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Case Report

Isolation and cultivation of *Borrelia lusitaniae* from the blood of a patient with multiple erythema migrans

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Abstract

Introduction: The region of Serbia is characterised by a high prevalence and diversity of *Borrelia* species, with *Borrelia lusitaniae* dominating, followed by *Borrelia afzelii*. Before this report, there were no data on *Borrelia* species causing Lyme borreliosis (LB) in Serbia.

Case presentation: We report the case of a 10-year-old boy with a clinical presentation of disseminated erythema migrans (EM). His results showed IgM antibodies at 6.27 (negative < 0.20; positive > 0.32) against *Borrelia burgdorferi* sensu lato, which was confirmed via ELFA. Except for skin lesions, the patient did not show any other clinical signs of systemic infection. His blood was taken to isolate and cultivate spirochetes and for molecular analysis. Antimicrobial therapy was prescribed according to the recommended treatment for patients with LB. A follow-up examination was conducted after nine days. The EMs on the skin had disappeared, and antibiotic therapy was continued for 14 days. A second follow-up was conducted one month after the end of therapy. The boy's health condition was normal. After 16 days of incubation in BSK-H medium, viable, motile, and spiral-shaped spirochetes were observed in the culture tube, and cultivation was prolonged for 29 days. PCR and sequencing were successful in both the blood sample and the culture and confirmed the presence of *B. lusitaniae*.

Conclusions: The results presented here is the first *Borrelia* isolate from the blood of a patient with the clinical manifestation of LB-disseminated EM. The presented results confirm the potential of *B. lusitaniae* for dissemination via the hematogenous route.

Key words: *Borrelia lusitaniae*; Lyme borreliosis; human blood; isolation and cultivation.

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Introduction

Lyme borreliosis (LB) is a multisystem disorder caused by the tick-borne spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex, and it remains the most widespread vector-borne disease in the Northern Hemisphere [1,2]. In Europe, *Borrelia afzelii* and *Borrelia garinii* are the two major agents of LB; *Borrelia bavariensis*, *Borrelia spielmanii,* and *Borrelia burgdorferi* sensu stricto (s.s.) are less common agents; and *Borrelia valaisiana*, *Borrelia lusitaniae*, and *Borrelia bissettii* have been identified in individual cases but are recognised as potential pathogens [1,2].

If left untreated, LB can pass through three stages with different clinical manifestations. A localised infection is typically characterised by an erythema migrans skin lesion. Early disseminated disease is usually characterised by two or more erythema migrans or neuroborreliosis and/or carditis. Late LB usually manifests as arthritis or acrodermatitis chronica atrophicans, but it can also include specific rare neurological manifestations [1].

If the infection persists, at least four pathogenic *Borrelia* species can cause disseminated infection: *B. afzelii* is mostly associated with skin manifestations; *B. garinii* and *B. bavariensis* are usually associated with nervous system disorders; and *B. burgdorferi* s.s. is often associated with arthritis [1-4]. So far, *B. lusitaniae* has only been isolated from the chronic skin lesions of one Portuguese patient with suspected LB [5] and from a patient with vasculitis syndrome with clinical signs that are not specific for LB [6]. In this case study, we report the first blood isolation of *B. lusitaniae* from a patient with a clinical manifestation of LB—disseminated erythema migrans.

Case report

In early June 2022, a 10-year-old boy with suspected LB was referred by a general practitioner to

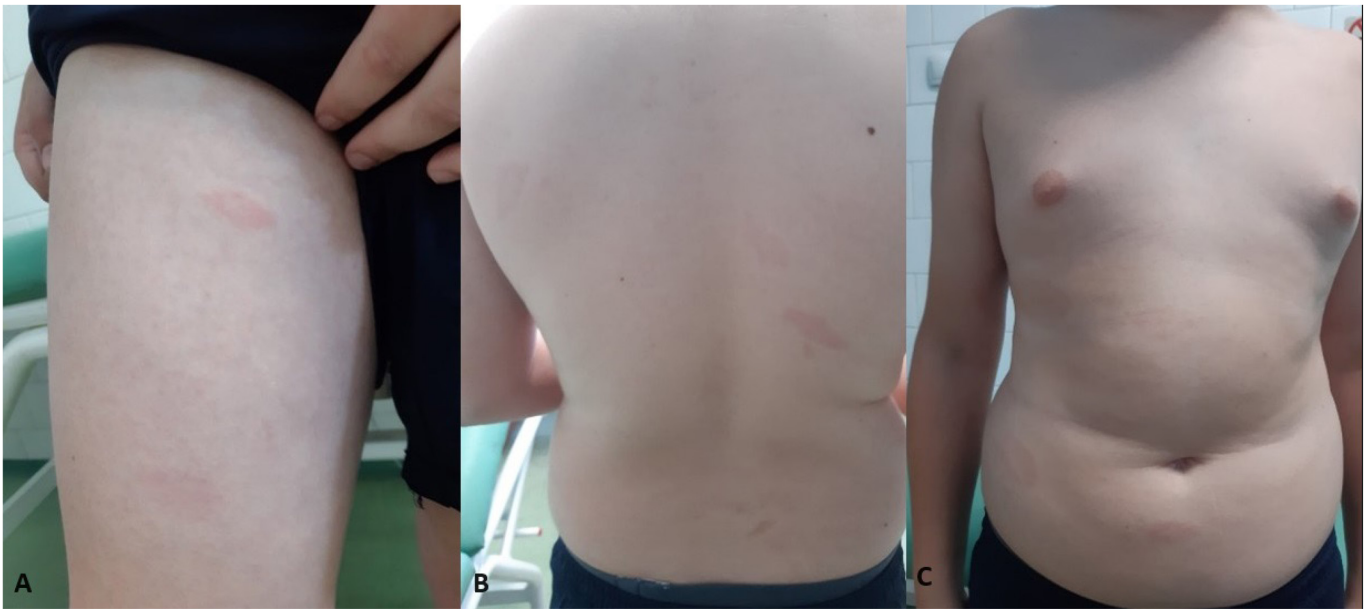
the outpatient department of the Clinic for Infectious and Tropical Diseases of the University Clinical Centre of Serbia. His results showed IgM antibodies at 6.27 (negative < 0.20; positive > 0.32) against *B. burgdorferi* s.l., which was confirmed via ELFA (Enzyme-Linked Fluorescent Assay). The boy had normal development and growth for his age; he was previously healthy without any signs of immunodeficiency and had no family history of immunodeficiency diseases or conditions. The patient lives in Belgrade (in an urban environment) and did not travel, and he and his mother could not recall if there had been a tick bite. Upon physical examination, the infectiologist diagnosed disseminated erythema migrans (multiple erythema migrans—EM) (Figure 1) and ten skin changes in the form of lesions. All EM lesions were ring-like and localized on the trunk and limbs. A few days before the examination, the mother noticed the first change—a skin lesion on the right upper arm that was 2 to 3 cm in diameter. After three or four days, the lesion expanded to about 10 cm, and around the same time, other lesions appeared on the patient's body. During the physical examination, we noted three lesions on the back, three on the abdomen, and two on the right upper leg. All of these EM lesions were at least 4 cm; one on the chest was 5 to 6 cm, and one on the right upper arm was around 10 cm. Except for these skin lesions, the patient did not show any other clinical signs of systemic infection. His blood was taken to isolate and cultivate spirochetes and for molecular analysis. Antimicrobial therapy was prescribed according to the recommended antibiotic treatment for patients with LB [1,2]: amoxicillin in a dose of 500 mg, thrice a day, for 2 weeks. A follow-up examination was conducted after nine days. The EMs on the skin had disappeared, and antibiotic therapy was continued for 14 days. A second follow-up examination was conducted one month after the end of therapy. The boy's health condition was normal, and blood was taken for serological analysis. The absence of IgM and IgG antibodies was confirmed using a commercial ELISA test (Alegria® Anti-Borrelia IgM Abs. and Alegria® Anti-Borrelia IgG, The ORGENTEC Anti-Borrelia Kits) at the Institute of Public Health "Dr. Milan Jovanovic Batut".

Since typical EM—and, therefore, multiple EM—is defined as two or more skin lesions, this was the clinical diagnosis [1,2]. Given that the boy did not present any systemic symptoms of this disease, no additional diagnostic procedures or biochemical analyses were performed during or after treatment. Written informed consent for a) blood sampling to detect *Borrelia* species in the patient's blood and to isolate and cultivate *Borrelia*; b) publishing clinical details; and c) the use of images was obtained from the patient's parents.

Total DNA was extracted from a blood sample according to previously published protocols [7,8], modified for this study.

Borrelia was cultivated and isolated using a published protocol [9] that was also modified for this study. After 16 days of incubation in Barbour–Stoenner–Kelly–H (BSK–H) medium (Sigma-Aldrich, St Louis, MO, USA), viable, motile, and spiral-shaped microorganisms were observed in the culture tube. Every second or third day, the culture was examined by

Figure 1. Disseminated erythema migrans localized on the A. right leg, B. back, C. chest and abdomen.



dark-field microscopy for the presence of spirochetes, their number, morphology, and motility, and incubation at 33 °C lasted 29 days.

After incubation, DNA was extracted from the culture-positive tube [10,11].

PCR amplification was successful for blood and culture samples. The app. 250 bp and 600 bp bands of the 5S–23S rDNA intergenic spacer and flagellin (*flaB*) gene, respectively, were observed on 2% agarose gels. The 5S–23S rDNA intergenic spacer fragment was amplified according to a "nested" PCR protocol [12], with the following primers (5'–3'):

RIS 1(CTG CGA GTT CGC GGG AGA) and RIS 2 (TCC TAG GCA TTCA CCA TA) for the first, and RIS 3 (GGA GAG TAG GTT ATT GCC AGG) and RIS 4 (GAC TCT TAT TAC TTT GAC C) for the second round of amplification, with annealing at 55 °C, using Go Taq® DNA Polymearse (Promega, Madison, WI).

The *flaB* gene fragment was amplified according to a "semi-nested" PCR protocol [13] with the following primers (5'–3'): primer A (TCT GAT GAT GCT GCT GCT GGT ATG), primer D (AAG TTT TCA ATA GCA TAC TC), and primer C (GCA GTT CAA TCA GGT AAC GG) [14].

The *flaB* gene fragment was amplified to a "semi-nested" PCR protocol [13] with the following primers (5'–3'): primer A (TCT GAT GAT GCT GCT GCT GGT ATG), primer D (AAG TTT TCA ATA GCA TAC TC), and primer C (GCA GTT CAA TCA GGT AAC GG) [14].

The PCR products were commercially sequenced (Macrogen, Amsterdam, the Netherlands). The sequences were analysed using FinchTV software (ver. 1.4.0) and the BLAST tool in GenBank (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST)

Sequencing was successful for all four samples. The sequences of both fragments (5S–23S rDNA intergenic spacer and *flaB* gene) were obtained directly from the DNA isolated from the patient's blood and then compared with the sequences obtained after cultivating the the same sample in BSK-H medium. Thus, their identity was 100% confirmed. The sequences were blasted against sequences in the GenBank database. The results showed 100% identity with sequences derived

Table 1. Results obtained by comparing sequences of cultured *Borrelia lusitaniae* in the present study with the deposited sequences in GenBank.

Sequences derived from this study	Description of the sequences in the GenBank	Host	Country	Sequence identity (%)	GenBank accession numbers
Flagellin gene, (flaB)partial cds, 527 bp, GenBank accession number: **PP654435**	*Borreliella lusitaniae* strain PoHL-1 chromosome, complete genome	*Homo sapiens*	Portugal	100.00%	CP132461
	Borreliella lusitaniae isolate 119 flagellin B (flaB) gene, partial cds	*Ixodes ricinus*	Serbia	100.00%	ON640808
	Borreliella lusitaniae isolate 76 flagellin (fla) gene, partial cds	*Ixodes ricinus*	Serbia	100.00%	MW901468
	Borrelia lusitaniae clone EP649-FN flagellin gene, partial cds	*Ixodes ricinus*	Poland	100.00%	KR782242
	Borrelia lusitaniae strain J3-2F-IR flagellin gene, partial cds	*Ixodes ricinus*	Poland	100.00%	KF422804
	Borrelia lusitaniae strain RB-Pm2N6 flagellin gene, partial cds	*Ixodes ricinus*	Germany	100.00%	DQ379486
	Borrelia lusitaniae strain 43ZLIF flagellin gene, partial cds	*Ixodes ricinus*	Slovakia	100.00%	DQ788618
	Borreliella lusitaniae isolate TR-Bo920 flagellin gene, partial cds	*Ixodes ricinus*	Turkey	100.00%	MK922619
5S-23S rDNA intergenic spacer region, partial sequence, 175 bp, GenBank accession number: **PP654436**	*Borrelia lusitaniae* strain PoHL1 5S ribosomal RNA gene, partial sequence	*Homo sapiens*	Portugal	100.00%	AY209179
	Borreliella lusitaniae isolate Pont12 5S-23S ribosomal RNA intergenic spacer	*Ixodes ricinus*	Spain	100.00%	MG245789
	Borreliella lusitaniae isolate IrOv10 5S-23S ribosomal RNA intergenic spacer region	*Ixodes ricinus*	Italy	100.00%	MG432676
	Borrelia lusitaniae DNA, 5S-23S rRNA intergenic spacer, strain: Tr161	*Ixodes ricinus*	Turkey	100.00%	AB091799
	Borreliella lusitaniae isolate J21/0 5S-23S rRNA intergenic spacer, partial sequence	*Lacerta viridis*	Czech Republic	100.00%	MZ561434
	Borrelia lusitaniae strain 11MIFMH 5S-23S intergenic spacer, partial sequence	*Ixodes ricinus*	Slovakia	100.00%	KF952598
	Borrelia lusitaniae genomic DNA containing 5S-23S IGS, isolate 11SS112	*Ixodes ricinus*	France	100.00%	HG798781
	Borrelia lusitaniae strain RB-Pm2N6 16S-23S ribosomal RNA intergenic spacer	*Ixodes ricinus*	Germany	100.00%	DQ379492

from humans, *I. ricinus* ticks, and the European green lizard (*Lacerta viridis*) in several European countries (Table 1). *Borrelia lusitaniae* DNA was confirmed in the DNA directly isolated from the blood sample, and the positive culture was obtained from the same sample after 29 days of incubation.

Representative sequences of a cultured *B. lusitaniae* were deposited in the GenBank database under the following accession numbers: PP654435 (*flagellin* gene) and PP654436 (5S–23S rDNA intergenic spacer).

Discussion

In this study, we presented the case of a patient with clinical manifestation of LB—multiple EM.

Before this report, there were no data on *Borrelia* species causing certain clinical manifestations of LB in Serbia. This region is characterised by a highly prevalent and diverse of *Borrelia* species in ticks and animals. Apart from the dominant *B. lusitaniae*, five other species have been detected: *B. afzelii*, *B. bavariensis*, *B. garinii*, *B. valaisiana*, and *B. burgdorferi* s.s. [14-19].

In analyses of the patient's blood, we detected *B. lusitaniae* via PCR amplification and sequencing. The *in vitro* isolation and cultivation of *Borrelia* from the blood was successful, and a molecular analysis of the strain confirmed the presence of the same species. In general, the sensitivity of PCR for detecting *Borrelia* DNA in blood samples from patients with LB is low, and according to European studies, the median sensitivity of PCR assays is 10% [20]. However, isolating and cultivating *Borrelia* species from an LB patient's clinical materials is a demanding, long (from nine to 12 weeks), labour-intensive, and expensive procedure with limited sensitivity [20]. Reported rates of *Borrelia* recovery from the blood of EM patients in Europe are less than 10% [20].

The pathogenic potential of *B. lusitaniae* is still unclear [1,2]. This species was isolated for the first time from a chronic skin lesion on a Portuguese patient with suspected LB, which was characterized by two ill-defined erythematous macules associated with local diffuse infiltration in the subcutaneous tissues [5]. The second strain was isolated from whole blood from a patient with vasculitis syndrome with clinical signs that are not specific for LB [6]. To date, no *B. lusitaniae* DNA has been detected in a human blood sample or isolated from the blood of a patient with any of the typical clinical manifestations of LB. Our results — both through direct molecular detection in blood and successful isolation and cultivation — confirm the presence of *B. lusitaniae* in a patient with multiple EM.

Multiple EM is one of the main representatives of the early disseminated stage of LB and is a consequence of the haematogenic spread of *Borrelia* from the initial skin lesion to other parts of the skin. *Borrelia afzelii*, *B. garinii,* and *B. burgdorferi* s.s. can haematogenously spread from the initial skin lesion, causing multiple EM lesions on the skin and other organs, particularly the peripheral and/or central nervous system (CNS), heart, or joints [1,2,4,21,22]. In Europe, the percentage of patients with multiple EM lesions — mostly caused by *B. afzelii* and *B. garinii* species—is lower in adult patients than in children, whereas in the United States, multiple EM lesions occur with a similar frequency in adults and children, caused by *B. burgdorferi* s.s., the main causative agent of LB in that country [1,2,4,21]. Furthermore, patients infected with *Borrelia mayonii* in the Upper Midwest region of the United States can also exhibit multiple and very small EM lesions [23].

In Europe, Slovenia is an endemic region for LB and has one of the highest incidences of this disease [24]. Arnež *et al.* [24] studied a large group of European children with EM and multiple EM. Over 5 years, 553 children with EM were diagnosed: 333 with EM and 220 with multiple EM. All patients were treated with antibiotics according to the European recommendations for treating early LB.

In that study, the incubation period from the bite to when the multiple EM patients first noticed their skin lesions was between 1 and 150 days (the median was 22 days). The duration (in the group with multiple EM), as noticed by the patient, was from 0 to 54 days (the median was 4 days) from the bite to the development of the lesions. Since our patient and his mother could not recall if there had been a tick bite or when, we could not compare our values with those above, except that the patient's mother noticed after three or four days that the first lesion expanded and other changes on his body appeared. During the physical examination in that period, ten skin changes–lesions were registered. In our study and that of Arnež *et al.*, the multiple EM lesions were ring-like in children. However, in the latter case [23], the lesion size ranged from 1 to 32 cm (median, 9 cm), whereas in our study, the size ranged from 2 to 10 cm.

Our patient was IgM-positive at the first examination, agreeing with the results of Arnež *et al.*, who reported positive IgM and/or IgG antibodies against *Borrelia* species [24] in 22% of patients with multiple EM. Together with a study by Gerber *et al.* [25], Arnež *et al.* state that skin lesions on children with multiple EM were more often located on the limbs than

the trunk; the patient presented in our case report had ring-like skin lesions localized on the trunk and limbs.

We noted an absence of any systemic symptoms in our patient compared with the children examined by Arnež *et al.* [24], who observed symptoms such as sore throat, enlarged liver, a temperature ≥ 38 °C, positive meningeal signs, otitis, and vomiting. This may be due to different *Borrelia* species that can cause multiple EM and be isolated from blood. The authors reported that *B. afzelii* was the most frequently isolated strain, followed by *B. garinii*, in contrast to our blood-isolated species, *B. lusitaniae*.

We isolated and cultivated *B. lusitaniae* from the blood of a patient with a clinical manifestation of LB (multiple EM) and performed sequencing to confirm this species in culture and the absence of co-infection.

Our results, thus, indicate that *B. lusitaniae* is another *Borrelia* species that can spread from the tick bite site to other skin sites, causing early disseminated infection in the form of multiple EM lesions.

Conclusions

Our findings represent the first human *Borrelia* isolate in Serbia and the first *B. lusitaniae* isolate from human blood obtained from a patient with an early stage of LB and clinical manifestation of multiple EM. This indicates that *B. lusitaniae* can disseminate via the hematogenous route and could be the causative agent of LB.

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Conflict of interests

No conflict of interests is declared.

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