extracted from samples. To confirm the identity of *Borrelia* species used in this study, a few PCR products were sequenced (Seqlab, Germany) and compared to 16SrRNA genes sequences of other RF *Borrelia* spp available in the GenBank database using the BLAST and phylogenetic analysis online software embedded in Pubmed (<http://www.ncbi.nlm.nih.gov/BLAST>). The sensitivity of the PCR assay in terms of the minimum number of in vitro-cultivated *B. persica* cells was determined with spirochetal solutions containing 1-3, 4-6, 7-10 and more than ten *Borrelia* spirochetes per 10 μ L solution.

The DNA of the 10 μ L extracted was diluted in 5 μ L ddH₂O and used for PCR. To test the course of time on success of PCR, the DNA of second group of ticks were isolated in various times (4 specimens for each interval) after taking blood meal including one hour, and 1, 2, 4, 8, 16, 32 days (complete gravid), and after complete blood digestion (empty) when females laid eggs.

ddH₂O, DNA of free pathogen lab strain of fed *O. tholozani* ticks, and blood DNA of none infected guinea pigs were used as negative controls. Also, blood DNA of the guinea pigs infected by inoculation was used as positive controls.

Results

Detection of *B. persica*

The result of utilities of PCR and xenodiagnosis methods has been shown in table 1. *Borrelia persica* DNA was detectable by PCR in 61 out of 62 (98.4%) of ticks specimens that assumed to acquire *B. persica* spirochetes by feeding on the infected animals. A 523 bp PCR band was produced (Fig. 1) for all positive samples. In xenodiagnosis method, *B. persica* was found in the blood samples of only 8 out of 32 guinea pigs that infected ticks were fed on them, however, after full digestion of blood meal in the ticks, 23 out of these 24 false negative ticks were PCR positive against the 16S-rDNA of *B. persica* (Table 1). To confirm the identity of the *Borrelia* species used in this study, the 16rDNA fragment of four PCR products were sequenced. These four samples had identical sequences and the consensus sequence was submitted to Genbank with the following accession number (U914141). The DNA sequences analyzed by BLAST was found to be 100% identical to that of isolates of *B. persica* from Iran (Genbank accession no. U42297). This confirmed that the RF spirochetes were used in this study was indeed *B. persica*.

Analytical sensitivity of the PCR assay in terms of the minimum number of in vitro-cultivated

B. persica cells showed that a reproducible amplification can be achieved when one spirochete was added to the PCR mixture (Fig. 1).

The present PCR assay equally amplified *B. persica* DNAs from infected male and female ticks. In addition, post digestion or course of

time had no effect on success of PCR and the present PCR assay equally amplified DNAs from samples either tested immediately or days after taking blood meal, fully gravid, or empty ones (Fig. 1).

Table 1: Comparison of PCR and xenodiagnosis methods to measure contamination of the *Borrelia persica* in *Ornithodoros tholozani* ticks

Method	Negative (%)	Positive (%)	Specimen	No tested
Xenodiagnosis (Microscopic)	24 (75.0)	8 (25.0)	Blood of Guinea pig	32
PCR following Xenodiagnosis	1 (3.1)	31 (96.9)	tick	32
PCR	0 (0)	32 (100)	tick	32

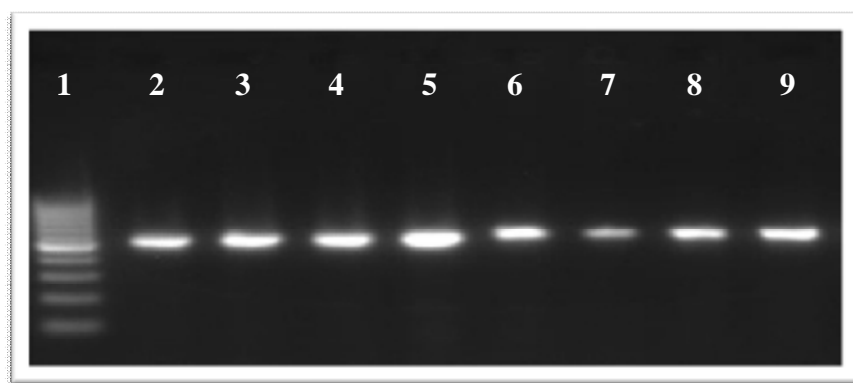


Fig. 1: PCR amplification of 523 bp of 16S rDNA of *Borrelia persica* in infected *Ornithodoros tholozani* ticks and in guinea pig bloods. No. 1: 100 bp Molecular weight marker (Cinnagen, Iran), 2 and 3: respectively male and female ticks following xenodiagnosis and full blood digestion, 4-6: post blood meal respectively in ticks after one hour, 15 days, and >30 days (full blood digestion), 7-9: PCR products of animal blood when respectively 1, 5, and 10 spirochetes were added to the PCR mixture

Discussion

This is the first report showing advantages of PCR method, in comparison with xenodiagnosis method, for diagnosis of *B. persica* in soft ticks. The diagnosis of *B. persica* in ticks is often difficult due to the low number of spirochetes found in tick organs. The PCR assay had 97-100% sensitivity; hence it might be useful tool for the diagnosis of *B. persica* in human patients and other host reservoirs. In compari-

son with xenodiagnosis, PCR-based identification is also faster, is less expensive and requires less sample preparation. The xenodiagnosis method is extremely laborious, and considering the fact that the density of *B. persica* in field ticks is even lower than that in experimentally infected ticks, the method is not useful in vector incrimination setting. The rate of infection detected by xenodiagnosis in this study was

25.0% which is comparable with 16% rate of infection detected by this method for detection of *B. afzelii* in *Ixodes ricinus* (L.) ticks (17).

Examination of ticks and other biological samples by culture on BSKH liquid medium followed by dark field microscopy is a reference standard for the demonstration of borrelial infection (12, 18). In this study we did not use this technique; however, this approach provides rather delayed results and is technically demanding and time consuming. In a study by Livesley et al. (19), the authors demonstrated that motile spirochaetes were not visible by dark-field microscopy in any of the cultures whereas 11/12 samples were PCR positive. Reller et al (20) designed a multiplex quantitative PCR (qPCR) assay to distinguish RF *Borrelia* from *Plasmodium falciparum* and *P. vivax* and specifically (100%) amplified pathogenic RF *Borrelia* (1 copy/reaction).

The 16S-rDNA is a highly conserved locus among the *Borrelia* spp and might be useful marker for detection of infection in ticks, animals, and patients infected with various RF *Borrelia* spp including *B. persica*, *B. microti*, *B. baltazardi* and *B. latyschevii* which are present in Central Asia and Middle-east countries (1, 3, 5). However, the initial PCR used in this study is not able to identify them to species level. However, there are a variety of PCR methods including qualitative (conventional PCR and nested PCR) or quantitative (competitive PCR and real-time PCR) which has been used for *Borrelia* detection and identification. Several factors including the gene target and primer set influence the effectiveness of a PCR assay. So far a number of genes including chromosomally carried genes such as rRNA genes (*rrs*), *flaB*, *recA*, *GlpQ*, and *p66* and the plasmid-carried gene *ospA* have been employed in research laboratories (10-15). Among these, *rrs* and *GlpQ* have been used as targets for PCR analysis of *B. persica* (13-15). Aguero-Rosenfeld et al. (12) summarized the sensitivities and specificities of PCR assays for the detection of *B. burgdorferi* DNA in different clinical samples and showed a

high range of sensitivities (36% to 100%) for different PCR assays.

In this study, xenodiagnosis method could not detect spirochetes in 75.0% of the animal blood. Although detailed biology of *B. persica* in guinea pig has not been properly studied yet, however, this low sensitivity is not in accordance with the result of another study showed that a single spirochete of *B. turicatae*, *B. hermsii*, or *B. duttonii* is sufficient to produce infection of laboratory animals and will appear in the blood as soon as 1 h after intraperitoneal inoculation of a large inoculum (21-23). It is shown that following presence of spirochetes in the blood, they multiply as often as once every 6 h and in susceptible animals, there may be as many as 10,000,000 spirochetes per ml of blood during peak spirochetemias (22). The absence of detectable spirochetes by microscopy in most of the animal blood of this study could be due to several reasons. It is shown that the transmission pathway employed to infect the host and the duration of systemic infection are important factors responsible for establishment or persistence of tick-borne pathogens in a given tick-host system (23).

One might suggest that due to long passaged in animals, they may lose the ability to infect and be transmitted by ticks (24-25). In one such strain of *B. duttonii* that was examined, the spirochetes were able to persist in the mid gut of *O. moubata* after feeding, but they could not enter the hemocoel (26). However, when the *B. duttonii* organisms were injected directly into the hemolymph in the hemocoel, ticks were then capable of transmitting the spirochetes to mice (27). It appears that the mouse-passaged borrelial variants were no longer capable of penetrating the tick's midgut wall (2). Recently, it is shown that there is a very early effect of tick saliva on the proliferation and distribution of *Borrelia* spirochetes in the host, probably due to the effect of saliva on the host innate immunity mechanisms (6). In contrast, long-term animal passage need not always result in loss of the ability to be transmitted by ticks; a strain of

B. hispanica was passed 71 times in guinea pigs and yet remained tick transmissible (27). The *B. persica* strain we used in this study usually, at least twice per year, is transmitted to bite of ticks on infected guinea pigs to keep its ability to infect ticks. One possible explanation for low rate of infection in xenodiagnosis method might be related to the environmental conditions and their effects on the aggregation of *Borrelia* spirochetes in ticks. It has been documented that Lyme borreliosis agents form aggregates during tick blood feeding, when they get transmitted from hard tick vector to mammalian hosts. The *Borrelia* aggregations are temperature, pH, and growth phase dependent and environmental conditions such high temperature, low pH, and high cell density favorable for their aggregation (28). However, favorable condition for *B. persica* in soft ticks is not or poorly characterized. Reznik (29) assessed efficiency of *B. persica* transmission by *O. papillipes* ticks and assumed two forms (mild and typical) of borreliemia in guinea pigs caused by ticks' bites. The mild form is not easily diagnosed by the analysis of animal blood preparations, being encountered in 40% of cases. More investigation needs to clarify which form of borreliemia in guinea pigs has been occurred in this study. Here we showed that PCR can equally amplify the DNA of *B. persica* in male and female and in immediately fed or days after taking blood meal even after completely empty abdomen ticks. This is important in epidemiological studies regarding to vector incrimination of tick borne diseases. Many studies have shown that male and female ticks are not equivalently infected by *Borrelia* spp and female ticks are more often infected than male ticks and thus transmit this pathogenic agent more often (30-33). The average number of spirochetes in female ticks is greater than males (6). Diagnostic value of PCR for detection of *B. burgdorferi* DNA using 16S ribosomal RNA in clinical specimens from patients with erythema migrans (EM) and Lyme neuroborreliosis was tested and compared with those obtained by in

vitro culture and serological testing (33). These authors showed that PCR of skin biopsy specimens is currently the most sensitive and specific test for the diagnosis of patients with EM, superior to culture and serological testing. In conclusion, the 16S-rDNA PCR was found as a highly reliable assay for the diagnosis of *B. persica*. However, its utility should be tested for the other *Borrelia* spp (*B. microti*, *B. baltazardi* and *B. latyschevii*) in the region. This rapid assay could facilitate the diagnosis of *B. persica* in different biological samples and provide an important tool for better understanding this yet poorly characterized spirochete.

Ethical Considerations

Ethical issue principles including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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