Comparison of isolation rate of *Borrelia burgdorferi* sensu lato in two different culture media, MKP and BSK-H

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Abstract

The aim of the study was to evaluate two culture media for *Borrelia burgdorferi* sensu lato isolation from a 5 × 2 × 2 mm skin biopsy that was dissected into two pieces and inoculated into modified Kelly–Pettenkofer (MKP) and Barbour–Stoenner–Kelly-H (BSK-H) medium. Samples were incubated at 33°C for up to 9 weeks. *Borrelia* species was determined by *Mlu*I-restriction of whole genome or by *Mse*I-restriction of PCR product. We determined the proportion of isolation rate, 'slow-growers', contaminated specimens and *Borrelia* species in the two media. In each of the two media 235 skin specimens were cultivated. We found 90/470 (19.1%) contaminated cultures (BSK-H 67/235, 28.5%; MKP 23/235, 9.8%; p <0.0001). *Borrelia* growth was ascertained in 59/235 (25.1%) BSK-H and 102/235 (43.4%) MKP cultures (p <0.0001); the corresponding values for non-contaminated cultures were 59/168 (35.1%) and 102/212 (48.1%); (p 0.003). Fourteen specimens were positive only in BSK-H, 57 solely in MKP, and 43 in both culture media. Slow growth was present in 8/59 (13.6%) BSK-H and in 4/98 (4.1%) MKP positive cultures (p 0.019). *Borrelia afzelii* was identified in 44/51 (86.3%) BSK-H and in 88/98 (89.8%) MKP culture-positive samples; the corresponding findings for *Boreelia garinii* and *B. burgdorferi* sensu stricto were 6/51 (11.8%) and 9/98 (9.2%), and 1/51 (1.9%) and 1/98 (1.0%), for BSK-H and MKP, respectively. Comparison of MKP and BSK-H medium for *Borrelia* culturing from skin specimens of European patients with erythema migrans revealed the advantage of MKP over BSK-H.

Keywords: *Borrelia burgdorferi* sensu lato, Barbour–Stoenner–Kelly-H medium, culture medium, isolation rate, modified Kelly–Pettenkofer medium

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Introduction

Lyme borreliosis is caused by the spirochetes of *Borrelia burgdorferi* sensu lato complex. The hallmark of the disease is erythema migrans, a skin manifestation that develops after a tick bite. Dissemination of *B. burgdorferi* sensu lato from the primary skin lesion may result in the involvement of other organs [1–5].

Isolation of *B. burgdorferi* sensu lato from clinical material represents the reference standard for demonstration of *Borrelia* infection and enables further molecular analysis of the isolated strains with a potential for enhancement of our knowledge on the relationships between *Borrelia* species, biological material (ticks, animals, humans) and clinical manifestations [1,6–10], as well as for the assessment of phenotypic and genotypic changes of the microorganism in different conditions [11–13]. However, with the exception of skin specimens obtained from patients with cutaneous manifestations, the yield of this approach in European Lyme borreliosis is usually < 10% [12,14]. The yield of *Borrelia* isolation depends not only upon the source of the specimen but upon several other factors including preparation of the optimal transport medium for clinical samples, stability of the culture medium during long-term incubation, and ability of the medium to perform optimal conditions for *Borrelia* adaptation and growth. Substantial practical requirements are simple preparation, low

expense and stability of a medium at different storage temperatures. Several *Borrelia* culture media were developed such as modified Kelly–Pettenkofer (MKP) medium, Barbour–Stoenner–Kelly-II (BSK-II) medium, and BSK-H, the only commercial medium [15–18]. Following good laboratory practice and standard operative procedures, preparation of culture medium, *Borrelia* culturing, isolation and maintenance may overwhelm the capacity of a routine laboratory; which is the reason why commercially available material is welcome.

The aim of the study was to evaluate and compare the performance of two culture media, homemade MKP and commercially available BSK-H for the isolation of *B. burgdorferi* sensu lato from clinical material.

Materials and Methods

Skin biopsies and strains

Skin specimens were obtained from 235 adult patients with solitary erythema migrans diagnosed according to CDC criteria [19] at the Department of Infectious Diseases of the University Medical Centre Ljubljana during 2007 and 2008. Skin biopsy was performed at the border of the skin lesion after disinfection with 70% alcohol and local anaesthesia with 2% xylocaine. The specimen (5 × 2 × 2 mm) was dissected into two equal pieces that were inoculated into MKP or BSK-H medium. The material was maintained at room temperature for up to 4 h until transported to the laboratory, where it was incubated at 33°C; tubes were subcultured several times and checked for motile spirochetes by dark-field microscopy weekly for up to 9 weeks [7,15,16,20]. Lack of viable spirochetes after 9 weeks of incubation was interpreted as a negative culture result.

Culture medium

The MKP was prepared in the laboratory following the original formulation of Preac-Mursic *et al.* [16], while commercially available BSK-H medium was purchased from Sigma (St Louis, MO; Cat. No. B-8291). The two media differed regarding to the concentration of some components and their origin (see Supporting information; Table S1). Additionally, BSK-H medium was supplemented with yeast extract at a concentration of about 2 g/L, whereas gelatine in a final concentration of about 10 g/L was added to MKP medium. Neither medium contained antibiotics. Each medium lot was checked for contamination at 2 days of incubation at 33°C; contaminated tubes were removed. Each medium lot was also checked for *Borrelia* growth by tube inoculation with a *Borrelia* strain of a particular species. After testing the quality of each medium lot (which lasted at least 1 week), media were distributed to clinicians.

Characterization of the isolated strains

Isolates giving good growth were analysed for their genotypic and phenotypic characteristics by pulsed-field gel electrophoresis (PFGE) and SDS–PAGE, while slow-growing isolates and visible but not growing strains were identified to species level by PCR.

Genotypic characteristics of well-growing strains. Isolated strains multiplied either in MKP or BSK-H medium, were centrifuged, mixed with agarose, distributed into moulds and solidified. Blocks were incubated overnight at 37°C in lysis buffer (containing ribonuclease 10 mg/mL and lysozyme 1 mg/mL), washed and incubated for 72 h at 50°C in digestion buffer (containing proteinase K 0.5 mg/mL). After exhaustive washing, blocks were stored at 4°C in the same buffer [8,20,21].

For plasmid profiling total genomic DNA was electrophoresed in 1% agarose gel with ramping time of 0.9–3 s for 37 h. Molecular weight marker size 8.3–145.5 kb from Sigma was used as a size marker [20,22].

For species identification, borrelial DNA was digested with 30 U of *Mlu*I restriction endonuclease overnight at 37°C. Restricted DNA fragments were separated by PFGE for 24 h with ramping time 1–40 s. A λ concatamer with monomer size of 48.5 kb from Sigma was used as a size marker. *Mlu*I-restriction fragment length polymorphism (*Mlu*I-RFLP) was applied for species identification according to previously reported data (*Borrelia afzelii*: fragments 440 and 90 kb; *Borrelia garinii*: fragments 220 and 80 kb; *B. burgdorferi* sensu stricto: fragment 140 kb) [8,20].

Phenotypic characteristics of well-growing strains. Isolated strains were subcultured in the same medium, centrifuged, washed three times in phosphate-buffered saline with $MgCl_2$ (PBS/Mg), resuspended in buffer containing 2.5% SDS and 2-mercaptoethanol, and boiled for 10 min. Proteins were separated using 12% PAGE at 100 V and stained with Coomassie brilliant blue. A low molecular weight marker from BioRad (Munich, Germany) was used as a size marker [11,20].

Comparison of phenotypic and genotypic characteristics of well-growing strains. Strains isolated from the same skin biopsy and grown in both media were compared regarding their species, species subtype, plasmid content and protein expression.

Genotypic characteristics of slow-growing isolates and visible but not growing strains. Strains that did not grow well enough to enable PFGE were identified to species level by a PCR-based method. After a prolonged period of incubation, tubes were centrifuged, and DNA was extracted from the pallet using a Qiagen