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Combined Infection of *Ixodes ricinus* with Three *Borrelia burgdorferi* sensu lato Genotypes

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ABSTRACT. *Ixodes ricinus* ticks were collected by random collections from western and central Slovakia during the years 1996–98. The midgut content of 240 ticks was examined by dark-field microscopy and submitted for cultivation for the presence of borrelias. Spirochetes were found in 21 unfed and host-seeking adults and nymphs (8.8 %). By the analysis of restriction fragment length polymorphism (RFLP) one sample from unfed *I. ricinus* male from western Slovakia was identified as a triple infection of *Borrelia burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. The simultaneous presence of different *B. burgdorferi* genospecies in one midgut sample (triple infection in the tick) could be observed only after the multipart amplification of denaturated DNA and subsequent pooling of the products for further analysis.

Lyme borreliosis (LB), the most prevalent tick-borne disease, has been presented in humans as a multisystemic disorder caused by the spirochete *Borrelia burgdorferi* sensu lato (s.l.). Clinical symptoms can vary from an acute skin rash (erythema migrans) to severe dermatological, arthritic, hematological, cardiac and neurological manifestations (Steere 1989). Genetic variation within *B. burgdorferi* s.l. has been shown to be responsible for some of the variable symptomatology of Lyme borreliosis (Assous *et al.* 1993; Wilske *et al.* 1993; Demaerschalck *et al*. 1995; Rijpkema *et al*. 1997; Ryffel *et al*. 1999; Baranton *et al*. 2001). On the basis of the previous findings of genetic and geographic variability in *Borrelia* genospecies and strains, it is possible to assume that the variable symptomatology of LB has been linked to the variation in *B. burgdorferi* strains (Guttman *et al.* 1996). In Europe, *B. burgdorferi* s.l. is diverse, including *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. lusitaniae* (Farlow *et al*. 2002). Spirochetes are transmitted to mammals primarily by ixodid ticks such as the *Ixodes ricinus* complex in Europe. Recently, it has been demonstrated by using PCR that DNA from more than one of the three *Borrelia* species associated with Lyme borreliosis in Europe can be present in the biological fluids of Lyme disease patients (Demaerschalck *et al.* 1997; Rijpkema *et al*. 1997). Ticks may be infected by more than one species (Guttman *et al.* 1996; Gern and Humair 2002). All double combinations of the species were observed but the association of three or more different genospecies in the infected ticks is more seldom (Guttman *et al.* 1996; Misonne *et al*. 1998).

The spirochete isolates from unfed or blood-seeking *I. ricinus* ticks were cultivated and characterized by PCR and restriction fragment length polymorphism (RFLP) analysis. In one of the isolate a triple infection of the *B. burgdorferi* s.l. complex was indicated but the standard amplification of denaturated DNA in PCR could recognize on a polyacrylamide gel only a weak pattern for *Borrelia* genospecies. We aimed to demonstrate that an infection with a few cells of *B. burgdorferi* s.l. can show a negative PCR (DNA not amplified or is in a low concentration), in spite of the presence of more than one genospecies in the host.

MATERIALS AND METHODS

Bacterial strains – control groups. Borrelia garinii strains K1, K10, K24 and K48 isolated from *I. ricinus*, and *Borrelia afzelii* strain MB20 isolated from *Apodemus* sp. were provided by Prof. E. Kmety (*Medical Faculty, Commenius University*, Bratislava). *Borrelia burgdorferi* s.s. strain MIL, isolated from *I. ricinus* in Slovakia, was provided by *Pasteur Institute* (Paris, France).

Collection of free-living ticks and culture. Unfed and host-seeking *I. ricinus* adults and nymphs were collected by flagging the vegetation and by direct collection from the host (humans, domestic animals) from the western and central Slovakia; 240 ticks were obtained by random collections in 1996–98. Ticks

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were maintained in tubes containing grass until use for isolation of spirochetes (Schwarzová and Čižnár 1996; Gern *et al.* 1999).

Cultivation. Ticks were briefly soaked in 70 % ethanol to reduce surface contamination, rinsed in sterile saline and the internal organs were cultivated in commercial BSK-H cultivation medium (*Sigma*) supplemented with 6 % rabbit serum.

Each midgut content was inoculated into two 1-mL cultivation Eppendorf tubes and cultivated for 4 weeks at 34 °C. After 1 week, the primary cultures were observed in a dark-field microscope (magnification 200×). After a 2-week incubation at 34 °C, each sample positive for the presence of spirochetal cells by dark-field microscopy (and without massive bacterial contamination) was placed into 10 mL of BSK-H medium and subcultured; subsequent isolates were stored at –70 °C. Inoculation dose for further cultivation was based on an estimation of the number of spirochetal cells in primary cultures (from 9 to >100 spirochetal microorganisms per μL of culture).

Electron microscopy. Spirochetal isolates (>100 microorganisms per μL of culture) were examined by electron microscopy according to Hovind-Hougen (1984). Ten to eleven-d-old cultures were harvested by centrifugation at 6000 ***g***, washed in phosphate-buffered saline (pH 7.2), fixed in 5 % glutaraldehyde, negatively stained with 3 % phosphotungstic acid and examined in an electron microscope (*Tesla Brno*, Czechia).

PCR and RFLP analysis. Spirochetes were identified according to Postic *et al.* (1994) using the unique structure of ribosomal genes in *B. burgdorferi* s.l. Primers in conserved regions of the *rrf* and *rrl* genes were used to amplify specifically the variable spacer between these two genes of *B. burgdorferi* s.l. Restriction analysis of PCR products by endonuclease *Mse*I led to different patterns specific for *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* which could be recognized on a polyacrylamide gel (Postic *et al*. 1994).

RESULTS

From 240 *I. ricinus* ticks (100 female, 50 male, 90 nymphs), unfed (180 individuals) or blood-seeking (60 individuals) 21 (8.8 %) ticks were positive for spirochetes by the cultivation procedure. The typical morphology of the spirochetal cells observed in electron-microscopic examination is shown in Fig. 1. The cells of the isolates were rather regularly waved and were covered by a surface layer in which a substructure was occasionally seen. Besides single isolated spirochetal cells we could observe colonic forms including blebs and gemmae associated with the cells.

Fig. 1. Typical spiral morphology of *Borrelia burgdorferi* s.l. in the electron microscope; negatively stained with 3 % phosphotungstic acid; ×4000.

Sample in which PCR analysis confirmed the presence of *B. burgdorferi* genospecies were further analyzed by RFLP. In strain ML1-96 PCR amplification of the denaturated DNA and subsequent RFLP analysis showed a weak pattern indicating to presence of bands characteristic for more than one genospecies. After comparison with the typical pattern of fragments from RFLP analysis of the three Lyme disease agents *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s. (Fig. 2; the distribution pattern shown corresponds to that described by Postic *et al*. 1994) we found that the presence of the DNA fragments was too low to be used for a species-specific characterization of ML1-96. This finding led us to use more concentrated PCR ML1-96 product.

This was achieved by using at least 9 tubes with PCR ML1-96 products that were pooled, concentrated and analyzed by RFLP. The pooling of 9 tubes provided sufficient amount of material for clearly separated bands in the electrophoresis. By this procedure the RFLP analysis revealed a triple-infection of *I. ricinus* with the three *B. burgdorferi* s.l. genotypes. RFLP fragments of all Slovak isolates including the triple-infected ML1-96 sample (the presence of all bands characteristic for three *B. burgdorferi* genospecies can be seen) is shown in Fig. 3.

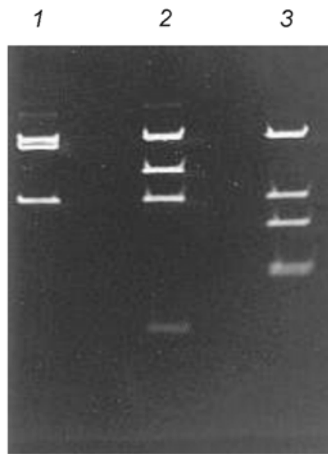

Fig. 2. Typical pattern of fragments from RFLP analysis using endonuclease *Mse*I; isolates of *Borrelia burgdorferi* s.l.; *1* – *B. garinii*, strain K48, *2* – *B. afzelii*, strain MB20, *3* – *B. burgdorferi* s.s., strain MIL.

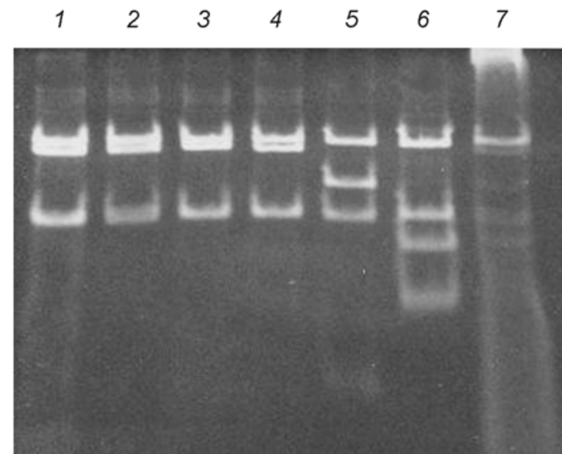

Fig. 3. RFLP fragments of all Slovak *Borrelia burgdorferi* s.l. isolates; *1–4* – *B. garinii*, strains K1 to K48, *5* – *B. afzelii*, strain MB20, *6* – *B. burgdorferi* s.s., strain MIL, *7* – *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, strain ML1-96.

DISCUSSION

The PCR based detection of *Borrelia* species in biological samples represents the most specific and sensitive method that is gaining a wide application in diagnostic and research laboratories. The dominating genospecies in our study were *B. afzelii*, less *B. garinii* and *B. burgdorferi* s.s. This rate corresponds to results of Gern *et al*. (1999) for western Slovakia and Hanincová *et al*. (2003). Slightly different rate was published by Tresová *et al*. (1997) for eastern Slovakia where *B. burgdorferi* s.s. was isolated in higher rate than the other two genospecies.

The geographic distribution of *B. burgdorferi* s.l. genospecies appears not to be uniform. In Western Europe, Belgium and Switzerland, *B. garinii* has been isolated more frequently, followed by *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana* (Rijpkema *et al.* 1997; Misonne *et al*. 1998; Saint Girons *et al.* 1998; Ryffel *et al.* 1999). In Slovenia, Scandinavia and the Netherlands, *B. afzelii* is probably the most common *Borrelia*, followed by *B. valaisiana*, *B. burgdorferi* s.s. and *B. garinii* (Ryffel *et al.* 1999; Ruzic-Sabljic *et al*. 2002). As the most prevalent genospecies in Ireland was described *B. valaisiana* followed by *B. garinii*, *B. burgdorferi* s.s. and *B. afzelii* (Gern *et al.* 1999). Whether this distribution is reflected in the profile of specific antibodies present in patients with LB has not yet been shown.

Several studies have demonstrated the presence of serum antibodies specific for *Borrelia* genospecies in patients with a clinical picture of neuroborreliosis even when the PCR test for *Borrelia* was negative (Demaerschalck *et al*. 1995). We demonstrated the presence of spirochetal microorganisms in *I. ricinus* (Schwarzová and Čižnár 1996). These spirochetes cultured *in vitro* showed morphological and immunochemical properties including cross-reacting antigens similar to *B. garinii*. However, the PCR for *Borrelia* was negative.

On the other hand, the diagnostic tests based on specific anti-borrelia antibodies are less reliable than PCR to confirm the infection with the mixture of *B. burgdorferi* genospecies. Due to different RFLP pattern of *B. burgdorferi* s.l., *B. garinii* and *B. afzelii* of PCR products it is possible to demonstrate the triple infection by these genospecies which is important with regard to the fact that mixed infections of *I. ricinus*

by *Borrelia* species and a high prevalence of triple infection in Lyme diseases was repeatedly described (Demaerschalck *et al*. 1995; Nakao and Miyamoto 1995; Rijpkema *et al*. 1996; Hubálek *et al*. 1997; Mateicka *et al*. 1997; Misonne *et al*. 1998).

After modification of the PCR procedure we were able to identify three *Borrelia* genospecies in the midgut of one *I. ricinus* tick collected in western Slovakia. The antibody response in the patient infected in such a case is usually very heterogeneous in terms of specificity (Stoitsova *et al.* 2003) and is also responsible for a lower confidence of serological tests.

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