

## Serologically Detected "New" Tick-borne Zoonoses in Eastern Croatia

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**Aim.** To establish serologically a contact with causative agents of human monocytic and granulocytic ehrlichiosis, human babesiosis, recently detected rickettsioses, and Lyme disease in individuals with a history of tick bite from three counties in eastern Croatia.

**Methods.** Seroepidemiologic testing was performed in 102 subjects with a history of tick bite, who either requested examination for a tick bite or were suspected of having tick-borne disease. The study was carried out during the 1998-1999 period in the area of the Vukovar-Srijem, Osijek-Baranya, and Brod-Posavina counties. Serum analysis was performed by indirect immunofluorescence assay for the detection of antibodies to causative agents of human monocytic and granulocytic ehrlichiosis (*Ehrlichia chaffeensis* and human granulocytic ehrlichiosis agent), human babesiosis (*Babesia divergens*), and rickettsiosis. Enzyme-linked immunosorbent assay was used for the detection of antibodies to the spirochete *Borrelia burgdorferi*, the cause of Lyme disease. The assays were performed at the Department of Microbiology, Osijek Public Health Institute in Osijek, and their results were confirmed at the Department of Microbiology and Immunology, School of Medicine in Ljubljana, Slovenia.

**Results.** *Ehrlichia chaffeensis* antibodies were detected in 5 sera, and antibodies to the causative agent of human granulocytic ehrlichiosis in 7 sera. A low titer of antibodies to the etiologic agent of babesiosis (*Babesia microti*) was detected only in a single serum. Eight sera that were positive for rickettsial antibodies contained rather high titers of antibodies against *Rickettsia conorii*, the agent of Mediterranean fever, and *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever. In six out of these 8 sera, antibodies to *Rickettsia typhi*, the cause of murine typhus, were detected possibly as a cross-reaction with some "newly detected" rickettsia circulating in this part of Europe, most likely *Rickettsia slovaca*. Positive titer of antibodies to *Borrelia burgdorferi* was detected in 15 sera.

**Conclusion.** The agents of human monocytic and granulocytic ehrlichiosis and of possibly newly detected rickettsiae were indirectly demonstrated to circulate in eastern parts of Croatia. The results obtained by IFA failed to provide definite evidence for the circulation of the human babesiosis agent, because the IFA used in our study detected *Babesia microti*, which prevails in the USA, but not *Babesia divergens*, which is the predominant cause of the disease in Europe. Serologic evidence for *Borrelia burgdorferi* infection was demonstrated in 80% of the subjects suspected of having the skin manifestation of Lyme disease.

**Key words:** babesiosis; Croatia; ehrlichiosis; fluorescent antibody technique, indirect; Lyme disease; tick; tick-borne diseases; rickettsia infections; zoonoses

Ticks are obligate hematophagous acarines that parasitize vertebrates, including humans, with an extremely widespread global distribution (1). Since the beginning of the 20th century, ticks have been known to transmit protozoal, viral, and bacterial diseases not only to animals, but also to humans. In addition to the recognized and well-described viral tick-borne encephalitis (tick-borne encephalitis), the identification of ticks serving as a vector for the spirochete *Borrelia burgdorferi* (the agent of Lyme disease) in 1982 pointed to the role of ticks in the occurrence of the disease in humans (1). Lyme disease is caused by the

bacterial species of the *Borrelia burgdorferi sensu lato* complex: *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia burgdorferi sensu stricto* (2,3).

Intensified monitoring and efforts invested in discovering new, to date unrecognized tick-borne diseases led to the identification of the causative agents of some important tick-borne diseases at the end of the 20th century. Two of these newly identified agents are *Ehrlichia (E.) chaffeensis*, the agent of human monocytic ehrlichiosis, and the pathogen causing human granulocytic ehrlichiosis, the bacterium antigenically and genetically closely related to the

veterinary pathogens *E. equi* and *E. phagocytophilla* (4). Ehrlichioses were previously known as diseases affecting domestic and wild animals. Human infection with *E. chaffeensis* was first recorded in 1986, and a similar disease caused by human granulocytic ehrlichiosis agent several years later (5). The genus *Ehrlichia* consists of obligate intracellular gram-negative bacteria characterized by high tropism for leukocytes. The genus belongs to the family *Rickettsiaceae* (6). In humans, both agents cause a disease with a mild flu-like clinical picture mostly accompanied by general symptoms, although a severe clinical picture and even lethal outcome may develop in immunocompromised patients, and only exceptionally in healthy individuals (7,8).

Recently, new rickettsiae have been detected, e.g., *R. mongolotimonae*, *R. helvetica*, *R. slovaca*, and the agent causing Astrakhan fever and some others (9). Rickettsioses have been known for a long time, the best known being Mediterranean spotted fever, also called Marseilles fever, caused by *R. conorii*; murine typhus caused by *R. typhi*; and Rocky Mountain spotted fever caused by *R. rickettsii*, occurring in South and North America (10).

Infections and diseases caused by protozoal agents of the genus *Babesia* have assumed epidemic proportions in North America, whereas in Europe they occur as sporadic cases (11). Babesiae are intracellular parasites that attack erythrocytes and cause a malaria-like disease in humans. The infection can be transmitted by a tick bite or via transfusion of infected blood and blood derivatives (12).

As the presence of these pathogens in reservoirs and vectors, along with cases of human affection, have been reported in the USA and some European countries with climate and geographic characteristics comparable to those in Croatia (5,8,12), we performed this study to assess their presence in our country. The study was based on the search for serologic evidence of the contact with newly recognized rickettsia, all potential etiologic agents of human illness, in individuals with a history of tick bite. The probability of such findings was supported by the fact that certain parts of eastern Slavonia represent an endemic area of known tick-borne disease, ie, tick-borne encephalitis (13,14). Another objective of the study was to confirm the hypothesis that eastern Slavonia is an endemic area of Lyme disease.

### Material and Methods

The study was conducted during the 1998-1999 period in the area of three eastern-Slavonian counties: Brod-Posavina, Osijek-Baranja, and Vukovar-Srijem. It included 102 subjects with a history of tick bite. Fifty-four subjects were symptom-free

but visited their family physician immediately upon a tick bite for a tick removal or advice on further intervention. The remaining 48 subjects were examined due to the symptoms of known tick-borne diseases. Twenty of these 48 patients underwent differential diagnostic procedure for cutaneous form of Lyme disease, whereas 28 were suspicious of having tick-borne encephalitis.

The subjects were informed about the purpose of the study, including the reason for taking their blood sample. Fifty-four symptom-free subjects were referred to the laboratory of biochemistry and hematology, where their blood samples were taken 3-4 weeks after their initial visit to a physician. Blood samples from 28 hospitalized patients were routinely collected at patient discharge from the hospital and referred to the laboratory for study purposes. In subjects examined for suspicion of Lyme disease, serum samples were routinely collected for differential diagnosis.

Upon routine treatment, blood samples (5 mL) were frozen and stored at -20 °C until analysis, which was performed at Department of Microbiology, Osijek Public Health Institute in Osijek in 2002. The commercially available indirect immunofluorescence assay (IFA; Focus Technologies Inc., Cypress, CA, USA) was used for the detection of antibodies against babesiae, rickettsiae, and agents of human monocytic and granulocytic ehrlichioses. VIDAL Lyme test (BioMerieux, Lyon, France) was used for the identification of causative agent of Lyme disease (15,16). Positive titers for the human monocytic ehrlichiosis IgG and IgM antibodies were 1:64 and 1:20, respectively; for the human granulocytic ehrlichiosis IgG antibodies 1:32; for the IgM and IgG antibodies against rickettsiae 1:64; and for the antibodies against babesiae 1:32. A questionnaire containing specific data was filled-out for each individual subject. The test results were confirmed at the Department of Immunology and Microbiology, School of Medicine from Ljubljana, Slovenia.

### Results

Out of 102 study subjects, 75 (73.5%) lived in the rural and 27 (26.5%) in urban setting. The mean age of the subjects was 41.7±19.0 (range, 5-76) years, and two-thirds were men (n=67).

Antibodies to the agent of human granulocytic ehrlichiosis were detected in 7 sera, 4 of these from subjects free from any clinical signs of the disease, and 3 from patients hospitalized with suspected tick-borne encephalitis (Table 1). A similar distribution of antibodies was recorded for *E. chaffeensis*, the etiologic agent of human monocytic ehrlichiosis. There were 5 positive sera: 2 from patients with tick-borne encephalitis and 3 from symptom-free subjects. Antibodies to both the agent of granulocytic and of monocytic ehrlichiosis, were detected in 3 sera. A low titer of antibodies to *Babesia microti*, which causes babesiosis, was detected in the serum sample from a patient with tick-borne encephalitis. Antibody titers to rickettsiae were found in 8 sera, 5 of them from patients with tick-borne encephalitis and 3 from asymptomatic subjects. These sera contained high titers (1:64 to more than 1:256) of antibodies to *Rickettsia conorii*, the causative agent of Mediterranean fever, and *Rickettsia rickettsii*, the causative agent of

**Table 1.** Results of serologic analysis for the presence of "newly detected" tick borne diseases in 102 subjects with a history of tick bite from three eastern Slavonia counties, 1998-1999

Clinical diagnosis	Sera positive for "newly detected" agents				
	HGE* agent	<i>Ehrlichia chaffeensis</i>	<i>Babesia microti</i>	<i>Rickettsia spp.</i>	<i>Borrelia burgdorferi</i>
Tick-borne meningoencephalitis (n = 28)	3	2	1	5	0
Lyme disease (n = 20)	0	0	0	0	15
Symptom-free subjects with a tick bite history (n = 54)	4	3	0	3	0
Total (n = 102)	7	5	1	8	15

\*HGE – human granulocytic ehrlichiosis.

Rocky Mountain spotted fever. Equally high titers of antibodies to *Rickettsia typhi*, the agent of murine typhus, were detected in 6 of these 8 sera, probably as a cross-reaction with some newly detected rickettsia circulating in this part of Europe, most likely *Rickettsia slovaca*. Thus, 15 out of 20 subjects suspected of Lyme disease had positive titers of antibodies to *B. burgdorferi* (Table 1).

### Discussion

Two human tick-borne diseases have been known in Croatia: tick-borne encephalitis and Lyme disease. Tick-borne encephalitis is a viral disease of a biphasic course; it was recorded in Croatia for the first time in 1953. According to definition, the disease occurs exclusively in the known natural foci in the hilly and deciduous forests of the northern and northwestern parts of Croatia, with the incidence of 0.4 to 3.5 per 100,000 (20 to 152 cases per year) and lethality below 1% (13,14).

Lyme disease was first recorded in Croatia in 1984, although some clinical entities (erythema migrans) of the disease have been occurring for decades (3,14). The disease is caused by a spirochete of the *Borrelia burgdorferi sensu lato* complex and, like tick-borne encephalitis, is transmitted by the *Ixodes ricinus* tick species. Every year, some 300 cases are recorded, mostly as a mild cutaneous form that largely contributes to the highest incidence of 7.4/100,000 population (3,14). The disease has not been fully investigated yet, but the spread of the disease in Croatia has been considered to coincide with the occurrence of tick-borne encephalitis (13,14) and increased number of vertebrate hosts of the tick – murine rodents and many other wild and domestic animals, such as deer, squirrel, and even dog. There are still many unknown factors related to the location and dissemination pattern of Lyme disease in Croatia. The areas of eastern Slavonia included in our study have been considered endemic for both tick-borne encephalitis and Lyme disease. We found antibodies to the etiologic agents of tick-borne encephalitis in 28 subjects, and verified the presence of etiologic agents of Lyme disease in 80% of suspected cases, thus confirming the known foci of the disease in eastern Slavonia.

The other hypothesis on the probable circulation of the “newly detected” tick-borne diseases in Croatia, based on the reported occurrence of these diseases in climatically and geographically comparable regions all over the world (5,8,12,17), also proved correct.

The assays we used in the study were manufactured in the USA, and found inappropriate for detection of the agents causing babesiosis in Europe. For example, the test for detection of *B. microti* cannot detect *B. divergens*, which is the predominant cause of the disease in Europe. Nevertheless, the circulation of other “newly detected” tick-borne diseases in eastern Slavonia was definitely demonstrated.

An intriguing finding was evidence of infection with multiple “newly detected” agents of tick-borne diseases, which is consistent with some literature data

on ehrlichioses (18,19). We found evidence of infection with the agents of granulocytic and monocytic ehrlichiosis in three sera. To our knowledge, evidence of infection with two pathogens, one causing “known” and the other “newly detected” tick-borne disease, has not been described before. Serological evidence of infection with the agent of human babesiosis was detected in the serum of one of our patients with tick-borne encephalitis. However, evidence of infection with three agents, which is a rather rare finding, seems even more intriguing: one of our patients diagnosed with tick-borne encephalitis on the basis of clinical picture showed concurrent infection with the agents of granulocytic and monocytic ehrlichiosis. If tick-borne encephalitis in this patient had not been verified serologically, we would have speculated that ehrlichiosis was the underlying disease, especially since the patient’s medical documentation was rather incomplete.

The agents causing “newly detected” tick-borne diseases have obviously been circulating in eastern parts of Croatia. To establish their distribution in other regions of Croatia, additional investigation should be undertaken including larger samples of patients, potential reservoirs, and vectors. Future studies should use high-quality tests, especially genetic methods of differentiation, to clarify with certainty the issue of “newly detected” rickettsiae, which has remained unsolved by the present study.

### Acknowledgment

We thank Prof Tatjana Županc-Avšič from the Department of Immunology and Microbiology, School of Medicine, Ljubljana, Slovenia, for her generous assistance with confirmation of our test results.

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Received: July 23, 2003

Accepted: September 5, 2003

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