Improved serodiagnosis of erythema migrans using novel recombinant borrelial BBK32 antigens

Pekka Lahdenne,¹ Jaana Panelius,² Harri Saxen,¹ Tero Heikkilä,¹ Heidi Sillanpää,² Miikka Peltomaa,^{3,4} Maja Arnez,⁵ Hans-Iko Huppertz⁶ and Ilkka J. T. Seppälä^{2,7}

Correspondence

Pekka Lahdenne

pekka.lahdenne@hus.fi

¹Hospital for Children and Adolescents, University of Helsinki, Stenbäckinkatu 11, 00290 Helsinki, Finland

²Department of Bacteriology and Immunology, Haartman Institute, Haartmaninkatu 3, 00014 Helsinki, Finland

^{3,7}Department of Otolaryngology³ and Laboratory Diagnostics⁷, Helsinki University Central Hospital, 00290 Helsinki, Finland

⁴Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA

⁵Department of Infectious Diseases, University Medical Center Ljubljana, Slovenia

⁶Professor-Hess-Children's Hospital Bremen and Children's Hospital of the University of Würzburg, Germany

The performances of recombinant borrelial BBK32 proteins as antigens in the serology of erythema migrans (EM) were evaluated in an ELISA. Serum samples were obtained from 75 patients from different geographic areas where three borrelial species, *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii* or *Borrelia garinii*, cause Lyme borreliosis. Antibodies to variant BBK32 proteins were compared with anti-flagella or with anti-IR₆ peptide antibodies. In IgG ELISA at presentation of EM, 65/75 (87 %) patients had antibodies to one or more variants of BBK32, 29/75 (39 %) had antibodies to flagella and 29/75 (39 %) had antibodies to the VlsE IR₆ peptide antigen. The immunoreactivity against variant BBK32 proteins differed in patients from different geographic regions. The present results suggest that the BBK32 proteins used in combination or in parallel may improve the laboratory diagnosis of EM.

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INTRODUCTION

Lyme borreliosis (LB) is caused by spirochaetes belonging to the complex *Borrelia burgdorferi sensu lato*, and is characterized by multistage skin, joint, neurological and cardiac manifestations (Steere, 2001). The earliest and most common manifestation of LB is erythema migrans (EM), which appears at the site of the tick bite days to weeks after exposure. Tick bites may easily be unrecognized, and the clinician has to rely on the appearance of the skin lesion. In a routine clinical setting, EM is considered to be pathognomonic for early LB. The classical appearance of EM is an enlarging, ring-like erythema with a central clearing. However, early in the course of LB, atypical lesions may occur and cause diagnostic problems (Oksi *et al.*, 2001; Smith *et al.*, 2002). The gold standard for the diagnosis of early LB is microbiological confirmation of LB by culture from biopsies taken from EM

lesions and/or from blood. PCR-based methods can also be used to detect *B. burgdorferi* DNA in skin biopsies. However, in routine clinical practice, these methods are not feasible. Therefore, accurate detection of borrelial infection requires other laboratory tests with sufficient sensitivities and specificities.

The drawback of the current serologic tests during early LB is their low sensitivity. The sensitivity of the IgM or IgG ELISA using borrelial flagella or whole-cell lysate antigens seldom exceeds 40–50 % (Mitchell *et al.*, 1994; Agüero-Rosenfeld *et al.*, 1996; Nowakowski *et al.*, 2001; Vaz *et al.*, 2001). New recombinant borrelial proteins or synthetic peptides have been tested in LB serology with promising results (Liang *et al.*, 1999; Gomes-Solecki *et al.*, 2001). We recently evaluated recombinant BBK32 (rBBK32) protein antigens in serologic assays for LB (Heikkilä *et al.*, 2002). The rBBK32 protein appeared to be a useful antigen in early and late LB, especially in IgG serology, but not in IgM serology. However, our recent

Abbreviations: EM, erythema migrans; LB, Lyme borreliosis.

study suggested species-specific variations in the immune responses to the variant BBK32 proteins in early LB (Heikkilä *et al.*, 2002). The rBBK32 antigen originating from a local *B. afzelii* strain appeared to be superior to the other heterologous rBBK32 variants.

In the present study, we have evaluated the performances of three variant BBK32 antigens in the IgG serology of EM in four different geographic areas where three pathogenic borrelial species, *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii*, are the common aetiological agents of LB. Antibodies to variant rBBK32 proteins were compared with antibodies to commercial flagella antigens (Dako) and to a recently described peptide antigen, VlsE IR₆ (Liang *et al.*, 1999).

METHODS

Patients. Serum samples were collected from patients with clinically documented or culture- or PCR-confirmed EM from Finland, Germany, Slovenia and the USA. All 23 Finnish sera were from culture (*n* = 18) and/or from PCR (*n* = 21) proven patients taken at the time of diagnosis (acute) and 1–3 months after treatment (convalescent) (Heikkilä *et al.*, 2002). In the 23 patients with EM, genotyping by PCR analysis (Junttila *et al.*, 1999) showed *B. afzelii* in 17 and *B. garinii* in 4 of the skin biopsies. In two biopsies, genotyping was not successful. As part of a regional study on LB, German samples were collected in Northern Bavaria (Huppertz *et al.*, 1999). Sera were collected from 22 patients with physician-diagnosed EM at the time of diagnosis and during the convalescence phase (Huppertz *et al.*, 1999). The clinical diagnosis of EM was confirmed by especially trained physicians. In addition, sera were collected from 10 US patients with culture-positive EM at the time of diagnosis and during convalescence 3–4 weeks after treatment (Vaz *et al.*, 2001). Serum samples were also available from 20 Slovenian patients in the acute stage of EM. Two out of nine Slovenian patients from whom skin biopsies were taken were culture-positive (*B. burgdorferi sensu lato*) (Strle *et al.*, 1996). The diameter of EM lesions in the Finnish, German or US patients varied between 4 and 27 cm (median 10 cm), 5 and 23 cm (median 10 cm) or 6 and 21 cm (median 10 cm), respectively. In the 20 Slovenian patients, the size of the EM lesions was not available, but the duration of EM prior to the diagnosis was from 1 to 30 days (median 7 days). All patients with EM were treated with oral antimicrobials.

Serum samples from 40 Finnish healthy blood donors were used as negative controls and to define the cut-off value for all ELISAs (cut-off = mean plus 3 SD).

ELISA. *bbk32* genes were cloned and sequenced from Finnish isolates of borrelial species representing *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*, as described previously (Heikkilä *et al.*, 2002). The respective rBBK32 antigens were produced as glutathione-S-transferase–BBK32 fusion proteins, and used as separate antigens in ELISA. The IgG BBK32 ELISAs were performed as described earlier (Heikkilä *et al.*, 2002). Briefly, serum samples were diluted 1:100 in 5 mg BSA ml^{–1} in 0.155 M NaCl–0.04 % Tween 20 buffer (BSA–NaCl–Tween). Alkaline-phosphatase-conjugated rabbit anti-human IgG (Jackson Immuno Research Laboratories) 1:5000 in BSA–NaCl–Tween was used as the secondary antibody. The reactions were visualized with 4-nitrophenyl-phosphate (Boehringer Mannheim), 1 mg ml^{–1} in diethanolamine buffer, pH 10.0. The OD₄₀₅ measurements were made after 10–20 min using a Multiscan photometer (Thermo Labsystems). The specificity of the BBK32 ELISA has been previously analysed with serum samples from patients with syphilis (*n* = 10), systemic lupus erythematosus (*n* = 8), rheumatoid factor positivity (*n* = 8), anti-streptolysin positivity (*n* = 8), Epstein–Barr virus infection (*n* = 10) and from healthy blood donors (*n* = 20). The total specificity of the BBK32 ELISAs with these samples was 93 % (Heikkilä *et al.*, 2002).

ELISAs for anti-flagella antibodies were performed as described earlier (Seppälä *et al.*, 1994). Briefly, IgM or IgG antibodies against *B. burgdorferi* were measured with the commercial flagellin-based ELISA kit K0416 (Dako) modified by end point titration of the antibodies. An end point titre was obtained at an optical density level determined by the cut-off control provided with the kit. The cut-off control material conformed with the level of the mean + 3 SD of the control samples from healthy blood donors living in southern Finland, an area endemic for LB.

The synthetic peptide antigen VlsE IR₆ was produced in the Core Facility of Protein Chemistry at the Haartman Institute, University of Helsinki. The amino acid sequence of IR₆ corresponded to the recently published invariable region 6 of the VlsE gene (Liang *et al.*, 1999). For IgG ELISA with the IR₆ peptide, the plate wells (Greiner) were initially coated with 0.05 µg streptavidin (Boehringer Mannheim) in PBS overnight at +4 °C. After washing, 20 ng per well of the amino-terminally biotinylated peptide in PBS–0.04 % Tween was added. The subsequent steps were performed as described above.

RESULTS AND DISCUSSION

Antibodies at diagnosis

At presentation of EM, 65 of the 75 (87 %) patients had IgG antibodies to one or more variants of rBBK32, 29/75 (39 %) had antibodies to flagella, and 29/75 (39 %) had antibodies to the IR₆ peptide antigen (Table 1; Fig. 1). The majority of

Table 1. Number of positive sera (%) for any of the three BBK32 variant antigens (BBK32-all), IR₆ peptide antigen and for flagella antigen (Dako) in patients with EM from Finland (FIN), Germany (GER), the USA and Slovenia (SLO)

Antigen	At diagnosis					At convalescence			
	FIN <i>n</i> = 23	GER <i>n</i> = 22	USA <i>n</i> = 10	SLO <i>n</i> = 20	Total <i>n</i> = 75	FIN <i>n</i> = 23	GER <i>n</i> = 22	USA <i>n</i> = 10	Total <i>n</i> = 55
BBK32-all-IgG	17 (74)	21 (95)	10 (100)	17 (82)	65 (87)	15 (65)	15 (68)	10 (100)	40 (73)
IR6-IgG	7 (30)	7 (32)	4 (40)	11 (55)	29 (39)	6 (26)	6 (27)	7 (70)	19 (35)
Flagella-IgG	6 (26)	9 (41)	4 (40)	10 (50)	29 (39)	6 (26)	11 (50)	8 (80)	25 (45)
Flagella-IgM	3 (13)	8 (36)	2 (20)	9 (45)	22 (29)	4 (17)	7 (32)	8 (80)	19 (34)
Flagella-IgM or IgG	6 (26)	13 (59)	4 (40)	12 (60)	35 (46)	8 (35)	15 (68)	10 (100)	33 (60)

Fig. 1. ELISA OD/cut-off values of patients with EM from Finland (FIN), Germany (GER), the USA and Slovenia (SLO). Serum samples were taken at diagnosis (a) and after antibiotic treatment in convalescence (c). IgG antibodies to rBBK32 from *B. afzelii* (BBK32-afz), *B. garinii* (BBK32-gar), *B. burgdorferi sensu stricto* (BBK32-sensu stricto) and to IR₆ peptide were assessed. Control samples (CO) were from 40 healthy blood donors. The level of positivity for OD/cut-off values (>1) is indicated with a horizontal line.

patients from Finland had antibodies to rBBK32 from *B. afzelii*, whereas, in the German and Slovenian sera, the most sensitive antigen was rBBK32 from *B. garinii*. All 10 samples from the USA reacted positively with BBK32 from *B. burgdorferi sensu stricto* and from *B. garinii*. In total, 31 of the 75 (41 %) patients were positive for rBBK32 from *B. afzelii*, 52/75 (69 %) were positive for rBBK32 from *B. garinii*, and 30/75 (40 %) were positive for rBBK32 from *B. burgdorferi sensu stricto*. All the control samples were negative for rBBK32 variant antigens.

The proportion of patients with IgM anti-flagella antibodies at the time of diagnosis varied from 13 to 45 % in the different regions. Of the total of 75 patients, 29 % had IgM anti-flagella antibodies. IgM and/or IgG anti-flagella antibodies were detected in 46 % of the patients (Table 1).

Antibodies in the convalescent phase

Forty of the 55 patients (73 %) in the convalescent phase had IgG antibodies to one or more rBBK32s, 25/55 (45 %) had antibodies to flagella and 19/55 (35 %) had antibodies to the IR₆ peptide. The pattern of seropositivity to variant rBBK32s in the convalescent and the acute sera was similar. In the

convalescence samples from Finland and Germany, the overall rate of IgG antibody positivity to rBBK32s had slightly decreased from that at diagnosis (Table 1; Fig. 1).

Recent reports from the USA and Europe (Oksi *et al.*, 2001; Smith *et al.*, 2002) have shown that the diagnosis of EM solely on clinical grounds may be difficult. During the first few days of infection, the pathognomonic expanding peripheral erythema with a central clearing appears to be far less common than lesions with homogeneous redness (Oksi *et al.*, 2001; Smith *et al.*, 2002). This implies that EM may be confused with other similar rashes, e.g. insect bites, etc. In order to avoid over- and underdiagnosis, there should be laboratory methods that would support or confirm the clinical diagnosis. We have previously shown that sera from the majority of Finnish patients with EM were positive for the rBBK32 antigen (Heikkilä *et al.*, 2002). In the present study, we have expanded our studies to EM patients from different countries. The high rate of antibodies to one or more variants of rBBK32 suggests that these antigens might be used universally and already at presentation in the serology of EM.

The immunoreactivity against variant rBBK32 proteins diverged in the sera of EM patients from different countries. The species specificity of the antibodies corresponded well

with the known aetiology of Finnish (*B. afzelii*) and American (*B. burgdorferi sensu stricto*) EM. We have recently reported that the sequence similarity of BBK32 between *B. afzelii* and *B. garinii* or *B. burgdorferi sensu stricto* is approximately 70 %, whereas the BBK32 proteins of *B. garinii* and *B. burgdorferi sensu stricto* are 93–100 % identical (Heikkilä *et al.*, 2002). It is evident that the differences in the antibody levels of patients from different areas may be explained by sequence heterogeneity of the BBK32 proteins, especially that between *B. afzelii* and the other borrelial species. This implies that in regions where LB is caused by different species of *B. burgdorferi sensu lato*, variant BBK32 antigens are probably needed to cover all the EM cases.

At the time of diagnosis of EM, the duration of the disease correlates with the antibody positivity in the serum. Patients with a longer duration of EM appear to be more frequently positive for anti-*B. burgdorferi* antibodies (Aguero-Rosenfeld *et al.*, 1996). This was also demonstrated in our study with the flagella antigen: convalescent sera contained antibodies more often than sera taken at presentation of EM. However, in clinical practice, diagnostic and therapeutic options of EM have to be considered at presentation. The present study suggests that the sensitivity of the rBBK32 ELISA in EM may be superior to that of the routinely used flagella or the new peptide antigen VlsE IR₆.

The VlsE IR₆ peptide has recently been shown to be a highly sensitive and specific antigen in the serology of LB (Liang *et al.*, 1999). In disseminated LB, our results concur with the published data (unpublished). However, the present results indicate that in EM, IgG IR₆ ELISA may be less sensitive. In our series, the immunoreactivity to IR₆ did not differ significantly between patients with culture- or PCR-confirmed EM and clinically diagnosed EM. At presentation of EM, the overall seropositivity rate to IR₆ was 39 %. This result differs from recent data by Liang *et al.* (1999), who reported 74–87 % sensitivity in specimens from culture-confirmed EM patients. A possible explanation for this discrepancy is that the serum samples in the present study were collected earlier during the course of LB than in the study by Liang *et al.* (1999). It is evident that further analyses of IR₆ sequences and reactivity with human LB sera, in both IgM and IgG serology, in European conditions are also needed.

The BBK32 protein is a borrelial lipoprotein preferentially expressed *in vivo*. Expression of *bbk32* is detectable in spirochaetes during tick feeding even before transmission to the host (Fikrig *et al.*, 2000). Thus if the spirochaetes gain access to the skin of a human, there may be an early antibody response. Currently, it is not known whether *bbk32* expression is down-regulated later during the course of infection. If EM is treated early on with antibiotics, antibodies to BBK32 might be useful markers of disease activity or response to therapy of EM. This hypothesis is supported by our observation that the proportion of anti-BBK32 antibody-positive samples was slightly lower at convalescence than at the acute phase. However, this decrease was only minor.

Therefore, further research with a greater number of samples is needed to confirm this possibility. So far, none of the current or novel Lyme disease tests seem to be able to differentiate active infection from a previous immune response.

In conclusion, this study with sera from epidemiologically diverse regions provides supporting evidence that the BBK32 proteins may be useful antigens in EM serology (Heikkilä *et al.*, 2002). At presentation of EM, the sensitivity of the BBK32 ELISAs appeared better than the anti-flagella or the new anti-IR₆ tests. BBK32 variant antigens combined or in parallel may improve the accuracy of serology of EM. Ongoing studies are aimed at optimization of the antigen–antibody conditions in BBK32 ELISA in order to improve differentiation between samples from EM patients and from controls.

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