NEW MICROBIOLOGICA, **30**, 399-410, 2007

Evaluation of a genotyping method based on the *ospA* gene to detect *Borrelia burgdorferi* sensu lato in multiple samples of Lyme borreliosis patients

Romina Floris[1], Giulia Menardi[1], Raffaela Bressan[1], Giusto Trevisan[2], Simona Ortenzio[2], Emanuela Rorai[3], Marina Cinco[1]

[1]Spirochete Laboratory, Dipartimento di Scienze Biomediche, Università di Trieste, Italy;
[2]Istituto di Dermatologia e Venereologia, Università di Trieste, Italy;
[3]Laboratorio di Analisi Cliniche, Ospedale di Cattinara, Trieste, Italy

SUMMARY

In this study we have developed a new Restriction-Fragment-Length-Polymorphism (RFLP) genotyping method for rapid detection and identification of *Borrelia* genospecies present as unique species or as co-infection in multiple specimens obtained simultaneously from 29 individual patients affected by early or late Lyme borreliosis (LB).
The target of the RFLP-genotyping was the heterogeneous plasmid located *ospA* gene, thus we developed a method able to detect and differentiate between six clinically relevant *Borrelia* genospecies circulating in Europe, *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana, B. bissettii* and *B. spielmanii.* In this study *Borrelia* DNA could be detected by PCR in at least one specimen of each patient, except in one case of neuroborreliosis (NB); blood samples gave the highest sensitivity in all patient groups. The genotyping indicated that *B. afzelii* was present in 8 patients with skin involvement, *B. garinii* in 2 cases of NB and 4 cases with skin involvement, *B. burgdorferi* sensu stricto was detected in one patient with skin involvement and another with Lyme arthritis. Different *Borrelia* species in distinct specimens were identified in one patient with EM. The RFLP analysis of 11 patients revealed mixed patterns, which suggested pluri-infection with different *Borrelia* species.

KEY WORDS: Borrelia burgdorferi, Genotyping, RFLP, Lyme borreliosis, *OspA*

Received February 07, 2007 *Accepted* April 24, 2007

INTRODUCTION

Lyme borreliosis (LB) is a multisystemic spirochetosis with dermatologic, neurologic and rheumatologic manifestations (Brouqui *et al.*, 2004; Steere and Glickstein, 2004; Halperin, 2005). The causative agent, *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato is quite heterogeneous: 11 genospecies have been identified (Michel *et al.*, 2004), but *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* have been detected as the main pathogenic for humans in Europe. *Borrelia* genospecies seems to be associated with distinct clinical syndromes. Indeed, infection with *B. burgdorferi* sensu stricto tends to lead to arthritis, whereas those by *B. garinii* and *B. afzelii* appear to cause neurological complications and cutaneous manifestations (van Dam *et al.*, 1993; Balmelli and Piffaretti, 1995).
The genospecies *B. valaisiana, B. lusitaniae, B. bissettii* and *B. spielmanii* were recently recognized in some cases of LB (Rijpkema *et al.*, 1997; Strle *et al.*, 1997; Wang *et al.*, 1999a; Ryffel *et al.*, 1999; Collares-Pereira *et al.*, 2004; Maraspin *et al.*, 2006). Generally, the genospecies identity of *Borreliae* infecting humans have been detected by

Corresponding author
Prof. Marina Cinco
Dipartimento di Scienze Biomediche
Laboratorio Spirochete
Università di Trieste
Via Fleming, 22 - 34127 Trieste
E-mail: cinco@dsb.units.it

isolation and typing the isolate. At present, PCR assays are the most sensitive techniques for *Borrelia*-specific DNA detection and genospecies identification (Dumler, 2001; Situm *et al*., 2002; Aguero-Rosenfeld *et al*., 2005). There are reports suggesting that the genospecies distribution in human tissues specimens, as determined by molecular methods, differs from that assessed by culture (Liveris *et al*., 1999).

Using PCR based methods, some authors managed to identify *B. burgdorferi* genospecies in clinical specimens (von Stedingk *et al*., 1995; Busch *et al*., 1996; Lunemann *et al*., 2001; Lebech and Hansen, 2002; Gooskens *et al*., 2006) while others demonstrated that co-infection can occur in human cases (Demaerschalck *et al*., 1995; Rijpkema *et al*., 1997; Liveris *et al*., 1999; Rudenko *et al*., 2005). In particular, Demaerschalck *et al*. (1995) found 8 out of 18 neuroborreliosis patients infected by more than one Lyme disease-associated genospecies, emphasizing the relatively high prevalence of pluri-infections in humans.

The aim of the present study was to identify the infecting *Borrelia* genospecies in multiple samples of patients with different clinical manifestations. By this approach we wanted to obtain more information on a correlation between genospecies and clinical symptoms, to provide epidemiological data, and ascertain the distribution of the *Borreliae* within tissues during infection. We used suitable primers homologous for the plasmid-located *ospA* gene described by Priem *et al*. (1997). Then we developed a Restriction-Fragment-Length-Polymorphism (RFLP) genotyping system on the *ospA* gene that permits the identification of the main pathogenic genospecies of *B. burgdorferi* circulating in Europe, *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B.valaisiana, B. bissettii* and *B. spielmanii*.

MATERIALS AND METHODS

Patients and samples

Twenty-nine patients (15 females and 14 males; median age 55) with clinically diagnosed LB were examined (Table 1). According to the prevalent symptoms they were divided into four groups. The first comprised 10 patients with Erythema Migrans (EM). The second included 10 subjects with late skin manifestations: acrodermatitis cronica atrophycans (ACA) and cases of unusual atrophic skin lesions such as anetoderma, lichen schlerosus et atrophicus (LSA) and atrophoderma Pasini-Pierini which have been associated with *B. burgdorferi* infection (Grigor'eva and Babkin, 1999; Breier *et al.*, 2001; Bauer *et al.*, 2003). A third group consisted of 3 patients with Lyme arthritis and a fourth group with 6 patients suffering from neuroborreliosis (NB). The majority of patients were clinically diagnosed at the Department of Dermatology of the University of Trieste, and some at the Department of Infectious Diseases of the University Medical Centre of Trieste from April 2004 to December 2004. All these subjects were from the Friuli Venezia Giulia region (Italy), an endemic area for LB, and the majority reported one or repeated tick bites. Sera were examined for IgM and IgG anti-*Borrelia* antibodies using two-tiered ELISA (recomWell Mikrogen, Martinsreid, Germany) and Western blotting (RecomBlot Borrelia Ig assay, Mikrogen) at the Clinical Research Laboratory, Cattinara Hospital of Trieste.

Clinical samples were collected simultaneously: skin biopsy, blood and urine from EM patients and subjects affected by late skin manifestations; blood, urine and synovial fluid (SF) from patients with Lyme arthritis; blood, urine and cerebrospinal fluid (CSF) from patients suffering of NB. All the specimens were processed within 24 hours.

Skin biopsies

Skin biopsies were obtained from each patient with dermal involvement prior to antibiotic therapy. The biopsies were immediately processed for DNA extraction and further PCR. A second skin sample was taken from eight patients (Table 2) and inoculated in 7 ml of modified Barbour-Stoenner-Kelly (BSK) II medium (Barbour, 1984), followed by incubation at 34°C for 1 week or more (skin biopsy tissue culture). Starting from the third day, 2 ml of the skin biopsy culture supernatant were centrifuged (13,000 rpm, 15 min) and the pellet resuspended in 200 µl of 1X PBS buffer. Cultures were also observed weekly at dark-field microscopy for spirochete visualisation.

B. burgdorferi strains and cultivation

Six European strains representing the main

TABLE 1 - *Nested-PCR on* ospA *gene and RFLP molecular typing results in clinical samples from 29 patients with different manifestations of Lyme borreliosis.*

Patient no.	*Tick bite*[a]	*Genotype*	*Diagnosis*	*Serology ELISA + WB*		*N-PCR*				
				IgM	*IgG*	*N-PCR blood*	*N-PCR urine*	*N-PCR biopsy*	*N-PCR CSF*	*N-PCR SF*
1	/	B. garinii	EM	-	+	+	+	-	/	/
2	/	B. afzelii	EM	-	-	-	+	-	/	/
3	+	B. afzelii	EM	+	-	+	+	+	/	/
4	/	B. afzelii	EM	-	-	+	-	-	/	/
5	+	B. garinii	EM	-	-	+	+	-	/	/
6	+	B. afzelii	EM	-	+	+	+	-	/	/
7	+	B. afzelii + B. b. sensu stricto	EM	-	-	+	+	-	/	/
8	+	B. afzelii	EM	-	-	+	-	-	/	/
9	+	B. afzelii	EM	-	-	+	+	+	/	/
10	+	B. afzelii (biopsy)+ B. b. sensu stricto (blood, urine)	EM	(+)	+	+	+	+	/	/
11	/	B. garinii+ B. afzelii	ACA	-	-	-	+	-	/	/
12	+	B. b. sensu stricto	ACA	-	+	+	+	-	/	/
13	+	B. garinii	ACA	-	-	+	+	-	/	/
14	/	B. garinii	LSA	-	-	+	+	-	/	/
15	++	B. garinii+ B. afzelii + B. b. sensu stricto	ACA	-	(+)	-	-	+	/	/
16	/	B. afzelii	Anet.	+	+	+	-	+	/	/
17	/	B. garinii+ B. afzelii + B. b. sensu stricto	LSA	(+)	(+)	+	-	+	/	/
18	/	B. garinii+ B. afzelii + B. b. sensu stricto	Atr. prof. P-P	+	-	+	-	+	/	/
19	/	B. garinii+ B. afzelii + B. b. sensu stricto	LSA	-	-	+	-	-	/	/
20	/	B. afzelii	Anet.+ACA	-	-	+	-	-	/	/
21	/	B. b. sensu stricto	Arthritis	+	+	+	+	/	/	+
22	++	B. garinii+ B. afzelii + B. b. sensu stricto	Arthritis	-	+	+	-	/	/	+
23	+	B. garinii+ B. afzelii + B. b. sensu stricto	Arthritis	+	+	+	-	/	/	+

→

→ TABLE 1 - *Nested-PCR on* ospA *gene and RFLP molecular typing results in clinical samples from 29 patients with different manifestations of Lyme borreliosis.*

Patient no.	*Tick bite*[a]	*Genotype*	*Diagnosis*	*Serology ELISA + WB*		*N-PCR*				
				IgM	*IgG*	*N-PCR blood*	*N-PCR urine*	*N-PCR biopsy*	*N-PCR CSF*	*N-PCR SF*
24	/	B. garinii	NB	+	-	-	+	/	-	/
25	+	B. garinii+ B. afzelii + B. b. sensu stricto	NB	+	+	+	-	/	+	/
26	/	B. garinii+ B. afzelii + B. b. sensu stricto	NB	-	-	+	-	/	+	/
27	/	B. garinii+ B. afzelii + B. b. sensu stricto	NB	-	+	+	-	/	+	/
28	/	B. garinii	NB	+	+	+	-	/	+	/
29	/	/	NB	+	-	-	/	/	-	/

Anet.: anetoderma; Atr. prof. P-P: atrofoderma profundumPasini-Pierini; NA: non available; +: positive result; (+): borderline-positive reaction; -: negative result; a -: no tick bite; +: one tick bite; ++: two or more tick bite.

TABLE 2 - *Comparison between isolation of* B. burgdorferi *s. l. from skin biopsy tissue cultures and nested-PCR performed on the supernatant of the same cultures.*

Patient n.	*Diagnosis*	*skin biopsy tissue culture*	*N-PCR skin biopsy tissue culture*
9	EM	+	+
10	EM	Contaminated	+
15	ACA	-	+
16	Anetoderma	+	+
17	LSA	Contaminated	+
18	Atr. Prof. P.-P.	Contaminated	+
19	LSA	Contaminated	-
20	Anet. + ACA	Contaminated	-

Anet.: anetoderma; Atr. prof. P-P: atrofoderma profundumPasini-Pierini.

genospecies of *B. burgdorferi* sensu lato (Table 3) were grown in modified Barbour-Stoenner-Kelly (BSK) II medium (Barbour, 1984) under microaerophilic conditions at 34°C and were subcultured twice a week. Two ml containing 10^8 cells were pelleted by centrifugation (13,000 rpm, 15 min), washed two times in 200 µl PBS buffer and finally resuspended in 200 µl PBS.

DNA extraction

DNA was extracted within 24 h from blood, skin biopsies, SF, CSF using a High Pure Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. *Borrelia* cultures and skin cultures resuspended in 200 µl of 1X PBS buffer were extracted using the same kit described

TABLE 3 - *Predicted RFLP-pattern of OspA gene for the different Borrelia burgdorferi species.*

Tested strains	*NCBI accession no.*	*Species*	*Predicted RFLP patterns (bp)*				
			MseI	*SpeI*	Alw*I*	Mnl*I*	Taq*I*
NANCY[a]	Z29087	*B. afzelii*	208/183	/	/	/	242/149
BITS[b]	Z29086	*B. garinii*	134/98/85/74	/	/	297/112	/
B31	11496892	*B. burgdorferi* s. s.	183/134/74	270/121	227/164	/	/
VS116[c]	Y10840	*B. valaisiana*	183/134/74	/	/	/	/
DN127[c]	Y10897	*B. bissettii*	183/134/74	/	165/165/62	224/167	/
A14S[c]	AF102057	*B. spielmanii*	165/148/60/18	/	/	/	/

[a]Used sequence of VS461 because the type strains appears to lack the OspA gene sequences. [b]Used sequences of G25 because the type strains appears to lack the OspA gene sequences. [c]Strains kindly provided by G. Baranton (Paris, France).

above. DNA from urine (ten-millilitre) was extracted with DNAzol according to Bergmann *et al*. (2002) and finally suspended in 100 µl of ultrapure water.

Nested-PCR

Primer set used for nested-PCR amplified specifically a portion of the *ospA* gene (Priem *et al*., 1997).

Each experiment included one negative control containing mastermix and sterile water instead of DNA template and another including DNA extracted from blood and urine of a healthy donor. In addition, a positive control consisting of extracted *Borrelia* DNA (100 pg) was included in each nested-PCR. Sensitivity was determined by serial dilutions. All six strains, B31 (*B. burgdorferi sensu stricto*), BITS (*B. garinii*), NANCY (*B. afzelii*), A14S (*B. spielmanii)*, VS116 (*B. valaisiana*) and DN127 (*B. bissettii*) were detectable with a sensitivity of 15 fg of DNA template (data not shown).

The PCR reaction mixture (total volume of 25 µl) contained 2.5 µl of the isolated DNA, 2.5 µl of 10-fold PCR buffer (Promega Corporation, Madison, USA), 0.3 µM concentrations of each primer (Sigma-Genosys Ltd., UK), 200 µM concentrations of each nucleotide (Amersham Biosciences, UK), and 0.8 U of *Taq* polymerase (*Taq* DNA Polymerase in Storage Buffer B, Promega Corporation, Madison, USA). The PCR was performed in an automated DNA thermal cycler (PTC 200, Biozym, Hessisch Oldendorf, Germany) as described previously by Priem *et al*. (1997).

Amplicons of 391 bp were visualised by agarose gel electrophoresis (3% SeaKem LE Agarose, FMC Bioproducts) stained with ethidium bromide in a final concentration of 0.5 µg/ml, UV illuminated, and photodocumented.

Genotyping

We developed a new RFLP genotyping system specific for the portion of the *ospA* gene amplified by nested-PCR previously described. *Borrelia* sequences available in GenBank were analysed with the Webcutter 2 program (www.firstmarket.com/cutter/cut2.html) and specific restriction enzymes able to distinguish among different *Borrelia* species were chosen (Table 3).

Aliquots of each amplification product (10 µl) were digested separately with 0.5 U of the restriction enzyme *Mse*I (Promega Corporation, Madison, USA).

The restriction with endonuclease *Mse*I was able to distinguish among *B. afzelii, B. garinii* and *B. spielmanii,* while *B. burgdorferi* sensu stricto, *B. valaisiana* and *B. bissettii* shared a common pattern (Figure 1) that needed a further restriction with 0.5 U of enzyme *Alw*I (Figure 2).

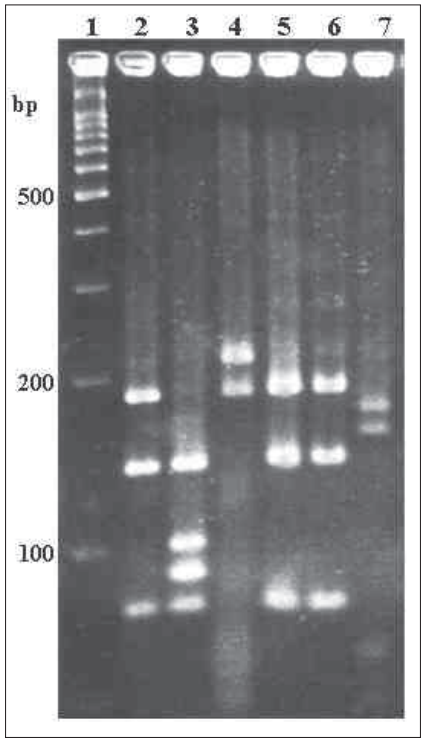

FIGURE 1 - Mse*I restriction patterns of the amplified* ospA *gene from the reference* B. burgdorferi *strains. Lane 1: 100 bp Molecular Ladder; lane 2: B31 (*B. burgdorferi sensu stricto*); lane 3: BITS (*B. garinii*); lane 4: NANCY (*B. afzelii*); lane 5: DN127 (*B. bissettii*); lane 6: VS116 (*B. valaisiana*); lane 7: A14S (*B. spielmanii*).*

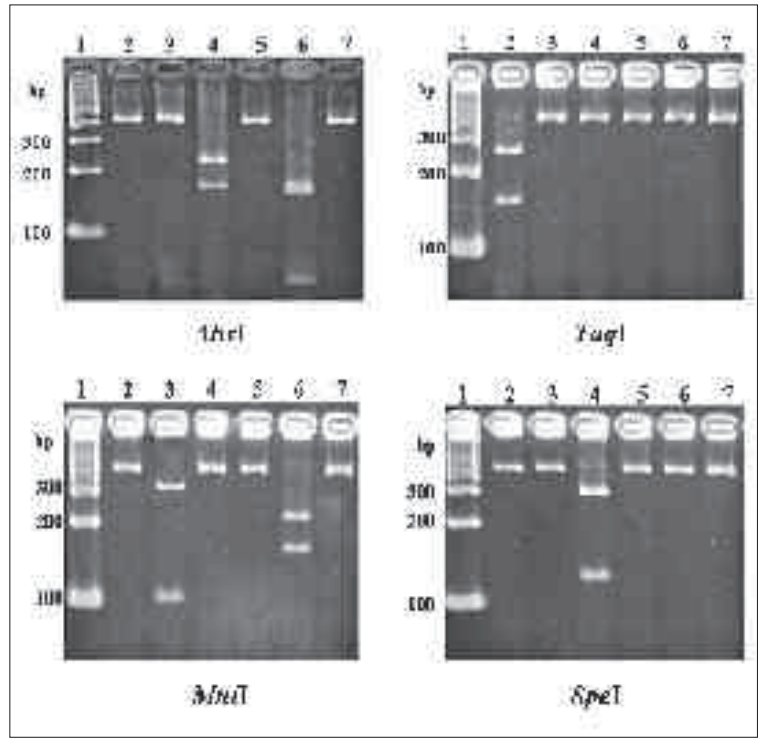

FIGURE 2 - *Restriction analysis with enzymes* Alw*I,* Taq*I,* Mnl*I and* Spe*I of the amplified* ospA *gene from the reference* B. burgdorferi *strains. Lane 1: 100 bp Molecular Ladder; lane 2: NANCY (*B. afzelii*); lane 3: BITS (*B. garinii*); lane 4: B31 (*B. burgdorferi sensu stricto*); lane 5: VS116 (*B. valaisiana*); lane 6: DN127 (*B. bissettii*); lane 7: A14S (*B. spielmanii*).*

To resolve mixed patterns, the amplicons were digested with a combination of restriction enzymes *Taq*I, *Mnl*I and *Spe*I, 0.5 U each (Promega Corporation, Madison, USA). Restriction with *Taq*I disclosed *B. afzelii*, while *Mnl*I resolved the different patterns of *B. garinii* and *B. bissettii* (Figure 2).

Co-infection with *B. burgdorferi* sensu stricto was detected using the enzyme *Spe*I instead of *Alw*I, able to restrict only this species (Figure 2); this was performed to avoid the double cut of *B. bissettii* with both enzymes *Alw*I and *Mnl*I. Digestions were carried out according to the manufacturer's instructions and digested products were visualized by agarose gel electrophoresis (4% MetaPhor Agarose, FMC Bioproducts), stained and documented as described for the PCR amplification products.

RESULTS

Amplification of B. burgdorferi *sensu lato DNA from clinical specimens*

We examined multiple specimens from patients with clinically defined Lyme borreliosis which included skin, blood, SF, CSF and urine. The nested-PCR method we used for the detection of *Borrelia* DNA (Priem *et al*., 1997) proved highly sensitive, detecting ≤3 borreliae/ sample. The positive nested-PCR was revealed as a clear band of 391 bp as shown in Figure 3.

Overall, *Borrelia* DNA was detected in at least one specimen of each patient, except the group of NB, where one subject presented all samples negative (Table 1). The amplification of samples from the EM group was positive for both blood and urine in 9 patients; the other three presented a positive

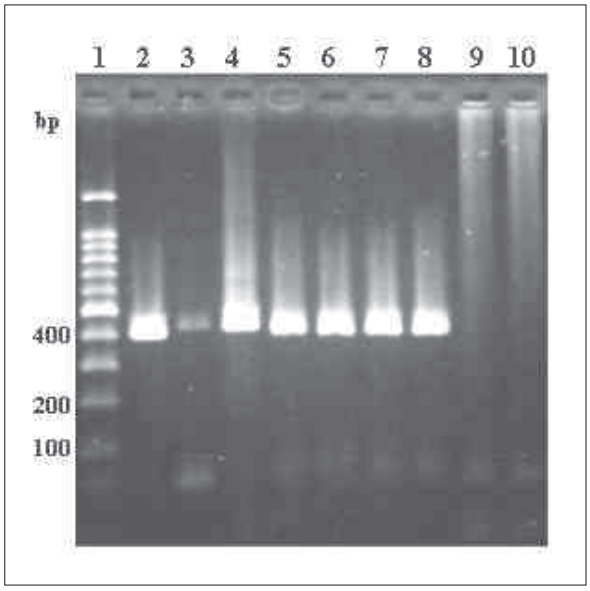

FIGURE 3 - *Nested-PCR with primers for* ospA *gene of strain NANCY (*B. afzelii*) and some clinical specimens from patients with Lyme borreliosis. Lanes 1: 100 bp Molecular Ladder; lane 2: strain NANCY (*B. afzelii*) 100 pg; lane 3: urine; lane 4: blood; lane 5: supernatant of skin biopsy tissue culture; lane 6: skin biopsy; lane 7: CSF; lane 8: SF; lane 9: blood of healthy subject; lane 10: negative control.*

PCR for all three samples (blood, urine and skin biopsy). A total of 10 patients with late skin manifestations were analysed. Six of them presented paired positive samples, three urine-blood and three blood-biopsy; of the others, two patients were PCR positive in blood, one in urine and one in biopsy. *Borrelia* DNA could be amplified in all paired blood-SF samples of patients with Lyme arthritis, and one of them was PCR positive also in urine. In four of the six analysed NB patients, both blood and CSF were PCR positive; one patient was positive only in urine and one patient with positive serology for IgM was PCR negative in all samples.

N-PCR of skin biopsy tissue culture

The skin biopsies placed in BSK medium (skin biopsy culture) obtained in a second sampling from eight patients, gave the following results: six were PCR positive and two negative (Table 2). By microscopic examinations of the 8 cultures, 2 developed visible *Borreliae*, one was *Borrelia* negative and 5 underwent contamination. The *Borrelia* containing cultures, patients n° 9 and 16, developed the spirochetes after 5 weeks of incubation, while the nested-PCR resulted positive already after 3 days. The contamination of the cultures of patients n° 10, 17 and 18 did not interfere with the sensitivity and specificity of the nested-PCRs (Table 2).

Genotyping of the amplified samples

The heterogeneity observed among *ospA* sequences of different European strains can be used for *Borrelia* genotyping of the amplification products (Moter *et al*., 1994; Wang *et al*., 1999b; Michel *et al*., 2004). All PCR positive specimens were analysed by RFLP method for *Borrelia* genotyping, as reported in Table 1, and some representative patterns were shown in Figure 4. Genospecies *B. afzelii* alone was clearly identified in all PCR positive samples of 6 patients with EM, in one case of anetoderma and in one patient with anetoderma + ACA; *B. garinii* alone was identified in 2 cases of NB, 2 patients with EM, in one case of ACA and in one case of LSA; *B. burgdorferi* sensu stricto alone was present in one patient with ACA and one patient with Lyme arthritis. A particular case of different *Borrelia* species was identified in the specimens of patient n° 10, affected by EM: *B. afzelii* was present in skin biopsy, while in blood and urine has been typed *B. burgdorferi* sensu stricto.

The *Mse*I-RFLP analysis of multiple specimens from 11 patients revealed mixed patterns (Fig. 4), which suggested the evidence of pluri-infection with different *Borrelia* species in the same specimen. These amplicons were further cut with endonucleases *Taq*I, *Spe*I and *Mnl*I in the same reaction mixture: with the concurrent cut of all three enzymes we have identified co-infections with B. *afzelii* and/or *B. burgdorferi* sensu stricto and/or *B. garinii* (Figure 5). As for the other patients, the co-infections were confirmed in all PCR positive specimens of the same patient. Therefore, RFLPs of both *B. afzelii* and *B. burgdorferi* sensu stricto were obtained in urine and blood of EM patient n° 7; urine sample of patient n° 11- affected by ACA- contained both *B. garinii* and *B. afzelii*; co-infection with B. *afzelii, B. burgdorferi* sensu stricto and *B. garinii* were detected in multiple samples of four patients with late skin manifestation, two patients with Lyme arthritis and three patients with NB (Figure 5). Genospecies *B. valaisiana*, *B. bissettii* and *B. spiel-*

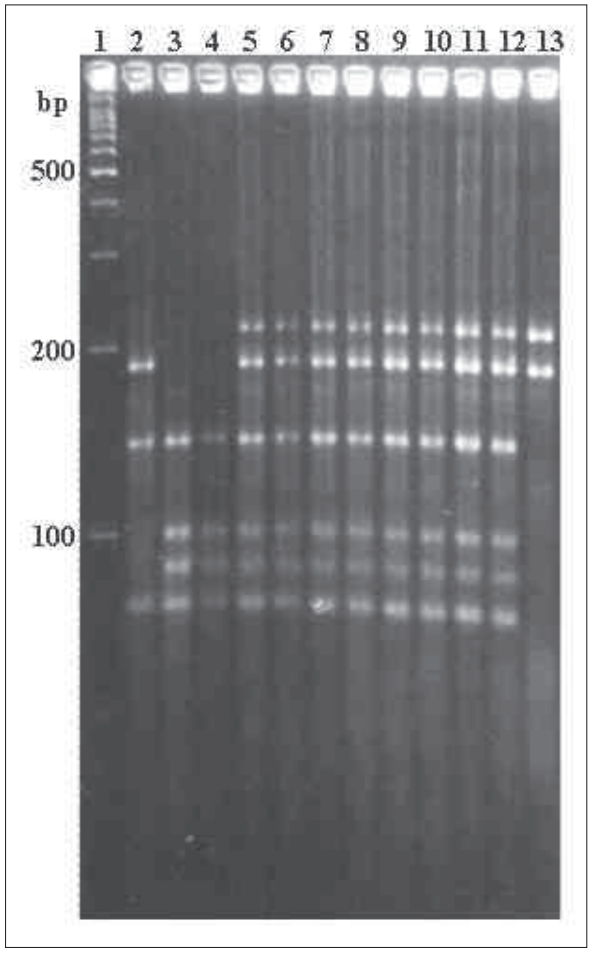

FIGURE 4 - Mse*I genotyping of the amplified* ospA *gene of* B. burgdorferi *strains in some clinical specimens. Lane 1: 100 bp Molecular Ladder; lane 2: pattern of* B. burgdorferi *sensu stricto; lanes 3,4: pattern of* B. garinii*; lanes 5-12: mixed patterns; lane 13: pattern of* B. afzelii.

manii were not detected in the analysed specimens.

DISCUSSION

In this work we detected *Borrelia* sequences in multiple specimens of LB patients using suitable primers homologous for *ospA* gene, as described by Priem *et al*. (1997). This nested-PCR disclosed fewer than 3 *Borreliae*/PCR reaction, confirmed in our setup of the method (data not shown). We increased the sensitivity of the nested-PCR in the clinical samples using a rapid and easy-to-perform DNA extraction (KIT Roche) or the protocol

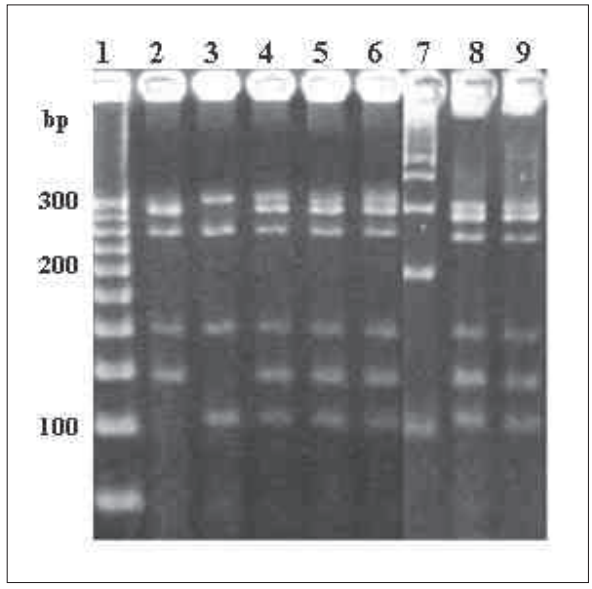

FIGURE 5 - *Concomitant restriction with enzymes* Taq*I,* Mnl*I and* Spe*I of some clinical specimens with* Borrelia *pluri-infections. Lane 1: 25 bp Molecular Ladder; lane 2: pattern of co-infection with* B. burgdorferi *sensu stricto and* B. afzelii*; lane 3: pattern of co-infection with* B. afzelii *and* B. garinii*; lanes 4-6 and 8,9: pattern of co-infection with* B. burgdorferi *sensu stricto,* B. afzelii *and* B. garinii*; lane 7: 100 bp Molecular Ladder.*

described by Bergmann *et al*. (2002) (used to process urine samples). These devices improved the detection of *Borrelia* DNA by avoiding several preparation steps used in other protocols with a considerable loss of *Borrelia* DNA. High sensitivity of the PCR is of paramount importance when the spirochete concentration in the host is near the detection limit of PCR, especially in CSF samples (Lebech *et al*., 2000; Liveris *et al*., 2002). Despite the excellent sensitivity of the nested-PCR, *Borrelia* DNA could not be detected in some skin biopsies of patients with proven (skin) borreliosis. A probable inhibition due to human DNA present in the extracted samples was excluded since *Borrelia* DNA could be detected in experimentally spiked biopsy. The concordance between amplification of skin biopsy and skin biopsy tissue culture of 8 patients led us to consider the possible failure of finding spirochetes in the advancing border of the EM lesion, as commonly performed in clinical practice. It was in fact reported that sometimes spirochetes are embedded in the middle of EM and not in the advancing border (Jurca *et al*., 1998).

The isolation of *B. burgdorferi* by cultivation requires from 1 to 6 weeks and contaminations can occur. This problem can be avoided by the use of PCR. In fact according to our data, the supernatant of skin biopsy tissue culture becomes PCR positive from the third day of culture, and *Borrelia* DNA can also be detected in the presence of contaminants.

All patients, except one affected by NB and a second with ACA, were PCR positive in at least one sample of blood and/or urine; overall, a positive PCR was obtained in 85.7% of blood, 50% of urine and 93.1% of paired blood-urine samples. Thus, PCR with blood specimens, mostly paired with urine, could be useful in diagnosis of Lyme borreliosis, especially when other more invasive sampling as biopsy, SF or CSF specimens are not available. In this study *Borrelia* DNA was detected by PCR in at least one specimen of each seronegative patients, showing that molecular analysis with PCR should contribute to formulate the diagnosis of Lyme disease in patients with well-defined clinical symptoms, but with a negative serology (Brettschneider *et al*., 1998; Aguero-Rosenfeld *et al*., 2005). The detection of *Borrelia* DNA in blood and/or urine of both early and late stages of LB indicates that some circulation of the microorganisms also occurs late in the infection as suggested by some authors (Schmidt, 1997; Mercier *et al*., 1997; Maraspin *et al*., 2002).

The RFLP genotyping method developed in this survey differentiated the species involved in the infection. *B. afzelii* was prevalent as single genospecies in all the PCR positive samples of patients with skin involvement, confirming the association of *B. afzelii* with cutaneous lesions (van Dam *et al*., 1993; Saint Girons *et al*., 1998). It is noteworthy that patient n° 16 with a diagnosis of anetoderma had *Borrelia* DNA both in blood and skin biopsy. *Borrelia* infection was also confirmed by positive serology and isolation of spirochete classified as *B. afzelii* from skin biopsy tissue culture. This is the second report of *B. burgdorferi* infection associated with anetoderma, after that described by Bauer *et al*. (2003). *B. burgdorferi* sensu stricto as single genospecies was present in one patient with Lyme arthritis, and in one with ACA. It is known that this genospecies can be the causative agent of Lyme arthritis (Eiffert *et al*., 1998), while the association with ACA is quite unusual, but not excluded (Picken *et al*., 1998). The same applies to *B. garinii*, which we detected not only in NB cases, but also in subjects with skin manifestations (Picken *et al*., 1998; Wang *et al*., 1999b). Interestingly, in patient n° 10, affected by EM, different genospecies were found in distinct specimens: *B. afzelii* was present in skin biopsy, and *B. burgdorferi* sensu stricto in blood and urine. A recent report by Ruzic *et al*. (2005) supports our findings: indeed, the authors isolated from distinct samples of individual LB patients *Borrelia* strains which belonged to different genospecies and shared phenotypic heterogeneity. Further, we obtained evidence of pluri-infection in multiple specimens of 11 patients. The RFLP genotyping based on restriction of enzyme *Mse*I revealed mixed patterns in the same specimens. A defined combination of species-specific enzymes identified the single species present in each mixed pattern.

We have observed that patients concurrently infected by the three genospecies *B. afzelii, B. garinii* and *B. burgdorferi* sensu stricto, shared an ambiguous clinical picture presumably due to occurrence of infection caused by distinct *B. burgdorferi* sensu lato. Hematogenous dissemination of different *B. burgdorferi* sensu lato species to the nervous system, joints or other skin areas, and occasionally to other organs, may give rise to a wide spectrum of overlapping clinical manifestations. The results of the present study support this hypothesis. In particular, patients n° 15, 17, 18 and 19 affected by chronic skin manifestations presented a miscellanea of other symptoms as chronic and/or migrans arthritis and paresthesias; patients n° 22 and 23 with Lyme arthritis recalled a past skin lesion; patients n° 25, 26 and 27 with NB reported other symptoms like arthralgias and dermatitis.

Pluri-infection with different *Borrelia* species is common in ticks collected in Europe, including our region FVG (Cinco *et al*., 1998; Schaarschmidt *et al*., 2001; van Dam, 2002). Thus, another matter of debate is if a pluri-infection in humans is due to an inoculum by a single tick bite carrying different *Borrelia* species, or, alternatively, humans may acquire different *Borrelia* species from repeated individual bites (Golde *et al*., 1998; Miller *et al*., 2006; Jarefors *et al*., 2006). Altogether, the RFLP analysis reported in this work showed that species present in the LB patients were *B. afzelii, B. garinii* and *B. burgdorferi*

sensu stricto, individually or in co-infection, with a particular prevalence of *B. afzelii*. These results are in agreement with previous reports on the prevalence of *Borrelia* genospecies detected in strains isolated from patient with Lyme disease from the north-eastern region of Italy (Ciceroni *et al.*, 2001) and in ticks collected on the field in FVG (data submitted).

Usually the infecting genotype is detected after isolation and cultivation of the infecting strain: since different strains and genospecies grow at different rates in isolation medium (Hubalek *et al.*, 1998; Wang, 2002; Aguero-Rosenfeld *et al.*, 2005), it is possible that only one strain/genospecies emerges overcoming the others, becoming predominant in the subsequent subcultures. Therefore the classification of isolates detects only a single genospecies corresponding to the predominant one. The use of PCR-genotyping allows the simultaneous detection in specimens of the infecting *B. burgdorferi* strains, in spite of their isolation in culture. For this reason, PCR directly on the clinical specimens, as we performed in this study, has more chance to disclose multiple infections (Demaerschalck *et al.*, 1995; Rijpkema *et al.*, 1997; Schaarschmidt *et al.*, 2001; Rudenko *et al.*, 2005). In view of its specificity, simplicity and rapidity, our method of genotyping offers the possibility of analyzing a large number of samples to further understanding of human disorders associated with *Borrelia* single- or pluri-infection. In conclusion, the RFLP genotyping method described in this study can greatly elucidate the distribution and prevalence of the different species among LB patients (and ticks), which could be of relevance in the evaluation of vaccines against *B. burgdorferi* sensu lato in a particular endemic region as FVG.

REFERENCES

Aguero-Rosenfeld, M.E., Wang, G., Schwartz, I., Wormser, G.P. (2005). Diagnosis of Lyme borreliosis. *Clin Microbiol.* **18**, 484-509.

Balmelli, T., Piffaretti, J.C. (1995). Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. *Res Microbiol.* **146**, 329-340.

Barbour, A.G. (1984). Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med.* **57**, 521-525.

Bauer, J., Leitz, G., Palmedo, G., Hugel, H. (2003). Anetoderma: another facet of Lyme disease? *J Am Acad Dermatol.* **48**, S86-88.

Bergmann, A.R., Schmidt, B.L., Derler, A.M., Aberer, E. (2002). Importance of sample preparation for molecular diagnosis of Lyme borreliosis from urine. *J Clin Microbiol.* **40**, 4581-4584.

Breier, F., Khanakah, G., Stanek, G., Kunz, G., Aberer, E., Schmidt, B., Tappeiner, G. (2001). Isolation and polymerase chain reaction typing of *Borrelia afzelii* from a skin lesion in a seronegative patient with generalized ulcerating bullous lichen sclerosus et atrophicus. *Br J Dermatol.* **144**, 387-392.

Brettschneider, S., Bruckbauer, H.N., Hofmann, H. (1998). Diagnostic value of PCR for detection of *Borrelia burgdorferi* in skin biopsy and urine samples from patients with skin borreliosis. *J Clin Microbiol.* **36**, 2658-2665.

Brouqui, P., Bacellar, F., Baranton, G., Birtles, R.J., Bjoersdorff, A., Blanco, J.R., Caruso, G., Cinco, M., Fournier, P.E., Francavilla, E., Jensenius, M., Kazar, J., Laferl, H., Lakos, A., Lotric Furlan, S., Maurin, M., Oteo, J.A., Parola, P., Perez-Eid, C., Peter, O., Postic, D., Raoult, D., Tellez, A., Tselentis, Y., Wilske, B. (2004). Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. *Clin Microbiol Infect.* **10**, 1108-1132.

Busch, U., Hizo-Teufel, C., Bohemer, R., Fingerle, V., Nitschko, H., Wilske, B., Preac-Mursic, V. (1996). Three species of *Borrelia burgdorferi* sensu lato identified from cerebrospinal fluid isolates by pulsefield gel electrophoresis and PCR. *J Clin Microbiol.* **34**, 1072-1078.

Ciceroni, L., Ciarrocchi, S., Ciervo, A., Mondardini, V., Guzzo, F., Caruso, G., Murgia, R., Cinco, M. (2001). Isolation and characterization of *Borrelia burgdorferi* sensu lato strains in an Area of Italy Endemic for Lyme Borreliosis. *J Clin Microbiol.* **39**, 2254-2260.

Cinco, M., Padovan, D., Murgia, R., Poldini, L., Frusteri, L., van de Pol, I., Verbeek de Kruif, N., Rijpkema, S., Maroli, M. (1998). Rate of infection of *I. ricinus* ticks with *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii* and group VS 116 in an endemic focus of Lyme disease in Italy. *Eur J Microbiol and Infect Dis.* **17**, 90-94.

Collares-Pereira, M., Couceiro, S., Franca, I., Kurtenbach, K., Schafer, S.M., Vitorino, L., Goncalves, L., Baptista, S., Vieira, M.L., Cunha, C. (2004). First isolation of *Borrelia lusitaniae* from a human patient. *J Clin Microbiol.* **42**, 1316-1318.

Demaerschalck, I., Ben Messaoud, A., De Kesel, M., Hoyois, B., Lobet, Y., Hoet, P., Bigaignon, G., Bollen, A., Godfroid, E. (1995). Simultaneous presence of different *Borrelia burgdorferi* genospecies in biological fluids of Lyme disease patients. *J Clin Microbiol.* **33**, 602-608.

Dumler, J.S. (2001). Molecular diagnosis of Lyme disease: review and meta-analysis. *Mol Diagn.* **6**, 1-11.

EIFFERT, H., KARSTEN, A., THOMSSEN, R., CHRISTEN, H.J. (1998). Characterization of *Borrelia burgdorferi* strains in Lyme arthritis. *Scand J Infect Dis.* **30**, 265-268.

GOLDE, W.T., ROBINSON-DUNN, B., STOBIERSKI, M.G., DYKHUIZEN, D., WANG, I.N., CARLSON, V., STIEFEL, H., SHIFLETT, S., CAMPBELL, G.L. (1998). Culture-confirmed reinfection of a person with different strains of *Borrelia burgdorferi* sensu stricto. *J Clin Microbiol.* **36**, 1015-1019.

GOOSKENS, J., TEMPLETON, K.E., CLAAS, E.C., VAN DAM, A.P. (2006). Evaluation of an internally controlled real-time PCR targeting the ospA gene for detection of *Borrelia burgdorferi* sensu lato DNA in cerebrospinal fluid. *Clin Microbiol Infect.* **12**, 894-900.

GRIGOR'EVA, L.A., BABKIN, A.V. (1999). The use of silver staining method in the diagnosis of late skin lesions in ixodid tick-borne borreliosis in man. Parazitologiia. **33**, 267-269.

HALPERIN, J.J. (2005). Central nervous system Lyme disease. Curr. Neurol. Neurosci. Rep. **5**, 446-452.

HUBALEK, Z., HALOUZKA, J., HEROLDOVA, M. (1998). Growth temperature ranges of *Borrelia burgdorferi* sensu lato strains. *J Med Microbiol.* **47**, 929-932.

JAREFORS, S., BENNET, L., YOU, E., FORSBERG, P., EKERFELT, C., BERGLUND, J., ERNERUDH, J. (2006). Lyme borreliosis reinfection: might it be explained by a gender difference in immune response? *Immunology.* **118**, 224-232.

JURCA, T., RUZIC-SABLJIC, E., LOTRIC-FURLAN, S., MARASPIN, V., CIMPERMAN, J., PICKEN, R.N., STRLE, F. (1998). Comparison of peripheral and central biopsy sites for the isolation of *Borrelia burgdorferi* sensu lato from erythema migrans skin lesions. *Clin Infect Dis.* **27**, 636-638.

LEBECH, A.M., HANSEN, K., BRANDRUP, F., CLEMMENSEN, O., HALKIER-SORENSEN, L. (2000). Diagnostic value of PCR for detection of *Borrelia burgdorferi* DNA in clinical specimens from patients with erythema migrans and Lyme neuroborreliosis. *Mol Diagn.* **5**, 139-150.

LEBECH, A.M., HANSEN, K. (2002). Detection of *Borrelia burgdorferi* DNA in urine samples and cerebrospinal fluid samples from patients with early and late Lyme boreliosis by Polymerase chain reaction. *J Clin Microbiol.* **30**, 1646-1653.

LIVERIS, D., VARDE, S., IYER, R., KOENIG, S., BITTKER, S., COOPER, D., MCKENNA, D., NOWAKOWSKI, J., NADELMAN, R.B., WORMSER, G.P., SCHWARTZ, I. (1999). Genetic diversity of *Borrelia burgdorferi* in lyme disease patients as determined by culture versus direct PCR with clinical specimens. *J Clin Microbiol.* **37**, 565-569.

LIVERIS, D., WANG, G., GIRAO, G., BYRNE, D.W., NOWAKOWSKI, J., MCKENNA, D., NADELMAN, R., WORMSER, G.P., SCHWARTZ, I. (2002). Quantitative detection of *Borrelia burgdorferi* in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. *J Clin Microbiol.* **40**, 1249-1253.

LUNEMANN, J.D., ZARMAS, S., PRIEM, S., FRANZ, J., ZSCHENDERLEIN, R., ABERER, E., KLEIN, R., SCHOULS, L., BURMESTER, G.R., KRAUSE, A. (2001). Rapid typing of *Borrelia burgdorferi* sensu lato species in specimens from patients with different manifestations of Lyme borreliosis. *J Clin Microbiol.* **39**, 1130-1133.

MARASPIN, V., RUZIC-SABLJIC, E., STRLE, F. (2006). Lyme borreliosis and *Borrelia spielmaniii*. *Emerg Infect Dis.* **12**, 1177

MARASPIN, V., CIMPERMAN, J., LOTRIC-FURLAN, S., RUZIC-SABLJIC, E., JURCA, T., STRLE, F. (2002). Cerebrospinal fluid findings in adult patients with multiple erythema migrans. *Wien Klin Wochenschr.* **114/13**, 505-509.

MERCIER, G., BURCHEL, A., LUCOTTE, G. (1997). Detection of *Borrelia burgdorferi* DNA by plymerase chain reaction in urine specimens of patients with erithema migrans lesions. *Mol Cell Prob.* **11**, 89-94.

MICHEL, H., WILSKE, B., HETTCHE, G., GOTTNER, G., HEIMERL, C., REISCHL, U., SCHULTE-SPECHTEL, U., FINGERLE, V. (2004). An *osp*A-polymerase chain reaction/restriction fragment length polymorphism-based method for sensitive detection and reliable differentiation of all European *Borrelia burgdorferi* sensu lato species and OspA types. *Med Microbiol Immunol (Berl).* **193**, 219-226.

MILLER, R.F., O'CONNELL, S., MANJI, H. (2006). Reinfection with Lyme borreliosis presenting as a painful polyradiculopathy: Bannwarth's, Beevor's and *Borrelia*. *J Neurol Neurosurg Psychiatry.* **77**, 1293-1294.

MOTER, S.E., HOFMANN, H., WALLICH, R., SIMON, M.M., KRAMER, M.D. (1994). Detection of *Borrelia burgdorferi* sensu lato in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by *osp*A-specific PCR. *J Clin Microbiol.* **32**, 2980-2988.

PICKEN, R.N., STRLE, F., PICKEN, M.M., RUZIC-SABLJIC, E., MARASPIN, V., LOTRIC-FURLAN, S., CIMPERMAN, J. (1998). Identification of three species of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) among isolates from acrodermatitis chronica atrophicans lesions. *J Invest Dermatol.* **110**, 211-214.

PRIEM, S., RITTIG, M.G., KAMRADT, T., BURMESTER, G.R., KRAUSE, A. (1997). An optimized PCR leads to rapid and highly sensitive detection of *Borrelia burgdorferi* in patients with Lyme borreliosis. *J Clin Microbiol.* **35**, 685-690.

RIJPKEMA, S.G., TAZELAAR, D.J., MOLKENBOER, M.J., NOORDHOEK, G.T., PLANTINGA, G., SCHOULS, L.M., SCHELLEKENS, J.F. (1997). Detection of *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. *Clin Microbiol Infect.* **3**, 109-116.

RUDENKO, N., GOLOVCHENKO, M., NEMEC, J., VOLKAERT, J., MALLATOVA, N., GRUBHOFFER, L. (2005). Improved method of detection and molecular typing of *Borrelia burgdorferi* sensu lato in clinical samples by polymerase chain reaction without DNA purification. *Folia Microbiol (Praha)*. **50**, 31-39.

RUZIC-SABLJIC, E., ARNEZ, M., LOGAR, M., LOTRIC-FURLAN, S., CIMPERMAN, J., STRLE, F. (2005). Comparison of *Borrelia* sensu lato strains isolated from specimens obtained simultaneously from two different sites of infection in individual patient. *J Clin Microbiol.* **43**, 2194-2200.

RYFFEL, K., PETER, O., RUTTI, B., SUARD, A., DAYER, E. (1999). Scored antibody reactivity determined by immunoblotting shows an association between clinical manifestations and presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. Valaisiana* in humans. *J Clin Microbiol.* **37**, 4086-4092.

SAINT GIRONS, I., GERN, L., GRAY, J.S., GUY, E.C., KORENBERG, E., NUTTALL, P.A., RIJPKEMA, S.G., SCHONBERG, A., STANEK, G., POSTIC, D. (1998). Identification of *Borrelia burgdorferi* sensu lato species in Europe. *Zentralbl Bakteriol.* **287**, 190-195.

SCHAARSCHMIDT, D., OEHME, R., KIMMIG, P., DIETER HESCH, R., ENGLISCH, S. (2001). Detection and molecular typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and in different patient samples from southwest Germany. *Eur J Epidemio* **17**, 1067-1074.

SCHMIDT, B.L. (1997). PCR in laboratory diagnosis of human *Borrelia burgdorferi* infections. *Clin Microbiol Rev.* **10**, 185-201.

SITUM, M., POJE, G., GRAHOVAC, B., MARINOVIC, B., LEVANAT, S. (2002). Diagnosis of Lyme borreliosis by polymerase chain reaction. *Clin Dermatol*. **20**, 147-155.

STEERE, A.C., GLICKSTEIN, L. (2004). Elucidation of Lyme arthritis. *Nat Rev Immunol.* **4**, 143-152.

STRLE, F., PICKEN, R.N., CHENG, Y., CIMPERMAN, J., MARASPIN, V., LOTRIC-FURLAN, S., RUZIC-SABLJIC, E., PICKEN, M.M. (1997). Clinical findings for patients with Lyme borreliosis caused by *Borrelia burgdorferi* sensu lato with genotypic and phenotypic similarities to strain 25015. *Clin Infect Dis*. **25**, 273-280.

VAN DAM, A.P. (2002). Diversity of *Ixodes*-borne *Borrelia* species-clinical, pathogenetic, and diagnostic implications and impact on vaccine development. *Vector Borne Zoonotic Dis.* **2**, 249-254.

VAN DAM, A.P., KUIPER, H., VOS, K., WIDJOJOKUSUMO, A., DE JONGH, B.M., SPANJAARD, L., RAMSELAAR, A.C., KRAMER, M.D., DANKERT, J. (1993). Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin Infect Dis*. **17**, 708-717.

VON STEDINGK, L.V., OLSSON, I., HANSON, H.S., ASBRINK, E., HOVMARK, A. (1995). Polymerase chain reaction for detection of *Borrelia burgdorferi* DNA in skin lesions of early and late Lyme borreliosis. *Eur J Clin Microbiol Infect Dis*. **14**, 1-5.

WANG, G. (2002). Direct detection methods for Lyme *Borrelia*, including the use of quantitative assays. *Vector Borne Zoonotic Dis.* **2**, 223-231.

WANG, G., VAN DAM, A.P., DANKERT, J. (1999a). Phenotypic and genetic characterization of a novel *Borrelia burgdorferi* sensu lato isolate from a patient with lyme borreliosis. *J Clin Microbiol.* **37**, 3025-3028.

WANG, G., VAN DAM, A.P., SCHWARTZ, I., DANKERT, J. (1999b). Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clin Microbiol Rev.* **12**, 633-653.