What Should Be Known about the Activity of the Reference Center of the Ministry of Health and Social Welfare of the Republic of Croatia for Diagnosis of Lyme Disease

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SUMMARY Lyme borreliosis is very important for dermatovenereologists because of its skin manifestations that include erythema chronicum migrans, lymphadenosis benigna cutis, and acrodermatitis chronica atrophicans. In Croatia, the spirochete *Borrelia burgodorferi* was first isolated in 1991 at University Department of Dermatology and Venereology, Zagreb University Hospital Center. In October 2003, the Laboratory of Clinical Immunology, Immunofluorescence and Serodiagnosis was appointed Reference Center of the Ministry of Health and Social Welfare of the Republic of Croatia for the Diagnosis of Syphilis and Lyme Disease. Results of immunofluorescence diagnosis of Lyme disease are reported and areas in Croatia endemic for Lyme borreliosis are presented.

KEY WORDS: Lyme borreliosis; erythema chronicum migrans; *Borrelia burgdorferi*; immunofluorescence diagnosis

Historical Background

Lyme disease (LD) is a tick-borne infection caused by the spirochete *Borrelia (B.) burgdorferi sensu lato.* LD originated from the USA and therefore the isolated borreliae most probably belong to the *B. burgdorferi sensu stricto* strain. Recent findings have revealed that specific genotypic subtypes of *B. burgdorferi sensu stricto* have different propensities for hematogenous dissemination in early LD, suggesting that these differences may influence the pathogenesis of the disease. The pathogenic mechanism of LD, particularly of the pathognomonic clinical sign of LD, is erythema migrans (EM). In several days to weeks or even months, *B. burgdorferi* may disseminate hematogenously or lymphogenously from skin to various organs leading to clinical manifestations of LD of the early disseminated and/or late infection (2). LD presents as early localized Lyme borreliosis (LB), early disseminated LB, and late chronic LB. Late-stage disease occurs rather frequently in patients who have received treatment at an early stage of LB (localized or disseminated) (1). Many factors seem to influence the presence of spirochete antigens in infected persons and the introduction of immune effects (e.g., increased number of antigens in complexes, surviving of spirochetes at the immunologically privileged sites, and maintaining of immunologic antigens). Determination of soluble (s) CD_4, CD_8 and CD_{25} antigens in patient sera may be useful in the evaluation of the state of immune activation, and might contribute to better assessment of the antibiotic treatment efficacy (1). Increased initial concentrations of sCD_8 and sCD_{25} indicate the activation of CD_8 and CD_{25} lymphocytes and their role in the immunopathogenesis of LD. Elevated levels of CD_8, before and after treatment suggest a significant involvement of CD_8 lymphocytes, indicating the role of long-lasting cellular response in early disseminated LB.

In Europe, *B. burgdorferi sensu lato* determined in the blood of patients with typical EM was low; the predominantly isolated strain was *B. afzelii* (2). Slovenia is an endemic country for LB with a vast majority of LB patients registered with EM and predominance of *B. afzelii* isolated from blood. In the majority of EM patients, spirochetemia was clinically silent, had no adverse prognostic significance upon therapeutic approach, and proceeded without clinical signs of disseminated borrelia infection (2). The course and outcome of EM patients was assessed according to clinical evaluation, laboratory serology, cultivation of *Borrelia* from skin specimens, and cultivation of *Borrelia* from blood. Literature search has revealed the frequency of isolation of spirochetes from the blood of patients with EM to be low, ranging from 2.5% to 5.5% in the majority of reports; however, in some reports it was as high as 21% (3-8). Clinical-epidemiologic studies support the view that there are clinically relevant differences regarding tissue tropism and perhaps virulence between *B. burgdorferi sensu stricto* and other *Borrelia* species such as *B. afzelii* and *B. garinii* (9). The cutaneous manifestation of infection with *Borrelia* species, EM, may be associated with systemic signs of the disease such as fever and malaise. Several studies have shown that systemic dissemination of spirochetes occurs during this phase of infection (1.2% in ref. 2); 25% of cases in Wormser *et al.* (10). Patients with disseminated LD have persistently elevated levels of (s) CD_8 and CD_{25} receptors, which indicate the presence of persistently activated CD_8 lymphocytes (1). Components of the cellular response such as T lymphocytes react to distinct spirochete proteins such as outer surface lipoprotein Osp A (9). Likewise, we need to learn more about the characteristics of the pathogen which is capable to survive in compartments for a prolonged period of time. Research on improved methods to diagnose and treat later stages of LD is needed.

Table 1. Frequency of early manifestations of Lyme disease at Dermatology and Venereology Health Services in central, north and east Croatia (1985-1994)

Dermatologic Service	ECM	Tick bites
Koprivnica	131	25
Karlovac	171	66
Čakovec (1991-1994)*	98	-
Slavonski Brod (1993-1994)	13	6
Ogulin	112	43
Sisak	101	74
Osijek	216	-
Total	848	214

*Data obtained from the Polyclinic for Infectious Diseases (1991-1994) and Dermatovenereology Service

Croatian Experience in Lyme Disease Research

In Croatia, the spirochete *B. burgdorferi* was first isolated in 1991 at University Department of Dermatology and Venereology, Zagreb University Hospital Center, from the skin of a patient with erythema chronicum migrans (ECM), and was designated as P1 Zagreb. As an early skin manifestation of LB, it was first described by Forenbacher in 1940, followed by Mohar in 1982, Maretić *et al.* in 1989, Curl in 1991, Kansky in 1992, and Bolanča-Bumber in 1997 (11). In 1998, Šitum reported on clinical and laboratory analysis performed in a group of 148 selected subjects (20 from a risk population of forestry workers from a nonendemic area, 82 from a risk population of forestry workers from an LB endemic area, and 46 LB affected subjects) (11,12). LB is a multisystem disease caused by the spirochete *B. burgdorferi*. Small rodents, hedgehog, wood mouse, field mouse and doormouse, birds, deer and vole are natural *B. burgdorferi* reservoirs, whereas domestic animals (cattle, sheep, horse, dog, etc.) play an important role in the spread of the disease in inhabited areas (11,12).

From *B. burgdorferi* designated as P1 Zagreb, electrophoretic analysis of *B. burgdorferi* protein content revealed six major proteins of different molecular mass (OspA, OspB, OspC, p41, p60 and p100) (11).

In northwest Croatia, an endemic area for Lyme borreliosis, four genomic *B. burgdorferi sensu lato* groups were identified in the *Ixodes ricinus* ticks: *B. afzelii, B. garnii, B. valaisiana (*group VSS116), and *B. burgdorferi sensu stricto* (11).

Table 2. Frequency of Lyme borreliosis infection during the 1992-1998 period

Year	Patients
1992	93
1993	315
1994	255
1995	268
1996	335
1997	229
1998	248

Population of Croatia: 4,501,149

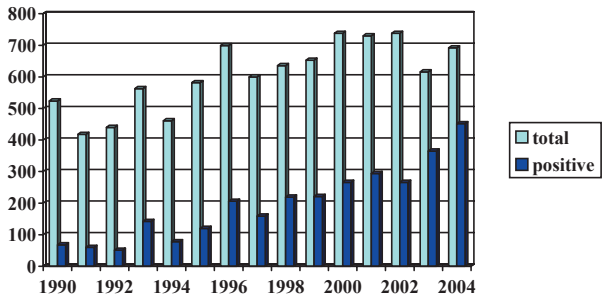

Figure 1. Indirect immunofluorescence test results in 9090 patient sera during 15 years at Laboratory of Lyme Disease Serodiagnosis

In her study Šitum included specific endemic areas for Lyme disease in Croatia and the results confirmed *B. afzelii* to be the main causative agent of ECM in the study group from the area (12).

As no similar studies have been conducted in other parts of Croatia, there is a dilemma whether *B. afzelii* is the causative agent of ECM, the most common manifestation of Lyme disease, in other parts of Croatia. There is the need of investigating other clinical types of Lyme disease such as Lyme arthritis and neuroborreliosis in order to determine whether other *B. burgdorferi* genospecies are also included in the etiology of extracutaneous types of Lyme disease (11).

Over the last 14 years, LD has grown into a public health problem, especially in the USA and Central Europe as well as in Croatia. Besides the skin, joints, heart and central nervous system can be involved. LD is very important for dermatologists for the three diseases described: ECM, lymphadenosis benigna cutis (LBC) and acrodermatitis chronica atrophicans (ACA).

From 1988 till 1990, 907 cases of ECM were recorded in central and north Croatia, the areas considered endemic for LB. In 1995, 216 cases of ECM were recorded in Osijek (east Croatia) (Table 1) (13). Data on LB prevalence in Croatia during the 1992-1998 period are shown in Table 2 (14).

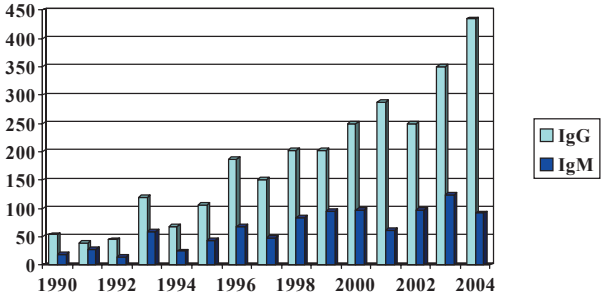

Figure 2. Distribution of IgG and IgM *Borrelia burgdorferi* antibodies in 2967 positive sera during 15 years at Laboratory of Lyme Disease Serodiagnosis

Table 3. Current results of indirect immunofluorescence (IIF) testing for *Borrelia burgdorferi* at Reference Center for Lyme Disease of the Ministry of Health and Welfare of the Republic of Croatia

IIF for Borrelia burgdorferi			
		2003	2004
Total		616	692
Negative		251	240
Positive		365	452
Positive			
IgM	IgG titer		
	IgM 1:64	64	45
	1:128	32	33
	1:256	12	10
	1:512	16	4
	IgG 1:64	47	56
	1:128	96	136
	1:256	110	143
	1:512	97	99

Immunofluorescence Diagnosis of *B. burgdorferi* Antibodies

At University Department of Dermatology and Venereology, Zagreb University Hospital Center, the Laboratory of Serodiagnosis and Immunofluorescence Diagnosis has now for years been performing immunofluorescence (IF) diagnosis of *B. burgdoferi*, with special reference to IgG and IgM titer in positive cases. Positive IF *B. burgdoferi* findings prevailed with IgG antibodies have been progressively seen since 1996. Results recorded during the 1990-2004 period are shown in Figures 1 and 2. Since the appointment of the Reference Center for the Diagnosis of Lyme Disease of the Ministry of Health and Welfare of the Republic of Croatia in 2003, the diagnosis, prevention and treatment of LD have become the most important tasks of the Center. Table 3 presents positive IF findings recorded during the last two years. IgG and IgM positive findings were similar during the last two years because target sera were referred to the Laboratory, verifying the diagnosis of LB infection.

In Croatia, the geographical and climate characteristics are favorable for the life cycle and spread of the *Ixodes* ticks (i.e. woodlands with humid climate). The central, north and east parts of Croatia are endemic areas for LB.

In Croatia, diagnostic workup for LD is performed by dermatovenereologists and infectologists. At University Department of Dermatology and Venereology, Zagreb University School of Medicine, laboratory diagnosis of antibodies to *B. burgdoferi* using indirect immunofluorescence has been done since 1988, and *B. burgdoferi* cultivation since 1991, whereas ELISA testing is performed at Dr. Fran Mihaljević University Hospital for Infectious Diseases in Zagreb, with close collaboration between the two institutions.

The Reference Center of the Ministry of Health and Welfare of the Republic of Croatia for the Diagnosis of Lyme Disease has an important role in accurate diagnosis of LB and treatment of ECM as well as in the prevention of possible late LB manifestation and proper education of general practitioners. Tight collaboration with dermatovenereologists all over Croatia is necessary to provide appropriate care and prevention, especially in the endemic areas.

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