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Evaluation of an internally controlled real-time PCR targeting the *ospA* gene for detection of *Borrelia burgdorferi sensu lato* DNA in cerebrospinal fluid

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ABSTRACT

This study reports the development and evaluation of an internally controlled real-time PCR targeting the *ospA* gene for detection of *Borrelia burgdorferi sensu stricto*, *Borrelia garinii*, *Borrelia afzelii* and *Borrelia valaisiana*. DNA was extracted using QIAamp DNA Blood Mini kit columns. DNA from 33 *B. burgdorferi sensu lato* strains reacted in the assay, whereas no reactivity was observed with DNA from four relapsing fever *Borrelia* spp., 11 unrelated spirochaetes, and 31 unrelated microorganisms. The quantitative sensitivity of the assay was 1–10 fg of *Borrelia* DNA and one to five cultured *Borrelia* spirochaetes. Cerebrospinal fluid (CSF) specimens from 70 patients sent for routine testing for neuroborreliosis, and three CSF specimens containing *B. garinii* were also tested. Positive PCR results were obtained with all three culture-confirmed neuroborreliosis specimens, five of ten neuroborreliosis specimens with specific antibodies in CSF and pleocytosis, none of nine specimens from possible cases of early neuroborreliosis (antibodies in serum, CSF pleocytosis, no antibodies in CSF), one of 15 specimens from patients with active or past Lyme disease with neurological signs (antibodies in serum, no pleocytosis or antibodies in CSF), and none of 36 specimens from patients without Lyme borreliosis (no antibodies in serum or CSF). Overall, the real-time PCR assay enabled sensitive and specific detection of all *B. burgdorferi sensu lato* species tested. The PCR had a sensitivity of 50% in patients with neuroborreliosis. The main diagnostic role of the assay could be to confirm neuroborreliosis in patients for whom the diagnosis is doubtful.

Keywords *Borrelia burgdorferi*, cerebrospinal fluid, detection, Lyme neuroborreliosis, *ospA* gene, real-time PCR

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INTRODUCTION

Lyme borreliosis (LB) is the most common tick (*Ixodes*)-borne human disease in North America and Europe [1,2] and is caused by spirochaetes belonging to the *Borrelia burgdorferi sensu lato* genetic complex. In North America, *Borrelia burgdorferi sensu stricto* is the only causative genospecies of LB, whereas LB in Europe is caused by *B. burgdorferi sensu stricto* and *B. burgdorferi sensu lato* species *Borrelia afzelii* and *Borrelia garinii*. Reports of detection of *Borrelia valaisiana* DNA from human skin, urine and cerebrospinal fluid (CSF) samples [3–5] and results from serological studies [6] suggest that *B. valaisiana* can probably also cause LB, and possibly even neuroborreliosis.

Current microbiological diagnosis of neuroborreliosis using antibody detection in serum and CSF is of limited efficacy because of differential expression, cross-reactivity and intra-species variation [7]. In addition, previous studies have described patients with positive PCR results, but without detectable antibodies in CSF [8–14]. Detection of spirochaetes in CSF by culture or PCR is desirable for confirmation of the clinical diagnosis, and is most sensitive in patients with acute disease (<2 weeks). However, culture is slow, labour-intensive, expensive and insensitive [15].

Molecular detection of *Borrelia* in CSF has been used to provide early diagnosis of neuroborreli-

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osis. Different genes have been selected for use as a target for detection, including the p66, 16S rRNA, flagellin and 2H1 genes, the 30-kb circular plasmid, and the plasmid-encoded *ospA/B* gene [8–14,16–27]. The highest sensitivities with CSF have been found in studies using the *ospA/B* plasmid gene [14,18,19,23,24]. The *ospA* gene is a fully sequenced plasmid gene specific for *B. burgdorferi sensu lato* species [1], including *B. valaisiana* [28].

Low numbers of spirochaetes in CSF may not be detected because of PCR inhibition. However, real-time PCR enables accurate monitoring of PCR inhibition by the use of internal controls. Real-time PCR assays for *Borrelia* have been developed for use with clinical specimens [27]. Clinical evaluation of real-time PCR with skin biopsies from patients with erythema migrans [29] showed good sensitivity (80%) in comparison with conventional nested PCR (64%) or culture (54%). In contrast, amplification of *Borrelia* DNA from urine using real-time PCR was not successful [30]. When a real-time PCR assay was used to investigate 56 CSF samples from patients suspected of suffering from neuroborreliosis, only one specimen was positive, implying low sensitivity [26]. However, clinical data were not provided and the specimens could have been obtained mainly from patients with a different final diagnosis. Therefore, the clinical value of real-time PCR for use with CSF samples has not been established. The present study aimed to validate an internally controlled, real-time PCR assay targeting the *ospA* gene for detection of *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii* and *B. valaisiana* in CSF specimens.

MATERIALS AND METHODS

Analytical sensitivity and specificity

Analytical sensitivity was determined using DNA extracted from 33 cultured *B. burgdorferi sensu lato* control strains described previously [31], comprising six *B. burgdorferi sensu stricto* (B31, HB19, A44S, VS293, VS130, M16), 12 *B. garinii* (A01C, A76S, A77C, A94S, PBi, VSDA, VSBM, VSBP, Ip89, 20047, NT29, M63), nine *B. afzelii* (PKo, VS461, A02S, A03S, A17S, A26S, A51T, A63T, A67T), four *B. valaisiana* (VS116, M19, M53, M57), one *Borrelia japonica* (H014) and one *Borrelia spielmani* (A14S). Specificity was determined using: (i) DNA extracted from four cultured relapsing fever (RF) *Borrelia* control strains (*Borrelia hermsii*, *Borrelia crocidurae* A124B, *B. crocidurae* A125B, *Borrelia anserina*) belonging to three phylogenetic clusters [32] and cultured in modified Kelly's medium at 33°C [31]; (ii) DNA extracted from unrelated spirochaetes, namely a clinical specimen containing *Treponema pallidum*, and ten *Leptospira* spp. belonging to different genetic clusters [33], i.e., *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Lai (Lai), *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni (M20), *L. interrogans* serogroup Canicola serovar Malaya (H6), *Leptospira borgpetersenii* serogroup Serjoe serovar Hardjobovis (Lely 607), *Leptospira santarosai* serogroup Shermani serovar Shermani (1342K), *Leptospira noguchii* serogroup Panama serovar Panama (CZ214K), *Leptospura weilii* serogroup Celledoni serovar Celledoni (Celledoni), *Leptospira kirschneri* serogroup Autumnalis serovar Bim (1051), *Leptospira alexanderi* serogroup Javanica serovar Mengla (A85), and *Leptospira meyeri* serogroup Ranarum serovar Ranarum (ICF); and (iii) DNA extracted from the following 31 unrelated microbial species and viruses: *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus salivarius*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Fusobacterium nucleatum*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Bacillus cereus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium abscessus*, *Candida albicans*, *Cryptococcus neoformans*, *Toxoplasma gondii*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, herpes simplex type 1, cytomegalovirus, Epstein–Barr virus and adenovirus.

The real-time PCR assay was also tested with a proficiency panel that formed part of the 2003 Second External Quality Assessment for the Molecular Detection of *Borrelia* (Belgian National Committee for Molecular Diagnostics; http://www.uia.ac.be/cmd/). The panel contained two negative samples, five body fluids spiked with spirochaetes (two CSF spiked with *B. burgdorferi sensu stricto* B31, two CSF spiked with *B. garinii* N34, and a synovial fluid spiked with *B. burgdorferi sensu stricto* B31), a cultured dilution of *B. burgdorferi sensu stricto* B31, and four DNA solutions (two weakly positive and two strongly positive; two *B. burgdorferi sensu stricto* B31, one *B. garinii* N34 and one *B. afzelii* ACA1).

Limiting dilutions

The quantitative sensitivity of the assay was determined initially using limiting dilution series of known DNA concentrations of *B. garinii* A94S (1.30 μg/μL), *B. afzelii* A03S (0.81 μg/μL) and *B. burgdorferi sensu stricto* A44S (1.25 μg/μL).

In a move towards clinical application, quantitative sensitivity was also determined using limiting dilutions of cultured *B. garinii* A87S (1.25×10^7 spirochaetes/mL) spiked in normal CSF samples. *B. garinii* A87S spirochaetes were cultured freshly in Barbour–Stoenner–Kelly medium and quantified using dark-field microscopy.

Clinical material

The real-time PCR was performed on clinical CSF specimens sent to the laboratory for testing for neuroborreliosis (collected between 1998 and 2004) and on three CSF specimens from which *B. garinii* had been cultured (collected between 1987 and 1990). CSF specimens were categorised into five groups: (I) culture-confirmed neuroborreliosis; (II) neuroborreliosis (antibodies in CSF and pleocytosis, but culture not performed); (III)