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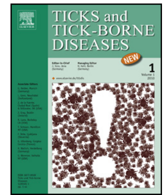

Original article

Borrelia persica: *In vitro* cultivation and characterization *via* conventional PCR and multilocus sequence analysis of two strains isolated from a cat and ticks from Israel

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ABSTRACT

Borrelia persica, one of the pathogenic agents of tick-borne relapsing fever, is transmitted by the soft tick *Ornithodoros tholozani*. It causes infections in humans as well as in animals. In this study, we developed a medium, termed Pettenkofer/LMU Bp, for reliable *in vitro* cultivation. Cell densities up to 5.2×10^7 viable cells/ml were achieved over at least 40 passages. The cultivable *B. persica* strain isolated from a cat was further analyzed by amplification of the *flaB* gene using conventional PCR. In addition, seven housekeeping genes (*clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA)* of this *B. persica* strain and a second strain isolated out of pooled ticks from Israel were amplified and the phylogenetic relationships among *Borrelia* species were analyzed. The results of the conventional PCR and the multilocus sequence analysis confirmed our isolates as *B. persica*.

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1. Introduction

Borrelia (*B.*) *persica*, a spiral shaped bacterium, is transmitted by the soft tick *Ornithodoros tholozani* (Masoumi Asl et al., 2009; Rafinejad et al., 2011) and causes tick-borne relapsing fever (TBRF) in humans (Dschunkowsky, 1913). This spirochete also shows pathogenicity in other mammals especially in guinea pigs, hedgehogs and rabbits (reviewed in Rodhain, 1976). In general, the occurrence of TBRF caused by *B. persica* depends on the distribution of its tick-vector and is found, for example, in the Middle East, Central Asia and Indian peninsula (reviewed in Assous and Wilamowski, 2009). Movement of hosts between TBRF endemic and TBRF-free areas poses the risk of pathogen introduction into new countries (Colin de Verdière et al., 2011; Kutsuna et al., 2013). TBRF presents with a variety of nonspecific clinical signs such as fever attacks, chills, headache, nausea, vomiting, sweating, abdominal pain, arthralgia, cough and photophobia (Arshi et al., 2002). Usually, fever episodes interrupted by fever-free intervals are characteristic for this illness (Adler et al., 1937; Kutsuna et al., 2013). Efficient and reproducible cultivation of *B. persica* is a basic requirement to improve research and further development of rapid and reliable diagnostic methods for TBRF. So far, the pathogen was maintained mainly in guinea pigs (Arshi et al., 2002; Ras et al., 1996) and was cultivated recently *in vitro* (Zamani et al., 2014). Further refinements of the *in vitro* cultivation methods for *B. persica* are essential to reduce or even replace animal experiments. Several culture media and conditions have been described for *in vitro* growth of relapsing fever borreliae. The most frequently used media are the Barbour Stoenner Kelly-II medium (BSK-II; Barbour, 1984), the commercially available BSK-H (Pollack et al., 1993) and the modified Kelly Pettenkofer medium (MKP; Preac-Mursic et al., 1986). However, in our experience *B. persica* did not propagate satisfyingly in these well-established media. Therefore, the aim of this study was to optimize a cultivation medium for *B. persica* that allows efficient and reproducible *in vitro* growth of this spirochete. In addition, we aimed to characterize two *B. persica* strains *via* conventional PCR and multilocus sequence analysis (MLSA). An MLSA system has been described for members of the Lyme borreliosis group of

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spirochetes (Margos et al., 2008, 2011). For immediate comparability, the same housekeeping genes located on the main linear chromosome were investigated (*i.e. clpA, clpX, pepX, pyrG, recG, rplB*, and *uvrA*; with the exception of *nifS*) to adjust the MLSA system to *B. persica*. This method is a powerful tool to identify new strains and allocate them to species (Margos et al., 2011; Richter et al., 2006).

2. Materials and methods

2.1. B. persica *strains*

A heparinized blood sample was collected from an ill cat in Jerusalem (Israel) and sent to the author's laboratory in Germany. The blood was positive for viable spirochetes by dark-field microscopy. From this sample 100 μl each were transferred into two glass vials containing 5 ml pre-warmed, sterile-filtered, donated human serum (RKS) and incubated at 33 °C in humidified air with 5 % CO_2. Dark-field microscopy of the cultures was performed daily. Nine days after inoculation the cultures were mixed with glycerol at a final concentration of 15 %, aliquoted and frozen at −80 °C as the culture of origin (P1). This strain was designated *B. persica* LMU-C01. An additional passage was produced and frozen as a new stock passage (P2, in donated human serum). This P2 was used in this study supplemental to the P1.

The second *B. persica* strain was isolated out of ticks collected in a cave in the Negev region (Israel). The ticks were pooled in five groups (each group containing four ticks), crushed and incubated in donated human serum as written above. Seven days after inoculation one culture out of five pools was positive for viable spirochetes by dark-field microscopy and was frozen at −80 °C. This strain was designated *B. persica* LMU-T01 and the extracted DNA of this culture was investigated by MLSA additionally to strain LMU-C01.

2.2. *Cultivation methods*

Cultivation of the *B. persica* strain LMU-C01 was further attempted in donated human serum as well as in human sera offered by commercial distributors (Human Serum, from human male AB plasma, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; Human Serum, Normal, Merck Chemicals GmbH, Schwalbach, Germany). All sera were sterile-filtered before use.

The medium for cultivation of *B. persica* strain LMU-C01 used in this study is based on an in-house medium which originated from the modified Kelly Pettenkofer medium (MKP; Preac-Mursic et al., 1986) and is now termed Pettenkofer/LMU Bp (Pett./LMU Bp, Table 1). Ingredients were added to slowly stirring autoclaved distilled water. The pH of the incomplete medium was adjusted to 7.6 with 6 N HCl. The medium was sterile-filtered through a 0.2-μm pore size bottle top filter (Nalgene™ Rapid-Flow™ Filters, VWR International GmbH, Ismaning, Germany). It was completed with a bovine serum albumin solution and with an autoclaved gelatin solution (115 °C, 15 min). The freshly prepared medium was stored at −20 °C until used.

For quality control of each new batch of Pett./LMU Bp, a 5-ml polypropylene tube (Sarstedt AG & Co., Nümbrecht, Germany) was filled with 3 ml of medium. Subsequently, 100 μl of a *B. persica* culture were inoculated. In this study *B. persica* cultures of the strain LMU-C01 were incubated at 37 °C in humidified air. The viability and morphology of the cultured spirochetes were inspected by dark-field microscopy from days three to six. Cell counts were performed in a Petroff–Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). To prove sterility, 100 μl of the medium were each streaked on a blood and a Sabouraud agar plate. These plates were incubated at 37 °C and visually checked after one, two and five days. Additionally, a 5-ml tube with 3 ml medium was incubated at 37 °C for at least five days as sterility control.

Table 1
Formulation of Pettenkofer/LMU Bp medium.

Ingredients	Quantity in medium
Distilled water	830 ml
HEPES sodium salt[a]	6 g
Bacto™ Neopeptone[b]	3 g
D-(+)-Glucose[a]	3 g
Sodium bicarbonate[a]	2 g
Sodium pyruvate[a]	0.8 g
Sodium citrate tribasic dihydrate[a]	0.7 g
N-Acetyl-D-glucosamine[a]	0.4 g
Rabbit serum; heat-inactivated 56 °C, 30 min[c]	140 ml
CMRL-1066 (10×), no glutamine[c]	100 ml
6 N HCl[d]	Adjust to pH 7.6
Bovine serum albumin solution, 35 % in 0.85 % sodium chloride, sterile[a]	35 ml
Gelatin from bovine skin, type B, powder; autoclaved 115 °C, 15 min[a]	14 g in 200 ml distilled water

[a] Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany.
[b] Becton, Dickinson and Company, Heidelberg, Germany.
[c] Life Technologies GmbH/Gibco ®, Darmstadt, Germany.
[d] Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.3. *Growth characteristics*

Growth behavior of *B. persica* strain LMU-C01 in Pett./LMU Bp medium was evaluated by inoculation 100 μl of P1 in 3 ml Pett./LMU Bp medium in duplicate and subsequent incubation. The samples were checked periodically. When bacterial growth reached the exponential phase, 100 μl of the culture were transferred into 3 ml of fresh medium. Passages were produced every three or four days up to passage P40.

Spirochete growth dynamic was studied in a triplicate: 100 μl *B. persica* stock passages (P2) were injected into 3 ml Pett./LMU Bp medium and incubated. The cultures (P3) were checked daily for eleven days and spirochetes were counted.

Growth analysis was carried out using cell count results of the growth behavior and growth dynamic studies and evaluated with the OriginPro 9.1 Software (Additive GmbH, Friedrichsdorf, Germany).

2.4. *Molecular analysis of the relapsing-fever* B. persica *strains*

A 3-ml culture of the strain LMU-C01 was transferred successive (3 × 1 ml) to a 1.5-ml safe-lock tube (Eppendorf Vertrieb Deutschland GmbH, Wesseling/Berzdorf, Germany) and centrifuged (19,000 × g, 25 °C, 10 min). The pellet was washed with 300 μl sterile phosphate-buffered saline (PBS) once and centrifuged again (19,000 × g, 4 °C, 10 min). The supernatant was discarded after each centrifugation step.

DNA-extraction of the pellets (strain LMU-C01) and of 100 μl of the thawed tick culture (strain LMU-T01) was done using the AS3000 Maxwell® 16 MDx Instrument (Promega GmbH, Mannheim, Germany) and the Maxwell® 16 LEV Blood DNA Kit (Promega GmbH). A modification of the original protocol on initial sample preparation was carried out: The pellet or 100 μl tick culture was filled up to 300 μl with sterile PBS. 300 μl Lysis Buffer and 30 μl Proteinase K were added. Following steps were done according to the manufacturer's protocol. DNA of pellets or tick culture was eluted in 100 μl or 60 μl Elution Buffer.

B. persica strain LMU-C01 was detected by conventional PCR targeting the *flaB* gene in the Mastercycler® pro (Eppendorf Vertrieb Deutschland GmbH). Primer design was done with Primer3Plus (Free Software Foundation, Inc., Boston, MA, USA; http://primer3plus.com; Untergasser et al., 2007). The primers and

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