POLYMERASE CHAIN REACTION (PCR) FOR DETECTION OF *BORRELIA BURGDORFERI* SENSU LATO

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ABSTRACT

POLYMERASE CHAIN REACTION (PCR) FOR DETECTION OF *BORRELIA BURGDORFERI* SENSU LATO

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The present study aimed detection of a human pathogen *B. bugdorferi* sensu lato species in suspected Lyme borreliosis (LB) patients in Turkey by PCR analysis and supportive serologic tests. The 152 clinical samples (140 serum and blood, 10 cerebrospinal fluid (CSF), 1 synovial fluid, 1 skin biopsy specimens) from 140 patients sent from 22 different cities of Turkey to The Spirochetal Diseases Diagnosis Laboratory of Central Veterinary Control and Research Institute were analysed.

Serum samples were subjected to ELISA with a commercial kit and all of the blood, CSF, synovial fluid and skin biopsy samples were examined by PCR. In PCR analysis two primer sets targeting the ospA gene located on the plasmid and ribosomal 23S rRNA gene of *B. burgdorferi* sensu lato were used. The results indicated that 32,1% (45 of 140) seropositivity was detectable by ELISA. Our results support that there is a risk of acquiring LB in different regions of Turkey. Although considerable positive detections were recorded using serologic tests,

none of the specimens were positive in PCR analysis. Further studies on PCR based methods for detection of *B. burgdorferi* sensu lato in patients with a high clinical probability of LB apparently may require that the specimen should be taken in the early phases and before the administration of any therapeutic agent.

Keywords: Lyme borreliosis, *Borrelia burgdorferi* sensu lato, PCR, ELISA.

ÖZ

BORRELİA BURGDORFERİ SENSU LATO'NUN POLİMERAZ ZİNCİR REAKSİYONU (PZR) İLE TEŞHİSİ

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Bu çalışmada Türkiye'de Lyme borreliozis (LB) şüpheli hastalarda insanlar için patojen olan *Borrelia burgdorferi* sensu lato türlerinin Polimeraz Zincir Reaksiyonu (PZR) ve destekleyici serolojik testler ile teşhisi amaçlanmıştır. Türkiye'deki 22 farklı ildeki 140 hastadan Merkez Veteriner Kontrol ve Araştırma Enstitüsü Spiroket Hastalıkları Teşhis Laboratuarına gönderilen 152 klinik örnek (140 serum ve kan, 10 beyin omurilik sıvısı (BOS), 1 eklem sıvısı, 1 deri biyopsisi) analiz edilmiştir.

Serum örnekleri ticari ELISA kiti ile çalışılmış ve tüm kan, BOS, eklem sıvısı ve deri biyopsisi örnekleri PZR ile incelenmiştir. PZR analizinde *Borrelia burgdorferi* sensu lato'nun plasmidinde bulunan ospA geni ve ribosomal 23S rRNA genini hedefleyen iki primer seti kullanılmıştır. Sonuçlar %32,1 (45/140) seropozitivitenin ELISA ile tespit edilebilir olduğunu göstermiştir. Sonuçlarımız Türkiye'nin değişik bölgelerinde Lyme borreliozis'e yakalanma riski olduğunu desteklemektedir. Serolojik testlerle yüksek oranda pozitiflik tespit edilmesine

rağmen PZR analizinde örneklerin hiçbirinde pozitiflik bulunmamıştır. Bundan sonra klinik olarak Lyme borreliozis olma olasılığı yüksek hastalarda *Borrelia burgdorferi* sensu latonun teşhisi için yapılacak olan PZR tabanlı çalışmalar için, klinik örneklerin hastalığın erken safhasında ve herhangi bir ilaç tedavisi uygulanmadan alınması uygun olacaktır.

Anahtar Kelimeler: Lyme borreliozis, *Borrelia burgdorferi* sensu lato, PZR, ELISA.

To my family...

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LIST OF ABBREVIATIONS

LB	Lyme Borreliosis
Osp	Outer Surface Protein
EM	Erythema Migrans
ACA	Acrodermatitis chronica atrophicans
IFA	Indirect Immunofluorescent Antibody Assays
EIA	Enzyme immunoassays
ELISA	Enzyme-Linked Immunosorbent Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
CSF	Cerebro-Spinal Fluid
BOS	Beyin-Omurilik Sıvısı
WIB	Western Immunoblot
PCR	Polymerase Chain Reaction
PZR	Polimeraz Zincir Reaksiyonu
qPCR	quantitative PCR assays

CHAPTER 1

INTRODUCTION

1.1. HISTORY

Lyme disease (Lyme borreliosis) (LB) is the most common tick-borne infection in both Europe and the United States (Schnarr *et al.*, 2006; Steere, 2001).

It was described as a new clinical entity in 1977 because of a geographic clustering of children with rheumatoid-like arthritis in Lyme Connecticut (Wang *et al*., 1999; Steere *et al*., 1978; Steere *et al*., 1977; Steere *et al*., 1977). Important cutaneous and neurological manifestations of LB were described in the late nineteenth century (Schnarr *et al*., 2006; Weber, 2001).

In Europe, where symptoms characteristic of the disease were first described by Afzelius (1921) in a patient in Sweden, this illness was originally known as Erythema Chronicum Migrans (ECM) (referring to the characteristic annular rash) (Garfield, 1989), but is now known as Lyme Disease or Lyme borreliosis. The term "Lyme disease" indicates the clinical condition, with its characteristic syndrome, whereas the term "Lyme borreliosis" indicates infection with the pathogenic agent regardless of whether or not symptoms are expressed (Sonenshine, 1993).

In 1982, the bacterium that causes LB was first isolated by Willy Burgdorfer and colleagues from the hard tick *Ixodes dammini* (now *Ixodes scapularis*) collected in Long Island, New York (Burdorfer *et al*., 1982; Wang *et al*., 1999). The isolate was subsequently identified as a new species of the genus *Borrelia* and was named *Borrelia burgdorferi* in 1984 (Johnson, 1984; Wang *et al*., 1999). Since then, hundreds of *B. burgdorferi* isolates have been cultured worldwide

from various geographic regions and biological sources, including *Ixodes* ticks, their reservoir hosts, and specimens from patients with different clinical syndromes. Molecular analysis of the agent indicated that these *B. burgdorferi* isolates are genetically and phenotypically divergent. The term "*B. burgdorferi* sensu lato" is now collectively used to refer to all *Borrelia* isolates within this cluster and to distinguish it from the species "*B. burgdorferi* sensu stricto" (strict sense of *B. burgdorferi*) (Baranton *et al*., 1992; Wang *et al*., 1999).

1.2. CAUSATIVE ORGANISM

The causative agent of LB is the Gram-negative, microaerophilic spirochete, *Borrelia burgdorferi*, a member of Spirochaetaceae family (Sonenshine, 1993). *B. burgdorferi* spirochetes have the same helical shape characteristic of all spirochetes, but are unusually long. The cells, configured with 3 to 10 loose coils, are 10 to 30 µm in length and 0.2 to 0.5 µm in width. Spirochetes found in tick tissues are considerably shorter in length (Barbour and Hayes, 1986; Sonenshine, 1993; Wang *et al.*, 1999; Aguero-Rosenfeld *et al.*, 2005). The bacteria have 7 to 11 periplasmic flagella (Aguero-Rosenfeld *et al*., 2005).

Living organisms can be visualized by dark-field or phase-contrast microscopy. They can also be recognized by light microscopy after silver staining or by fluorescent microscopy methods (Aguero-Rosenfeld *et al*., 2005).

B. burgdorferi has about 30 major proteins in its body; two of these are outer surface membrane proteins (Osp) A and B, 31 and 34 kDa respectively, which are antigenic and in turn useful in differentiating these organisms from other spirochetal species (Sonenshine, 1993).

The genomic sequence of the *B.burgdorferi* B31 chromosome was determined in 1997 by Fraser *et al*. The genome size of the type strain *B. burgdorferi* sensu stricto B31 is 1,521,419 bp. This genome consists of a linear chromosome of 910,725 bp, with a G-C content of 28.6%, and 21 plasmids (9 circular and 12

linear) which have a combined size of 610,694 bp (Fraser *et al*., 1997; Aguero-Rosenfeld *et al*., 2005). Comparative analysis of the genome of the recently sequenced *Borrelia garinii* strain PBi with that of *B. burgdorferi* B31 reveals that most of the chromosome is conserved (92.7% identity with regard to both DNA and amino acids) in the two species. The chromosome and two linear plasmids (lp54 and cp26), which carry approximately 860 genes, seem to belong to the basic genome inventory of the Lyme *Borrelia* species (Glöckner *et al*., 2004). Not all strains of *B. burgdorferi* have the complete complement of plasmids, and thus the cumulative genome size may vary among different *B. burgdorferi* isolates (Aguero-Rosenfeld *et al*., 2005).

Genome analysis has revealed that *B. burgdorferi* possesses certain genetic structures that are uncommon among prokaryotes (Fraser *et al*., 1997; Aguero-Rosenfeld *et al*., 2005). A small number of protein with bioactivity have been found, therefore, the organism depends on the host for most of its nutritional requirements. In contrast to other bacteria, *B. burgdorferi* has no toxins, no lipopolysaccharides, and no requirement for iron. This may allow the spirochete to circumvent the usual host defense of limiting the availability of iron (Steere *et al*., 2004; Schnarr *et al*., 2006). Therefore, *B. burgdorferi* is an elusive organism that uses both tick and mammalian host factors for survival. The most remarkable aspect of the huge borrelial genome is the abundance of lipoproteins (lp > 150). Moreover, the genome is unusual in that more than 40% is represented on linear and circular plasmids. This allows the organism to rapidly adapt in response to different environments and to evade the immune response of the host by antigenic variability (Schnarr *et al*., 2006).

LB is a globally distributed tick-borne zoonosis. Human cases occur predominantly in the northern hemisphere (Wang *et al*., 1999).

Eleven *Borrelia* species within the *B. burgdorferi* sensu lato complex have been described worldwide. Of these, three species (*B. burgdorferi* sensu stricto,

Borrelia andersonii, and *Borrelia bissettii*) have been identified in North America, six species (*B. burgdorferi* sensu stricto, *B. garinii*, *Borrelia afzelii*, *Borrelia valaisiana*, *Borrelia lusitaniae, Borrelia bissettii* sp. nov) have been documented in Europe, and seven species (*B. garinii*, *B. afzelii*, *B. valaisiana*, *Borrelia japonica*, *Borrelia tanukii*, *Borrelia turdi*, and *Borrelia sinica*) have been identified in Asian countries (e.g., China, Japan, or Korea) (Wang *et al*., 1999; Aguero-Rosenfeld *et al*., 2005). *B. garinii* and *B. afzelii* are the most frequently cultured species in Europe (Wang *et al*., 1999).

Recently *Borrelia burgdorferi* sensu lato strains were isolated and characterized from *Ixodes ricinus* ticks in Turkey (Güner *et al*., 2003). In that study *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* (Eurasian type), *Borrelia afzelii*, *Borrelia lusitaniae* and *Borrelia valaisiana* have been identified. However, no Asian-type *B. garinii* has been found. These results provide first evidence for the existence of the LB agent in Turkey (Güner *et al*., 2003).

LB cases in the southern hemisphere including South America (Azulay *et al*., 1991; Wang *et al*., 1999), Africa (Schafrank *et al*., 1990; Mhalu and Matre, 1996; Wang *et al*., 1999), and Australia (McCrossin, 1986; Wang *et al*., 1999) have been reported. However, these cases were based only on serological studies. *B. burgdorferi* sensu lato has not been isolated from local *Ixodes* ticks or any other suspected vectors or patients (Burgdorfer *et al*., 1991; Russell, 1995; Wang *et al*., 1999). Recently, *B. garinii* was isolated from a patient with LB in Australia; however, the infection may have been acquired in Europe (Hudson *et al*., 1998; Wang *et al*., 1999).

At least three strains of *Borrelia* species are pathogenic in humans: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. In the USA, only *B. burgdorferi* sensu stricto is encountered, whereas all three species can be found in Europe. In humans the different genospecies tend to cause distinct manifestations affecting different systems: *B. burgdorferi* sensu stricto tends to be present in joint fluid and cause arthritis, *B. afzelii* is present in skin and

induces cutaneous manifestations and *B. garinii* is present in cerebrospinal fluid and is responsible for neurologic troubles (Humair and Gern, 2000). This organotropism may explain the different clinical pictures of Lyme disease in the USA as compared with Europe (Wang *et al*., 1999; Steere, 2001; O'Connell, 2005; Schnarr *et al*., 2006). *B. valaisiana*, another *Borrelia burgdorferi* sensu lato species, is suspected to be pathogenic for humans as it has been isolated from skin and cerebrospinal fluid in a single case in Europe (Rijpkema *et al*., 1996; Diza *et al*., 2004; Schnarr *et al*., 2006). A novel species, designated A14S, isolated from patients with erythema migrans in Southern Germany (Wang *et al*., 1999; Schnarr *et al*., 2006). Further analyses are necessary to assess the relevance of these new species (Schnarr *et al*., 2006).

1.3. TICK VECTOR

Lyme disease spirochete, *Borrelia burgdorferi*, is transmitted to human hosts via the hard-shelled ticks. The epidemiologically most important ticks in transmitting *B. burgdorferi* sensu lato to humans are *Ixodes persulcatus* (northern mid Asia), *Ixodes scapularis* (eastern North America), *Ixodes pacificus* (western North America) and *Ixodes ricinus* (Europe and some adjacent areas) (Steere *et al*., 1977; Burgdorfer *et al*., 1982; Hengge *et al*., 2003; Templeton, 2004; Kahl *et al*., 2002). They are common in woodland, heath and moorland, but can also live in semi-rural areas bordering large population centres (O'Connell, 2005).

I. ricinus was recognized as a vector of all three human pathogenic *Borrelia* species, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. *I. scapularis* and *I. pacificus* in the United States and *I. ricinus* in Europe are vectors of *B. burgdorferi* sensu stricto. Both *I. ricinus* and *I. persuclatus* can be infected with *B. garinii* and *B. afzelii.* Other *Borrelia* species may also be transmitted by several vector species of the *I. ricinus* group. Although *B. valaisiana* and *B. lusitaniae* are cultured mainly from European *I. ricinus* ticks, the former species

has also been cultured from *I. columnae* ticks in Japan (Wang *et al*., 1999).

All ticks have 4 biological stages, the embryonated egg and the three active stages; the larvae, the nymphal stages, and the adult. In most species, each active stage seeks a host, feeds and drops off to develop in the natural environment (3-host life cycle) (Sonenshine, 1991). Tick larvae and nymphs feed primarily on small rodents and birds, whereas adult ticks feed on a variety of mammals (deer, domestic and wild carnivores, and larger domestic animals) (Brouqui *et al*., 2004).

Ixodes ticks take a blood meal, which can last several days, during each of their larval, nymph, and adult stages, attaching themselves to their hosts by barbed mouth-parts (Templeton, 2004; O'Connell, 2005). *I. ricinus* ticks acquire *B. burgdorferi* sensu lato infection during an infective meal on a reservoir host (Figure 1.1.) (Humair and Gern, 2000). *Borrelia* transmission is typically initiated by spirochete ingestion and midgut colonization during a larval stage feed on an infected mammalian host. Reservoir hosts for the spirochaetes include small and medium-sized mammals (e.g. field mice, hares), and birds (e.g. blackbirds, pheasants and gulls). This is followed by a period of spirochete relative dormancy through the larvae-nymph molt (Templeton, 2004), during fall and winter times the spirochaetes survive in a dormant state in the nymphal tick midgut. During this period, they express primarily the outer surface protein (Osp) A which is required for attachment and survival in the tick's midgut (Schnarr *et al*., 2006).

When the ticks feed in late spring and summer, the spirochaetes undergo changes that enable them to move into the new host. During the tick's feeding period of 24-72 hours, the spirochaetes replicate and move from the midgut to the salivary glands, triggered by the down-regulation of *OspA* and the reciprocal up-regulation of another surface lipoprotein, *OspC*. The transmission of the spirochaetes is the final step of the feeding period. It explains why ticks attached

for less than 24 hours do not transmit *B. burgdorferi* (Schnarr *et al*., 2006).

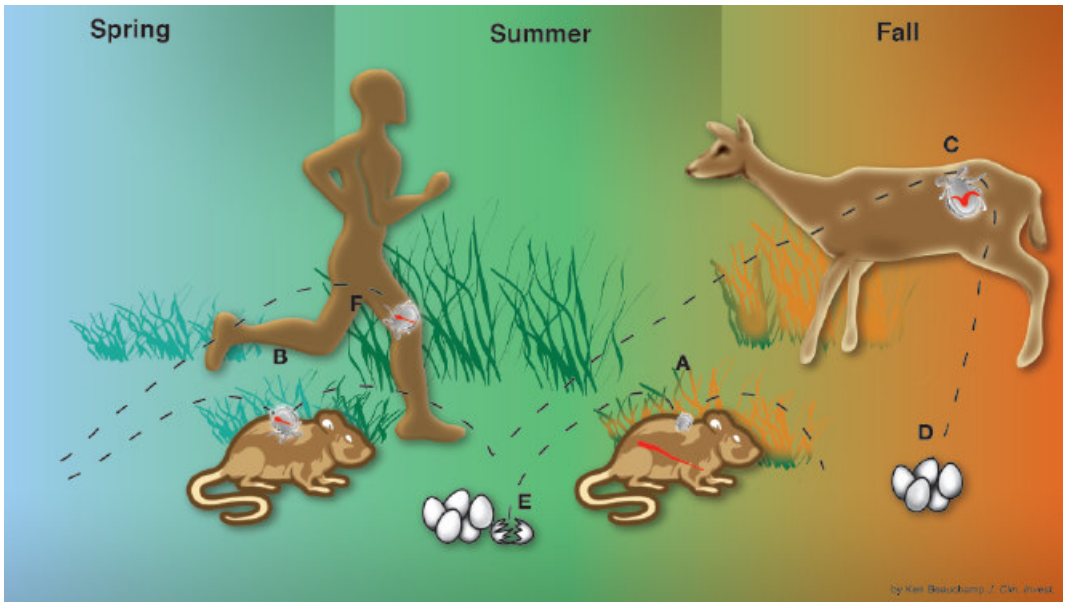

Figure 1.1: The enzootic cycle of *B. burgdorferi* infection. *I. scapularis* ticks feed once during each of the three stages of their usual 2-year life cycle. Typically, larval ticks take one blood meal in the late summer (**A**), nymphs feed during the following late spring and early summer (**B**), and adults feed during the fall (**C**), after which the female tick lays eggs (**D**) that hatch the next summer (**E**). It is critical that the tick feeds on the same host species in both of its immature stages (larval and nymphal), because the life cycle of the spirochete (wavy red line) depends on horizontal transmission: in the early summer, from infected nymphs to certain rodents, particularly mice or chipmunks (**B**); and in the late summer, from infected rodents to larvae (**A**), which then molt to become infected nymphs that begin the cycle again in the following year. Therefore, *B.burgdorferi* spends much of its natural cycle in a dormant state in the midgut of the tick. During the summer months, after transmission to rodents, the spirochete must evade the immune response long enough to be transferred to feeding larval ticks. Although the tick may attach to humans at all three stages, it is primarily the tiny nymphal tick (~1 mm) that transmits the infection (**F**). This stage of the tick life cycle has a peak period of questing in the weeks surrounding the summer solstice. Humans are an incidental host and are not involved at all in the life cycle of the spirochete (Adapted from Steere *et al*. (2004)).

Humans are incidental hosts for ticks, and infections occur mainly in late spring, early summer and autumn – the peak periods for the tick's feeding. The annual incidence of LB can vary, depending on climatic factors affecting tick population density and activity. Nymphal ticks are the main source of human borrelial infection, but are very small and may be overlooked. Tick bites may not be recognized because they do not usually cause significant pain, irritation or itch.

Those who may be exposed to ticks can minimize their risk of infection by wearing protective clothing (light-coloured long-sleeved shirts and long trousers) and considering use of insect repellents. They should check their skin regularly and gently remove any attached ticks, preferably using tweezers as close as possible to the skin. It is particularly important to check the head and neck of young children. Borrelial infection is unlikely to occur when ticks are attached for less than 24–36 hours, so prompt removal is an additional preventive measure (O'Connell, 2005).

Ixodid ticks may also carry other organisms, including ehrlichiae, babesiae, bartonellae and Central European tick-borne encephalitis virus. Co-infections have been documented and may cause atypical presentations (O'Connell, 2005).

1.3.1. Reservoir Hosts

There is an association between certain *B. burgdorferi* sensu lato spp. and specific vertebrate hosts (Humair and Gern, 2000; Brouqui *et al*., 2004). *B. burgdorferi* sensu stricto in the United States and *B. garinii* in Eurasia were isolated from a large diversity of mammalian hosts and birds. In contrast, *B. afzelii* in Eurasia and *B. burgdorferi* sensu stricto in Europe were isolated mainly from rodents. Many species of mammals are hosts of *B. japonica* in Japan. However, birds are assumed to be the only hosts for *B. valaisiana* in Europe, since no *B. valaisiana* isolate was cultured or detected from mammals or rodents to date (Wang *et al*., 1997; Wang *et al*., 1999). Only a few *B. lusitaniae* strains were isolated from *I. ricinus* in southern, central and eastern Europe (Le Fleche *et al*., 1997; Wang *et al*., 1999). Data for hosts of this species are currently not available. *B. andersonii* and *B. Bissettii* sp. nov. in North America seem to involve particular and narrow host spectra, i.e., cottontail rabbits (*Sylvilagus floridanus*) and wood mice (*Neotomae fuscipes*), respectively, although further studies are necessary to clarify these unique relationships. The relationships between Borrelia species associated with LB and vertebrate hosts acting as

reservoirs need to be investigated more extensively in Europe (Wang *et al*., 1999; Humair and Gern, 2000).

1.4. CLINICAL FEATURES

In general, clinical manifestations of Lyme disease are similar worldwide. However, there are considerable regional variations in frequency and appearance of certain symptoms and some findings have specific associations with certain genospecies of *B. burgdorferi* (Table 1.1) (Schnarr *et al*., 2006).

Table 1.1.: Clinical features of Lyme borreliosis in North America and Eurasia (Adapted from Piesman and Gern (2004)).

Region	Vector	Aetiologic Agent	Clinical Features
North America	*I. scapularis,* *I. pacificus*	*B. burgdorferi* sensu stricto	Erythema Migrans, arthritis, facial palsy, meningitis, peripheral radiculoneuropathy, atrioventricular block
Eurasia	*I. ricinus,* *I.persulcatus*	*B. burgdorferi* sensu stricto, *B. afzelii*[1], *B. garinii*[2]	Erythema Migrans, acrodermatitis chronica atrophicans[1], lymphocytoma, arthritis, facial palsy[2], meningitis[2], peripheral radiculoneuropathy[2], atrioventricular block

[1] *B. afzelii* is associated with skin disease, including specifically acrodermatitis chronica atrophicans. [2] *B. garinii* is associated with neurological disease, including facial palsy, meningitis, and peripheral radiculoneuropathy.

The clinical course of LB may be divided into an early disease (also called early infection or 'acute phase') due to local and early dissemination of *Borrelia burgdorferi* infection, and a late or chronic disease (chronic phase) with persistent infection. The traditionally used grouping into three stages may be misleading since LB does not necessarily develop in stages (Schnarr *et al*., 2006).

The infection begins with the rash of erythema migrans and flulike sympthoms and may progress, after days to weeks, to a disseminated stage and in months to years, to a late (chronic) stage (Sternbach and Dibble, 1996).

In early localized infection the most common clinical manifestation is erythema migrans (EM), a localized, erythematous rash appearing after 2–30 days (usually 5–15 days) at the site of a bite. There may be local lymphadenopathy (O'Connell, 2005).

Early disseminated disease results from the spread of the bacterium from the inoculation site through blood and lymphatics and localization in many organs and tissues. Frequent findings include multiple EM lesions, facial nerve (Bell's) palsy, radiculoneuritis, meningitis, carditis with conduction abnormalities, and migratory oligoarticular joint pains, arthritis and synovitis. In Europe, *Borrelia garinii* is most strongly associated with neuroborreliosis, but other *B. burgdorferi* sensu lato species may also cause these findings. The presence of spirochetes in these tissues during the early phases of LB incites the inflammatory response leading to clinical signs and symptoms (Dumler, 2001).

Late phases of LB include a variety of findings. Chronic Lyme arthritis is the most common manifestation in the USA. In some patients, inflammation continues for months or even several years after antibiotic treatment. Chronic neurological manifestations include radiculo-neuropathy, presenting mainly with sensory symptoms. Lyme encephalopathy is uncommon. Patients may complain of poor memory and concentration, and have subtle learning difficulties. Acrodermatitis chronica atrophicans (ACA) is a chronic skin manifestation. The lesions are usually found on the limbs, are initially violaceous and may last for years, eventually becoming atrophic. They are strongly associated with *B. afzelii* infection, which has been cultured from lesions years after onset, despite a strong antibody response. The condition is often accompanied by peripheral neuropathy (O'Connell, 2005).

The disease may become manifested at any stage, earlier stages may be skipped (less than one third of patients with Lyme arthritis recall an erythema migrans), missed by patients and doctors or erythema migrans may coincide with manifestations of disseminated infection. Moreover, it is rather unusual for patients to develop several different organ manifestations of disseminated and

chronic infections. For example, Lyme arthritis has rarely been observed in a patient after acute neuroborreliosis and a patient with Lyme arthritis is at very low risk of developing chronic neuroborreliosis (Schnarr *et al*., 2006).

1.5. EPIDEMIOLOGY

LB is recognized as an important infectious disease in the northern hemisphere, with foci in North America and Eurasia, including China and Japan (Campbell *et al*., 1998; Schnarr *et al*., 2006). The risk of infection is highly variable depending on the density of infected ticks and on their feeding habits and annual hosts; these have evolved differently in different geographic locations, a fact which is believed to explain the geographic and seasonal distribution of LB (Schnarr *et al*., 2006).

Infection can occur at any age with similar frequencies in men and women, but is most likely in individuals whose residence or occupational or recreational activities place them at high risk of tick bites (Hengge *et al*., 2003; O'Connell, 2005). LB was documented in many occupational groups, including forestry workers, farmers, veterinarians, military recruits, orienteers and outdoor workers in general (Wilske, 2005; Schnarr *et al*., 2006).

In 2000, 17,730; 2001, 17,029; 2002, 23,763 cases were reported in United States. The 2001-2002 incidence were 5,98-8,24 per 100,000 of the population respectively. During 2003-2004, 41,077, in 2005, 23,305 cases of LB were reported yielding a national average of 7.9 cases for every 100,000 persons (Marshall *et al*., 2002; Bacon *et al*., 2004; Bacon *et al*., 2007).

In Europe, Lyme borreliosis is mostly established in forest areas. The disease is present in most parts of Europe except the hot South (Sicily, southern Spain) and the cold North (northern Scandinavia and northern Russia) (Stanek *et al*., 1993; Schnarr *et al*., 2006). The highest estimates of the prevalence and incidence of

LB are reported from middle Europe and Scandinavia, particularly from Austria, Germany, Sweden and Slovenia; there is marked regional variability and differences of frequencies even within the same geographical area (Piacentino and Schwartz, 2002; Schnarr *et al*., 2006). In the UK, approximately 500 cases are serologically confirmed each year (Hengge *et al*., 2003; O'Connell, 2005).

Approaches to collecting data on the incidence of LB vary considerably across Europe. Very few countries have made LB a mandatorily notifiable disease and there are currently no plans to add this to the listed diseases covered by European Community-coordinated disease surveillance (Smith *et al*., 2006).

Fewer than 20 Lyme borreliosis cases have been published in Turkey since 1990 (Çakır *et al*., 1990; Köksal *et al*., 1990; Ergül *et al*., 1996; Aksu *et al*., 1997; Öztürk *et al*., 1997; Demirkaya *et al*., 1998; Leblebicioglu, 1999; Ulus *et al*., 2001; Ceylan *et al*., 2005; Karcıoğlu *et al*., 2005). These cases were diagnosed as LB based on clinical symptoms such as arthralgia, myalgia, extreme fatigue, migratory pain, erythema migrans and positive ELISA results with antigens derived from European isolates (Güner *et al*., 2003).

Apart from these, Utaş *et al*. (1994) studied Kayseri region during April-July 1992. 5/50 (10%) cases were detected as positive for *B. burgdorferi* IgM-IgG antibodies in this study. In Ankara region, Birengel *et al*. (1999) reported 13% (7/54), 6% (3/50) and 4% (2/50) Lyme seropositivity in patient groups, in risky group and healty control group respectively, whereas Hızel *et al*. (1997) reported 10,4% seropositivity in patients with Lyme disease sympthoms. In Antalya region, Mutlu *et al*. (1995) reported 35,9% seropositivity, Tuncer *et al*. (1999) found 6,4% and 22,1% IgG prevalence in city center and in rural areas respectively, additonally among the collected ticks 80,4% were typified as *Ixodes ricinus*. In 2004, Tuncer *et al*. studied the seasonal activity of populations of ticks during one-year period in Antalya region and 608 of 3338 (18,2%) ticks were specified as *Ixodes ricinus*. The 93 of them had been dissected and one *B. burgdorferi* was seen by immunofluorescence assay. Tünger and Büke (1995)

reported 7,8% seropositivity in İzmir region. Demirci *et al*. (2001) studied patients with tick-bite and who did not remember tick-bite, and they reported 17% - 2,3% seropositivity respectively in Isparta region. Aydin *et al*. (2001) reported 6,6% seropositivity in Trabzon region whereas Çelik *et al*. (2001) reported 18,9% seropositivity in Denizli region , and Erensoy (2002) reported 6,43% seropositivity in the rural areas of Elazığ. These studies are important since Lyme seropositivity was shown in our country, and also important for being fundamental studies of epidemiological investigations. In a recent study, Güner *et al.* (2003) isolated *B. burgdorferi* sensu lato strains from *Ixodes ricinus* ticks in Trakya region (prevalence rate of 4,0% by cultivation). In the same year Güner *et al.* isolated a novel, fast growing *Borrelia* sp. (*Borrelia turcica* sp.nov.) from the hard tick *Hyalomma aegyptium* (family *Ixodidae*, subfamily *Metastriata*) in northwestern Turkey. DNA-DNA hybridization results showed that this strain was distinct from Lyme-disease-related *Borrelia, Borrelia burgdorferi* (Güner *et al*., 2003; Güner *et al*., 2004).

1.6. LABORATORY DIAGNOSIS

Patient's history (e.g. tick bite, stay in an endemic area), characteristic clinical features, and laboratory results all contribute to the diagnosis of LB (Schnarr *et al.*, 2006).

To meet the demand for laboratory-based diagnosis, various new tests for direct detection of the etiologic agent or for detection of specific antibodies have been introduced into the clinical laboratory (Aguero-Rosenfeld *et al.*, 2005).

1.6.1. Antibody detection methods

The immune response to *B. burgdorferi* sensu lato infection begins with the appearance of specific immunoglobulin M (IgM) antibodies, usually within the first several weeks after initial exposure. The IgM response may persist for many

months or years despite effective antimicrobial therapy. Thus, the presence of specific IgM antibodies cannot be used as the sole criterion to diagnose a recent infection. Most patients will have detectable IgG antibodies after 1 month of active infection. Like that of IgM, the IgG response can persist for years after LB symptoms have resolved and there is no role for the routine use of serologic testing to monitor response to therapy. Both IgG and IgM responses can be greatly diminished or absent in patients receiving antimicrobial therapy early in the course of disease (Reed, 2002). The presence of specific antibodies does not prove the presence of disease; a positive antibody test may also be due to clinical or subclinical infections in the past. The more nonspecific the symptoms, the lower the predictive value of a positive serological test (Wilske *et al*., 2007).

Several methods have been used for detection of antibodies to *B. burgdorferi* sensu lato.

1.6.1.1. Indirect Immunofluorescent Antibody Assays (IFA)

IFA uses cultured organisms fixed onto glass slides. The presence of antibodies is detected by fluorescence microscopy (Aguero-Rosenfeld *et al*., 2005). Specimens testing reactive at screening dilutions are serially diluted and titers of 1/128 or 1/256 for IgM or IgG respectively are usually considered positive (Magnarelli *et al*., 1984; Russell *et al*., 1984). Limitations of this assay include the need for fluorescence microscopy and for well-trained personnel and the subjectivity in reading and interpreting fluorescence microscopy (Aguero-Rosenfeld *et al*., 2005).

1.6.1.2. Enzyme immunoassays (EIA)

Enzyme-Linked Immunosorbent Assay (ELISA) is the most frequently used method to test for antibodies to *B. burgdorferi* sensu lato. Most c ommonly,

antigen mixtures comprised of whole-cell sonicates of *B. burgdorferi* sensu lato are used as the source of antigen for the detection of IgG, IgM or IgA antibodies individually or in combination (most frequently IgG-IgM combinations) (Aguero-Rosenfeld *et al*., 2005).

One of the limitations of ELISA for detection of *B. burgdorferi* sensu lato antibodies is lack of standardization among the numerous commercial kits marketed for LB diagnosis in the United States and Europe. Variations exist between assays in terms of antigenic composition and in the detection of specific immunoglobulin classes, particularly in the detection of IgM antibodies. Such variations may occur among different commercial kits as well as between the lots of the same kit (Aguero-Rosenfeld *et al*., 2005). When results from different laboratories for well-characterized proficiency samples are compared, significant differences in the sensitivities and specificities of ELISA and IFA have been observed (Bakken *et al.,* 1997; Reed, 2002).

The numerous antigens present in whole-cell assays can result in cross-reaction with antibodies to other microorganisms or tissue components. Many diseases have been reported to cause significant cross-reactivity in IgM and/or IgG assays. Among such diseases are autoimmune disorders, Epstein-Barr virus infection, bacterial endocarditis, syphilis, other spirochetal infections and *Helicobacter pylori* infection (Reed, 2002).

ELISA and IFA have the drawback of lacking sensitivity for early disease (Reed, 2002). Kaiser (2000) investigated the risk of obtaining false-negative results in serological assays in serum and cerebro-spinal fluid (CSF) specimens in patients with neuroborreliosis, concluded that there is a small, but real, risk of false-negative serological findings at the time of initial clinical presentation in patients with typical sympthoms.

1.6.1.3. Western Immunoblot (WIB)

WIB is used currently as a confirmatory assay in the serodiagnosis of LB, but is usually only employed following a positive screening assay (Brouqui *et al*., 2004). It allows detection of antibodies to individual antigens of *B. burgdorferi* sensu lato and is more specific than ELISA or IFA. Antigens can be derived from whole-cell preparations of *B. burgdorferi* sensu lato or from expressed proteins taken from recombinant DNA. Both IgM and IgG immunoblotting kits are available, but IgM immunoblotting is less specific than IgG immunoblotting and patients with symptoms lasting longer than 4 weeks should have only IgG antibody testing done (Reed, 2002). On the other hand Cooke and Bartenhagen (1994) studied to determine background levels and specifity of antibody to *Borrelia burgdorferi* by WIB in an area nonendemic for LB and to correlate antibody specificity with clinical or serologic findings. They conclude that significant levels of antibody to *B. burgdorferi* may be seen on WIB in patients who have not been exposed to this organism by clinical or epidemiologic criteria.

In an attempt to standardize serologic diagnosis of LB, criteria for WIB interpretation were established in the United States. According to these criteria, a positive IgM blot is defined by the presence of two of three particular immunoreactive bands (OspC, 41 or 39 kDa). The IgG criteria require the presence of at least 5 of 10 particular bands (93, 66, 58, 45, 41, 39, 30, 28, 21 [OspC], or 18 kDa). Guidelines for WIB interpretation in Europe have recently been published, but consensus on criteria has not been reached. Criteria applicable to each species causing LB may be needed in Europe (Aguero-Rosenfeld *et al*., 2005).

There is no single optimum test for the serodiagnosis of LB. The existing methods must therefore be combined logically in order to achieve the highest possible diagnostic efficiency. A stepwise diagnostic protocol is recommended in which a screening assay is used as the first step. If the result of the screening

assay is positive or borderline, a confirmatory WIB should be used. A two-tier protocol has also been recommended in the USA by the Centers for Disease Control (CDC) (Brouqui *et al*., 2004).

1.6.1.4. Newer EIA antibody tests

Because of the above-described limitations of current ELISA and WIB testing, there is interest in developing simplified but accurate new approaches for serodiagnosis. The principal focus has been on the use of purified, recombinant, or synthetic peptides as the source of antigens in immunoassays. Unfortunately, so far no single antigen has demonstrated sufficient sensitivity and specificity. Antigenic variability among *B. burgdorferi* sensu lato species and the temporal appearance of antibodies to different antigens at various stages of LB make the choice of a single antigen a difficult task. Several immunoassays using recombinant antigens have been developed and evaluated for the serodiagnosis of LB. In serodiagnosis, recombinant antigens have been used alone or in combination. Particularly in Europe they have been prepared from different *B. burgdorferi* sensu lato species and used in both ELISA and WIB formats in an attempt to increase sensitivity (Aguero-Rosenfeld *et al.,* 2005).

1.6.1.5. Borreliacidal antibodies

Borreliacidal antibodies have been used in the immunodiagnosis of early and late LB (Aguero-Rosenfeld et al., 2005). Borreliacidal antibodies are detected by incubating viable *B. Burgdorferi* organisms with serum and complement for 16 to 24 h. If serum contains borreliacidal antibodies, spirochetes will be readily killed (Jobe *et al*., 1999). Growth inhibition of *B. burgdorferi* sensu stricto can be determined by visual inspection of the percentage of nonmotile spirochetes, colour changes by use of a pH indicator, or flow cytometry after staining with acridine orange (Aguero-Rosenfeld *et al*., 2005). The use of live spirochetes increases the specificity by eliminating the detection of cross-reactive antibodies that bind to the organism but are incapable of killing the spirochetes (Jobe *et al*.,

1999). The advantage of these assays is their high specificity in untreated patients. Major disadvantages include the need for cultured live *B. burgdorferi* sensu stricto, interference from antimicrobials that might be present in patient sera, and the relatively cumbersome nature of the assays (Aguero-Rosenfeld *et al*., 2005).

1.6.2. Direct Detection of *B. burgdorferi*

Different approaches have been used in the clinical laboratory: microscope-based assays, detection of *B. burgdorferi*-specific proteins or nucleic acids and culture.

Direct microscopic detection of *B. burgdorferi* sensu lato has limited clinical utility in laboratory confirmation of LB due to the sparseness of organisms in clinical samples (Aguero-Rosenfeld *et al.,* 2005).

1.6.2.1. Culture Technique

This method should be attempted only in certain clinical circumstances and should be carried out by reference laboratories (Brouqui *et al.,* 2004). Culture of *B. burgdorferi* sensu lato involves incubating a specimen in Barbour-Stoenner-Kelly medium (BSK) (or modifications of BSK) and detecting the presence of characteristic spirochetes by dark-field microscopy or by fluorescent microscopy with acridine orange or a specific fluorescent antibody (FA) (Preac-Mursic *et al*., 1991; Reed, 2002). As this approach is time-consuming (requiring up to 12 weeks of incubation before being considered negative.), expensive and labour-intensive, it is rarely used for the routine diagnosis of LB. Attempted isolation of borreliae is potentially of most diagnostic value in patients with suggestive clinical presentations, but no detectable antibody response (seronegative Lyme borreliosis), e.g., atypical EM, suspected neuroborreliosis without detection of intrathecal antibodies or suspected LB combined with immune deficiencies. Such seronegative cases are often characterised by a short duration of disease. Occasionally, culture attempts may be justified in seropositive patients, e.g.,

patients with dermatological disease manifestations that cannot be attributed unambiguously to LB (Aguero-Rosenfeld *et al.,* 2005; Brouqui *et al.,* 2004).

1.6.2.2. Molecular methods of detection of *B. burgdorferi* sensu lato

For laboratory diagnosis of LB, the utilization of molecular techniques has focused mainly on Polymerase Chain Reaction (PCR) based methods (Aguero-Rosenfeld *et al.,* 2005). More than 100 research studies involving PCR in the diagnosis of *Borrelia burgdorferi* infections, with a variety of different target genes, primer pairs, techniques, extraction procedures and detection methods, have been published (Schmidt, 1997).

In the first PCR paper, submitted for publication in April 1989 by Rosa and Schwan, a chromosomal gene, clone 2H1, to code for a surface-associated 66-kDa protein, was used as target. Although primers were able to amplify 26 *B. burgdorferi* strains isolated in America, even in this first publication, it was shown that a German isolate (G2) failed to be amplified. Therefore, the primers had to be redesigned and only in the second publication (Rosa *et al.,* 1991) was successful amplification of the German strain described. In 1990, Persing *et al.* described the amplification of *B. burgdorferi*-specific sequences from museum specimens by using a target located on a 49-kb plasmid, coding for the outer surface proteins OspA and OspB. Nielsen *et al.* (1990) amplified a 145-bp DNA fragment of the OspA gene. Goodman *et al.* (1991) were able to detect *B. burgdorferi*-specific sequences (*Ly* gene) in the urine of infected patients with late LB and Wallich *et al.* (1990) sequenced the gene coding for flagellin, which later proved to be an excellent target for PCR (Picken, 1992). Lebech *et al.* (1992) developed a PCR for use in the identification of the flagellin gene in urine and
CSF from patients with Lyme neuroborreliosis and they concluded that urine is a more suitable sample source than CSF for use in *B.burgdorferi* DNA detection by PCR. Schwartz *et al.* (1992) evaluated the sensitivity and specifity of PCR in skin biopsy specimens, used primer targeting 23Sr RNA gene, concluded that

PCR is a promising technique for the diagnosis of early LB. In 1995 Demaerschalck *et al.* have designed primers based on *Borrelia burgdorferi* sensu lato *ospA* gene sequences for use in the PCR to type all or each of the *B. burgdorferi* sensu lato genospecies involved in LB. Priem *et al.* (1997) performed nested PCR with primer sets targeting the plasmid-located *ospA* gene and a chromosomal gene segment encoding a 66-kDa protein, among these, more positive results were obtained with the p66 gene primer than with the *ospA* primer and they can detect DNA from *B.burgdorferi* sensu lato species sensitively and specifically. Brettschneider *et al.* (1998) used primer sets targeting 23S rRNA and 66-kDa protein genes. They concluded that a combination of two different primer sets achieves high sensitivity with skin biopsies and in early erythema migrans infection, culture and PCR are more sensitive than serology. Schaarschmidt *et al.* (2002) used species specific primers targeting 16S rRNA gene to obtain more information about possible correlation between the distribution of *Borrelia* species and clinical syndromes of LB. Cyr *et al.* (2005) developed a primer targeting 16S rDNA sequences of *B.burgdorferi* sensu lato, amplifying strains of *B.burgdorferi* sensu stricto, *B.afzelii* and *B.garinii* but not the non-Lyme causing *B.hermsii* or *B.turicatae*, facilitating detection of causative agents of LB in infected ticks and human skin samples, and potentially allowing for a more rapid diagnosis of the disease. Picha *et al.* (2005) used three primer sets targeting ospC, chromosomal flagellin, 16SrRNA genes, achieved the highest sensitivity of PCR in the acute period of neuroborreliosis.

The number of spirochetes in infected tissues or body fluids of patients is very low, appropriate procedures for sample collection and transport and preparation of DNA from clinical samples are critical for yielding reliable and consistent PCR results. A variety of clinical specimens from patients with suspected LB have been analyzed by PCR assays (Schmidt, 1997). Of these, skin biopsy samples taken from patients with EM or ACA have been the most frequently tested specimens (Wang, 2002). Depending on the clinical manifestations of the

patients, appropriate body fluid samples (e.g., blood, CSF, or synovial fluid) can be collected and analyzed by PCR (Aguero-Rosenfeld *et al.,* 2005).

The efficiency of a PCR assay is determined by several factors. Among these, the selections of an appropriate gene target and primer set for PCR amplification are the most important in development of any new PCR protocols (Aguero-Rosenfeld *et al.,* 2005). The most important consideration in selecting a target for amplification is genetic stability. Loss or alteration of the target sequence may result in a loss of reactivity. For the diagnosis of LB by PCR, attention must also be paid to test specificity (Schmidt, 1997).

The sensitivity of PCR can be increased by using a nested PCR procedure. Here, two rounds of amplifications are performed. After a first standard PCR, a small sample is removed and put into a master mix containing primers specific for an inner part of the generated amplicon. After a second round of amplification cycles, the sensitivity is increased, as expected (Haff, 1994; Schmidt, 1997).

Recently, the number of spirochetes in clinical specimens of patients with LB was determined by real-time quantitative PCR assays (qPCR) (Schwaiger *et al.,* 2001; Liveris *et al.,* 2002). In one study, *B. burgdorferi* sensu stricto-specific *recA* DNA was detected by qPCR assay in 40 (80%) skin biopsy samples from 50 untreated adult U.S. patients with EM (Liveris *et al.,* 2002). In another study, *B. burgdorferi* sensu lato *flagellin* gene sequence was amplified in 5 of 28 (17.9%) synovial fluid specimens and 1 of 5 (20%) synovial membrane biopsies obtained from 31 patients with arthropathies in Switzerland (Schwaiger *et al.,* 2001). Furthermore in 2006 *B. burgdorferi* sensu lato *ospA* gene was detected in clinical specimens including CSFs by real-time PCR, the PCR had a sensitivity of 50% in patients with neuroborreliosis (Gooskens *et al.,* 2006).

In this study, detection of *B. burgdorferi* sensu lato from clinical specimens, received from different hospitals in Turkey to The Spirochetal Diseases

Diagnosis Laboratory of Central Veterinary Control and Research Institute, by Polymerase Chain Reaction and ELISA were aimed.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

Table 2.1. Materials and suppliers.

Chemicals	**Suppliers**
Agarose	Sigma
Borrelia burgdorferi ELISA Kit	Genzyme Virotech
DNA Extraction Kit	Qiagen
DNA Loading Buffer	Dr. Zeydanlı
DNA Size Marker	BioLabs
dNTP	GeneMark
Ethidium Bromide	Sigma
Primers	AlphaDNA
Taq DNA Polymerase	Sigma
10X TBE	CLP

2.1.2. Equipments

Table 2.2. Equipments and suppliers.

Equipments	**Suppliers**
Centrifuge	Eppendorf
Deepfreezer	Bosch
Electrophoretic Gel System	ThermoEC

Table 2.2. Cont'd.

Equipments	**Suppliers**
Thermal Imaging System	Pharmacia Biotech
UV Transilluminator	UVP
Incubator	Dedeoglu
Power Supply	Gibco BRL
Thermal Cycler	MJ Research Inc.
Various Micro Pipets	Eppendorf
Vortex	Heidolph
Dark-Field Microscope	Olympus
Spectrophotometer	NanoDrop ND-1000

2.1.3. Media

Table 2.3. Media and supplier.

Media	**Supplier**
BSK H Medium Complete	Sigma

2.1.4. Control Strains

- *Borrelia burgdorferi* sensu stricto B31 **ATCC 35210**
- *Borrelia afzelii* VS461 (kindly provided by Olivier Peter, Institut Central des Hôpitaux Valaisans, Sion, Switzerland.)
- *Borrelia garinii* VS102 (kindly provided by Olivier Peter, Institut Central des Hôpitaux Valaisans, Sion, Switzerland.)

Borrelia strains were grown in BSK-H complete medium at 35ºC for 1 to 2 weeks and checked via dark-field microscope.

2.1.5. Patient Samples

Serum, blood, cerebrospinal fluid (CSF), synovial fluid and skin biopsy samples of patients with suspected LB sent from different hospitals in Turkey admitted to The Spirochetal Diseases Diagnosis Laboratory of Central Veterinary Control and Research Institute between 2003 and 2007 were screened. Specimens from one hundred and forty two patients were examined for this study.

2.2. METHODS

2.2.1. Enzyme-Linked Immunosorbent Assay

Genzyme Virotech Borrelia burgdorferi ELISA IgG/IgM test kit were used for ELISA according to the manufacturer's instructions. Patient sera were diluted with dilution buffer as 1+100 (e.g. 10 µl serum+ 1 ml dilution buffer). For each test run, 100 µl each of ready to use dilution buffer (blank), IgG- and IgM-positive, negative, cut-off controls and diluted patient sera were put into the wells, incubated 30 minute at 37ºC with cover. After the incubation period, microtiter strips were washed 4 times with 350-400 µl washing solution per well. The 100 µl of ready to use conjugate was pipetted into each well covered and incubated 30 minute at 37ºC. The washing process was repeated 4 times with 350-400 µl washing solution per well. The one hundred microliters of ready to use TMB (substrate) was put into each well and incubated in a dark place for 30 minute at 37ºC with cover. Substrate reaction was stopped by adding 50 µl of citrate stopping solution into each well and measured at 450/620 nm within 1 hour after adding the stopping solution. The test was evaluated as mentioned in the kit procedure.

2.2.2. Polymerase Chain Reaction

Table 2.4. Primers for PCR.

Target gene	Primer	Sequence*	Reference
OspA	SL 1	AAT AGG TCT AAT AAT AGC CTT AAT AGC	Demaersch-alck *et al.,* 1995
	SL 2	CTA GTG TTT TGC CAT CTT CTT TGA AAA	
23S rRNA	BslF (Outer Primer)	AGC ATA GAA GTG CTG GAG TCG AAG CGA	Schaarschm-idt *et al.,* 2002
	BslR (Outer Primer)	TCA ATT AGT GCT CTA CCT CTA TTA A	
	nBslF (Nested Primer)	TTA GTT AGA TGT GGT AGA CCC GA	
	nBslR (Nested Primer)	CTA AAA TAA GGC TGA ACT TAA ATC CA	

*Sequences are shown from 5' to 3'.

2.2.2.1. DNA extraction

Bacterial DNA was extracted from cultures and clinical specimens (blood, CSF, skin biopsy) using commercial kits, QIAamp DNA Blood Mini Kit and QIAamp DNA Mini Kit (Qiagen, Germany). The procedure was accomplished according to the manufacturer's instructions (QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook; protocol for blood and body fluid, bacteria and tissue 02/2003). DNA concentrations were determined spectrometrically by measuring the A260 (NanoDrop ND-1000, USA). Extracts were stored at -20ºC until use. For PCR amplifications, 100 ng of the preparations was used as template DNA.

2.2.2.2. PCR

The detection of *Borrelia burgdorferi* DNA in the human samples were attempted by using conventional and nested PCRs. We used two primer sets, which sequences are shown in Table 2.4.

An aliquot of 7 µl of template DNA was added to 50 µl of PCR mixture consisiting of:

10X PCR buffer
1,5 mM $MgCl_2$
200 µM (each) dATP, dCTP, dGTP and dTTP
20 pmol of primers
1,25 U Taq DNA polymerase.

The first round of PCR was performed with outer primer pairs (Table 2.4.). The 5 µl of this reaction was used for the second round of nested-PCR which was performed with the nested primer pairs.

Extraction, mixture preparation and amplification steps were performed at different rooms to avoid contamination.

For the SL primer set, the samples were subjected to 35 PCR cycles, each consisting of: 1 min. at 93 ºC
1 min. at 65 ºC
1 min at 72 ºC was performed.

For the 23S rRNA outer primer set, 40 cycles of PCR amplification, each consisting of: 1 min. at 94 ºC
1 min. at 50 ºC
1,5 min. at 72 ºC was performed.

For the 23S rRNA nested primer set, 35 cycles of PCR amplification, each consisting of: 30 sec. at 94 ºC
30 sec. at 58 ºC
1 min. at 72 ºC was performed.

The PCR products were analyzed with electrophoresis on 2% agarose gels with visualization of the amplicon with ethidium bromide over UV illumination.

The 30 ml 1XTBE buffer was mixed with 0,6 mg agarose, and boiled to prepare 2% agarose gel. The 3 µl ethidium bromide was added into agarose gel, and replaced into the electrophoresis tank. The 5 µl PCR product was mixed with 2-3 µl 6Xloading buffer, and subjected to agarose gel electrophoresis. DNA size marker was loaded into one of the wells. Electrophoresis was performed at 120 volt for 20 minutes into the 1X TBE buffer solution. The amplicons were visualized over a UV light box. DNA size marker, positive controls and negative control were compared to confirm the results.

CHAPTER 3

RESULTS

In this study, a total of 152 clinical specimens (140 serum and blood, 10 cerebrospinal fluid, 1 synovial fluid, 1 skin biopsy specimens) collected from 140 suspected LB patients were subjected to ELISA and PCR analysis. The results are shown on table 3.1. Samples were sent by from different hospitals in Turkey.

Table 3.1. Results of ELISA and PCR analysis.

NO	Location	Gender	Sample	ELISA IgM	ELISA IgG	PCR I*	PCR II**
1	Ankara	Male	Blood, Serum	+	-	-	-
2	Tokat	Female	Blood, Serum	?	-	-	-
3	Ankara	Female	Blood, Serum	-	-	-	-
4	Ankara	Female	Blood, Serum	-	?	-	-
5	Ankara	Female	Blood, Serum	?	-	-	-
6	Çorum	Female	Blood, Serum	-	-	-	-
7	Kastamonu	Male	Blood, Serum	+	+	-	-
8	Ankara	Female	Blood, Serum	-	-	-	-
9	Ankara	Female	Blood, Serum	+	-	-	-
10	Ankara	Female	Blood, Serum	+	?	-	-
11	Ankara	Female	Blood, Serum	-	-	-	-
12	Ankara	Female	Blood, Serum	-	-	-	-
13	Ankara	Female	Blood, Serum	?	-	-	-
14	Bolu	Female	Blood, Serum	-	-	-	-
15	Yozgat	Male	Blood, Serum	?	-	-	-
16	Zonguldak	Male	Blood, Serum	-	-	-	-
17	Çankırı	Female	Blood, Serum	-	-	-	-
18	Kırşehir	Female	Blood, Serum	+	-	-	-
19	Zonguldak	Male	Blood, Serum	-	-	-	-
20	Yozgat	Male	Blood, Serum	+	-	-	-
21	Ankara	Male	Blood, Serum	-	+	-	-
22	Ankara	Male	Blood, Serum	-	-	-	-
23	Kastamonu	Female	Blood, Serum	-	-	-	-
24	Ankara	Male	Blood, Serum	?	-	-	-
25	Çorum	Female	Blood, Serum	-	-	-	-
26	Ankara	Male	Blood, Serum	?	-	-	-

* OspA primer PCR, **23SrRNA primer PCR

Table 3.1. Cont'd.

NO	Location	Gender	Sample	ELISA IgM	ELISA IgG	PCR I*	PCR II**
27	Karabük	Male	Blood, Serum	+	-	-	-
28	Ankara	Female	Blood, Serum	+	-	-	-
29	Amasya	Female	Blood, Serum	-	-	-	-
30	Bolu	Female	Blood, Serum	-	-	-	-
31	Çankırı	Female	Blood, Serum	+	-	-	-
32	Amasya	Female	Blood, Serum	?	-	-	-
33	Yozgat	Male	Blood, Serum	-	-	-	-
34	Trabzon	Female	Blood, Serum	+	-	-	-
35	Ankara	Male	Blood, Serum	+	-	-	-
36	Ankara	Female	Blood, Serum	+	-	-	-
37	Ankara	Female	Blood, Serum	+	-	-	-
38	Ankara	Female	Blood, Serum	+	-	-	-
39	Diyarbakır	Female	Blood, Serum	+	-	-	-
40	Yozgat	Male	Blood, Serum	+	-	-	-
41	Ankara	Male	Blood, Serum	+	-	-	-
42	Ankara	Male	Blood, Serum	?	-	-	-
43	Çorum	Female	Blood, Serum, CSF	+	-	-	-
44	Ankara	Male	Blood, Serum	+	-	-	-
45	?	Male	Blood, Serum	-	-	-	-
46	Kırıkkale	Male	Blood, Serum	+	-	-	-
47	Sinop	Female	Blood, Serum	+	-	-	-
48	Ankara	Male	Blood, Serum	+	-	-	-
49	Kastamonu	Female	Blood, Serum	+	-	-	-
50	Ankara	Female	Blood, Serum	+	-	-	-
51	Ankara	Female	Blood, Serum	?	-	-	-
52	Ankara	Male	Blood, Serum	+	-	-	-
53	Ankara	Female	Blood, Serum	?	-	-	-
54	Ankara	Female	Blood, Serum	?	-	-	-
55	Ankara	Male	Blood, Serum	+	-	-	-
56	Ankara	Female	Blood, Serum	+	-	-	-
57	Ankara	Male	Blood, Serum	?	?	-	-
58	Ankara	Female	Blood, Serum	+	-	-	-
59	Ankara	Female	Blood, Serum	+	-	-	-
60	Ankara	Female	Blood, Serum	+	-	-	-
61	Ankara	Female	Blood, Serum	?	?	-	-
62	Ankara	Male	Blood, Serum	?	-	-	-
63	Ağrı	Male	Blood, Serum	+	-	-	-
64	Ankara	Male	Blood, Serum	+	-	-	-
65	Şırnak	Male	Blood, Serum, SF	+	-	-	-
66	Ankara	Male	Blood, Serum	+	-	-	-
67	Ankara	Female	Blood, Serum	+	-	-	-
68	Ankara	Female	Blood, Serum	-	-	-	-
69	Ankara	Female	Blood, Serum	?	-	-	-
70	Bolu	Female	Blood, Serum	-	-	-	-
71	Çorum	Male	Blood, Serum	-	-	-	-
72	Çorum	Female	Blood, Serum	+	-	-	-
73	?	Female	Blood, Serum	-	-	-	-

* OspA primer PCR, **23SrRNA primer PCR

Table 3.1. Cont'd.

NO	Location	Gender	Sample	ELISA IgM	ELISA IgG	PCR I*	PCR II**
74	?	Male	Blood, Serum	-	-	-	-
75	Ankara	Female	Blood, Serum, CSF	-	-	-	-
76	Kastamonu	Male	Blood, Serum	-	?	-	-
77	Ankara	Male	Blood, Serum	-	-	-	-
78	Ankara	Male	Blood, Serum	-	-	-	-
79	Ankara	Male	Blood, Serum	-	-	-	-
80	Ankara	Male	Blood, Serum	?	+	-	-
81	Ankara	Male	Blood, Serum	-	-	-	-
82	Ankara	Female	Blood, Serum	-	-	-	-
83	Çorum	Male	Blood, Serum	-	-	-	-
84	Yozgat	Male	Blood, Serum	-	-	-	-
85	Çorum	Male	Blood, Serum	?	-	-	-
86	Kastamonu	Male	Blood, Serum	-	-	-	-
87	Ankara	Female	Blood, Serum	-	-	-	-
88	Kastamonu	Male	Blood, Serum	-	-	-	-
89	Kastamonu	Male	Blood, Serum	-	-	-	-
90	Ordu	Male	Blood, Serum	-	+	-	-
91	Yozgat	Male	Blood, Serum	-	-	-	-
92	Ankara	Male	Blood, Serum	-	-	-	-
93	Yozgat	Female	Blood, Serum	-	-	-	-
94	Ankara	Female	Blood, Serum	-	-	-	-
95	Yozgat	Male	Blood, Serum	-	-	-	-
96	Ankara	Male	Blood, Serum	+	-	-	-
97	Ankara	Female	Blood, Serum	-	-	-	-
98	Kastamonu	Female	Blood, Serum	-	-	-	-
99	Karabük	Male	Blood, Serum	-	-	-	-
100	Ankara	Male	Blood, Serum	-	-	-	-
101	Ankara	Female	Blood, Serum	?	-	-	-
102	Ankara	Female	Blood, Serum	-	+	-	-
103	Yozgat	Male	Blood, Serum, CSF	-	-	-	-
104	Çorum	Female	Blood, Serum	-	-	-	-
105	Yozgat	Male	Blood, Serum	-	+	-	-
106	Çorum	Male	Blood, Serum	-	-	-	-
107	Kastamonu	Male	Blood, Serum	-	-	-	-
108	Bolu	Female	Blood, Serum	-	-	-	-
109	Çorum	Male	Blood, Serum	-	-	-	-
110	Kastamonu	Female	Blood, Serum	-	-	-	-
111	Artvin	Male	Blood, Serum	-	-	-	-
112	Kastamonu	Female	Blood, Serum	-	-	-	-
113	Çankırı	Female	Blood, Serum	-	-	-	-
114	Yozgat	Male	Blood, Serum	-	-	-	-
115	Çankırı	Male	Blood, Serum	-	-	-	-
116	Ankara	Female	Blood, Serum	-	-	-	-
117	Ankara	Female	Blood, Serum	-	-	-	-
118	Zonguldak	Female	Blood, Serum	-	-	-	-
119	Muş	Female	Blood, Serum	-	-	-	-
120	Çorum	Male	Blood, Serum	-	-	-	-

* OspA primer PCR, **23SrRNA primer PCR

Table 3.1. Cont'd.

NO	Location	Gender	Sample	ELISA IgM	ELISA IgG	PCR I*	PCR II**
121	Samsun	Male	Blood, Serum	-	-	-	-
122	Samsun	Male	Blood, Serum	-	-	-	-
123	Ankara	Male	Blood, Serum	-	-	-	-
124	Ankara	Male	Blood, Serum	-	-	-	-
125	Kastamonu	Male	Blood, Serum	-	-	-	-
126	Bolu	Female	Blood, Serum	-	-	-	-
127	Kastamonu	Female	Blood, Serum	+	-	-	-
128	Çorum	Female	Blood, Serum	+	-	-	-
129	Çorum	Female	Blood, Serum	+	-	-	-
130	Kastamonu	Male	Blood, Serum	?	-	-	-
131	Yozgat	Female	Blood, Serum	?	-	-	-
132	?	Female	Serum, CSF	-	-	-	-
133	Giresun	Female	Serum, CSF	-	-	-	-
134	?	Male	Serum, CSF	-	-	-	-
135	?	Female	Serum, CSF	?	-	-	-
136	?	Female	Serum, CSF	-	-	-	-
137	Ankara	Female	Serum, Skin biopsy	?	-	-	-
138	Samsun	Male	Serum, CSF	-	-	-	-
139	Samsun	Female	Serum, CSF	-	-	-	-
140	Ankara	Male	Serum, CSF	-	-	-	-

* OspA primer PCR, **23SrRNA primer PCR

The 45 of 140 samples were found positive and 25 of 140 samples were found uncertain in ELISA tests. In terms of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, 39 and 5 of the 45 positive specimens were found IgM and IgG positive respectively, 20 and 3 of 25 uncertain specimens were found IgM and IgG uncertain respectively. One of 45 positive samples were found both IgM and IgG positive and 2 of 25 uncertain samples were found both IgM and IgG uncertain. Table 3.2. concludes ELISA results.

Table 3.2. ELISA results.

	Positive	Uncertain
IgM ELISA (%) (n=140)	39 (27,9 %)	20 (14,3 %)
IgG ELISA (%) (n=140)	5 (3,6 %)	3 (2,1 %)
IgM/IgG ELISA (%) (n=140)	1 (0,7 %)	2 (1,4 %)
Total	45 (32,2 %)	25 (17,8 %)

Seropositivities in terms of the region which samples were sent are shown in figure 3.1. According to our study, in Ankara region 25/62 (40,3%), in Çorum region 4/13 (30,8%), in Kastamonu region 3/14 (21,4%) and in Yozgat region 3/12 (25%) cases were positive for *B. burgdorferi* IgM-IgG antibodies.

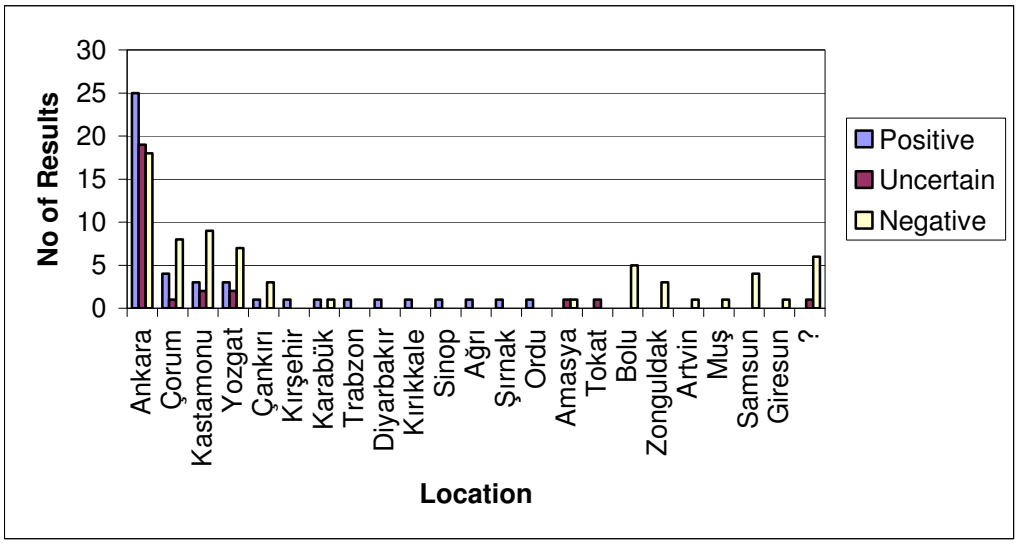

Figure 3.1. Positive, uncertain and negative ELISA results of samples in terms of region they were sent.

To evaluate the sensitivity of our PCR assay, 10-fold serial dilutions of the DNA of different *B. burgdorferi* strains were analyzed with the two primer sets. Equal sensitivities were achieved with the plasmid ospA primer and the ribosomal 23S rRNA primers, each detecting $\geq 4{,}4 \times 10^{-4}$ ng DNA/µl (Figure 3.2.b). The strains of the three species, *B. burgdorferi* sensu stricto B31, *B. garinii* VS102, and *B. afzelii* VS461, were detected with similar sensitivities.

All of the blood, CSF, SF and skin biopsy samples were examined by PCR with two primer sets. The PCR products were visualized by UV illumination and photographed (Figure 3.2., Figure 3.3.). There were no positive results in PCR analysis as shown in Table 3.1.

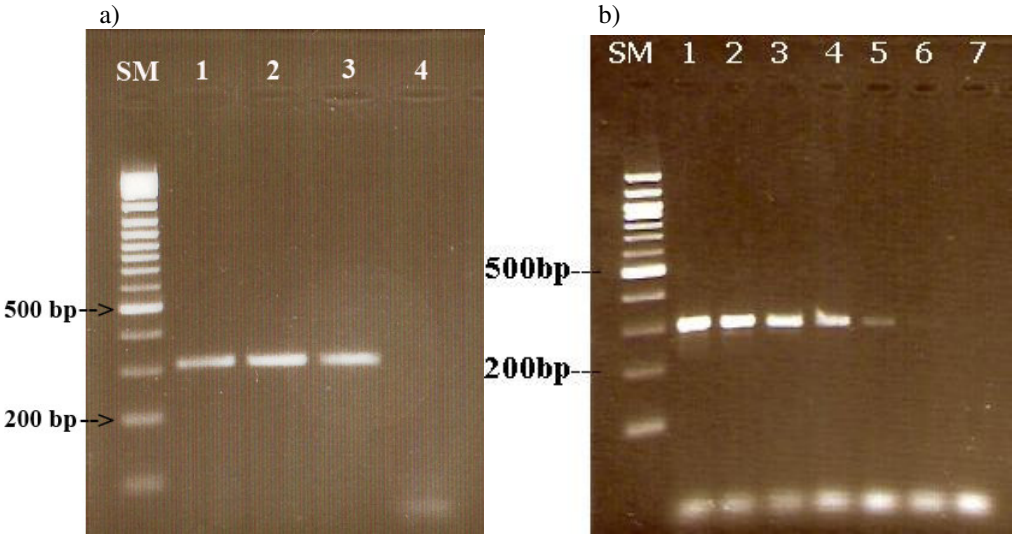

Figure 3.2. PCR amplification with ospA based SL primers (position on DNA sequence; 21→47, 302←328). a) Three representative Lyme disease isolates belonging to each of three genospecies were amplified by the PCR as described in Materials and Methods. Lane SM, molecular size marker (BioLabs 100 bp DNA Ladder yielding the following fragments, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 bp), and the sizes of 200 and 500 bp are indicated on the left. Lane 1, 2 and 3 are *B. burgdorferi* sensu stricto B31, *B. afzelii* VS461 and *B.garinii* VS102 positive controls respectively; Lane 4 is negative control of *B. burgdorferi* sensu lato. b) Sensitivity of PCR amplification with 10-fold serial dilutions are shown; lane SM indicates molecular size marker, lane 1, 2, 3, 4, 5, 6, and 7 specify amplifications of DNA from 4,4 ng/µl, $4{,}4x10^{-1}$ ng/µl, $4{,}4x10^{-2}$ ng/µl, $4{,}4x10^{-3}$ ng/µl, $4{,}4x10^{-4}$ ng/µl, $4{,}4x10^{-5}$ ng/µl dilutions and negative control respectively.

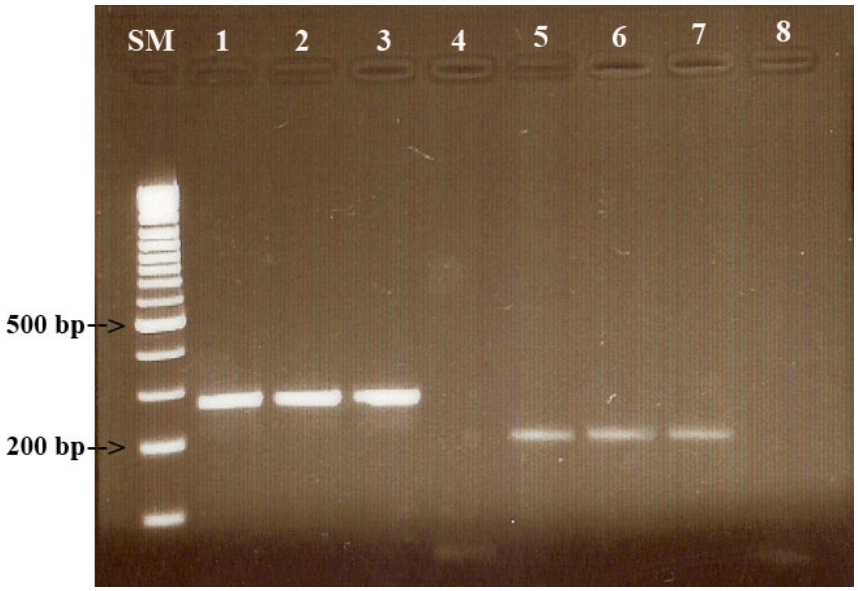

Figure 3.3. PCR amplification with 23SrRNA based Bsl-nBsl primers. Lane SM indicates molecular size marker. Lane 1, 2 and 3 are *B. burgdorferi* sensu stricto B31, *B. afzelii* VS461 and *B.garinii* VS102 positive controls respectively; Lane 4 is negative control of *B. burgdorferi* sensu lato in outer PCR with Bsl primers (269 bp). Lane 5, 6 and 7 are *B. burgdorferi* sensu strictoB31, *B. afzelii* VS461 and *B.garinii* VS102 positive controls respectively; Lane 8 is negative control in nested PCR with nBsl primers (216 bp).

CHAPTER 4

DISCUSSION

LB is a globally distributed tick-borne zoonosis. Human cases occur predominantly in the northern hemisphere (Wang *et al.,* 1999). LB represents a new global public health problem. It is now the most common vector-borne disease in North America and Eurasia. (Wang *et al.,* 1999; Steere, 2001; Schnarr *et al.,* 2006). The agent of LB, *Borrelia burgdorferi* sensu lato, is a gram-negative, highly motile, corkscrew-shaped bacterium (Sonenshine, 1993; Schnarr *et al.,* 2006). At least three species are pathogenic in humans: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* (Schnarr *et al.,* 2006; Humair and Gern, 2000). In the USA, only *B. burgdorferi* sensu stricto occurs, whereas all three species can be found in Europe. The different species preferentially invade different organs (organotropism), which may explain the different clinical pictures of LB in the USA as compared with Europe (Wang *et al.,* 1999; Humair and Gern, 2000; Steere, 2001; O'Connell, 2005; Schnarr *et al.,* 2006). The diagnosis of LB is currently based on the presence of a characteristic clinical picture, exposure in an area of endemic infection, an elevated antibody response to *B. burgdorferi* and direct detection of the etiologic agent. These methods have advantages and disadvantages according to each other (Schmidt, 1997; Aguero-Rosenfeld *et al.,* 2005; Schnarr *et al.,* 2006).

For the diagnosis of LB, IFA, ELISA and WIB assays have been commonly utilized for detection of specific antibodies against *Borrelia burgdorferi* sensu lato (Reed, 2002; Brouqui *et al.,* 2004; Aguero-Rosenfeld *et al.,* 2005). One of the limitation of ELISA and IFA is lack of standardization among numerous commercial kits marketed for LB in the USA and Europe (Bakken *et al.,* 1997; Reed, 2002). Furthermore, ELISA and IFA have the drawback of lacking

sensitivity for early stage of disease (Kaiser, 2000; Reed, 2002). Also the presence of specific antibodies does not prove the presence of disease because of antibody persistence for months to years (Reed, 2002; Wilske *et al.,* 2007). Another disadvantage of antibody assays is that of cross-reactivity with many diseases (Reed, 2002). There is no single optimum test for the serodiagnosis of LB (Brouqui *et al.,* 2004). As a direct detection of the causative agent of LB, culture technique is time-consuming, labour-intensive and expensive, consequently it is rarely used for the routine diagnosis of LB (Brouqui *et al.,* 2004; Aguero-Rosenfeld *et al.,* 2005).

Because of the above-described limitations of serological testing and culture technique, there is interest in developing new approaches for diagnosis of LB by using molecular techniques which mainly includes PCR-based methods (Rosa and Schwan, 1989; Nielsen *et al.,* 1990; Persing *et al.,* 1990; Rosa *et al*., 1991; Goodman *et al.,* 1991; Picken, 1992; Lebech *et al.,* 1992; Demaerschalk *et al.,* 1995; Schmidt, 1997; Priem *et al.,* 1997; Brettschneider *et al.,* 1998; Schwaiger *et al.,* 2001; Schaarschmidt *et al.,* 2002; Wang, 2002; Liveris *et al.,* 2002; Cyr *et al.,* 2005; Picha *et al.,* 2005; Aguero-Rosenfeld *et al.,* 2005; Gooskens *et al.,* 2006).

Since specific antibodies can generally be detected only weeks after a tick bite (Reed, 2002), in most of the studies it has been concluded that, the sensitivity of serology was somewhat lower then PCR in patients with early stage of disease (Schwartz *et al.,* 1992; Schmidt, 1997; Brettschneider *et al.,* 1998; Picha *et al.,* 2005; Aguero-Rosenfeld *et al.,* 2005). However, in patients with late disease, the sensitivity of serologic testing is high, but the persistence of antibodies after treatment can cause problems in the diagnosis of patients with ongoing symptoms, and antibody-negative patients have been reported (Cooke and Bartenhagen, 1994; Schmidt, 1997; Aguero-Rosenfeld *et al.,* 2005).

In this study ELISA and PCR results were quite different. Since it is reported

that antibody assays show cross-reactivity with many diseases (Reed, 2002) there is a possibility of false-positivity in ELISA results. Apart from this, since antibodies can persist months to years in body fluids (Reed, 2002; Wilske *et al.,* 2007), positive ELISA results in this study may also be due to clinical or subclinical infections in the past (Wilske *et al.,* 2007) and does not indicate presence of causative agent in body fluids. On the other hand, negative PCR results in this study does not exclude the disease. A published meta-analysis demonstrated that PCR is a very sensitive approach when it is employed to detect *B. burgdorferi* sensu lato DNA in skin biopsy and synovial fluid specimens from patients with LB, whereas the diagnostic value of PCR assays for detection of *B. burgdorferi* sensu lato DNA in blood (plasma or serum) and CSF specimens is low (Lebech *et al.,* 1992; Brettschneider *et al.,* 1998; Dumler, 2001; Aguero-Rosenfeld *et al.,* 2005; Picha *et al.,* 2005). In blood, spirochetemia is transient, and in other body fluids, spirochetes might simply not be present in sufficient numbers at the time samples were taken for analysis. Generally, borreliae, like all spirochetes, seem to be trophic for tissues, avoiding body fluids (Schmidt, 1997). From another point of view, we have not had any knowledge about the clinical context of patients involved in this study and whether or not they have been started an antibiotic therapy when the clinical samples were taken. Results showed that antibiotic treatment may also hamper detection by the PCR in accordance with Schwartz *et al.* (1992), Schmidt (1997), Brettschneider *et al.* (1998).

Considering that LB is a globally distributed tick-borne zoonosis, different studies have been attempted in Turkey. In serological investigations, seropositivities heve been reported in Trabzon region as 6,6% (Aydın *et al.,* 2001), in Antalya region 35,9% (Mutlu *et al.,* 1995), 6,4% and 22,1% (Tuncer *et al.,* 1999), in İzmir region 7,8% (Tünger and Büke, 1995), in Denizli region 18,9% (Çelik *et al.,* 2001). Additionally, in Kayseri region 10% (Utaş *et al.,* 1994), in Isparta region 17% and 2,3% (Demirci *et al.,* 2001) and in Elazığ region 6,43% (Erensoy, 2002) seropositivity were reported. In Ankara region

Hızel *et al.* (1997) reported 10,4% Lyme seropositivity while Birengel *et al.* (1999) reported 13% and 6%. Furthermore, Birengel *et al.* (1999) found 4% seropositivity in healthy control group. In our study, samples were mostly taken from Ankara region and 25/62 (40,3%) seropositivity were found for *B. burgdorferi* IgM-IgG antibodies, which is relatively higher than that of Hızel *et al*. (1997) and Birengel *et al.* (1999). In Çorum region 4/13 (30,8%), in Yozgat region 3/12 (25%) cases were detected as seropositive in our study. Our results show relatively higher seropositivities than that of Utaş *et al.* (1994) in Kayseri region and Demirci *et al.* (2001) in Isparta region. In Kastamonu region 3/14 (21,4%) cases were found seropositive in this study which is comparatively higher than in Trabzon region (Aydin *et al.,* 2001). Fewer than 20 Lyme borreliosis cases have been published since 1990 (Çakır *et al.,* 1990; Köksal *et al.,* 1990; Ergül *et al.,* 1996; Aksu *et al.,* 1997; Öztürk *et al.,* 1997; Demirkaya *et al.,* 1998; Leblebicioglu, 1999; Ulus *et al.,* 2001; Ceylan *et al.,* 2005; Karcıoğlu *et al.,* 2005). These cases were diagnosed as LB based on clinical symptoms and positive ELISA results with antigens derived from foreign isolates.

Recently *Borrelia* species were isolated and characterized from hard ticks in Trakya region of Turkey (Güner *et al.,* 2003; Güner *et al.,* 2003; Güner *et al.,* 2004). To our knowledge, there are no reports of isolation and detection of *B. burgdorferi* sensu lato species from clinical specimens in Turkey. In summary, we could not detect the bacteria in any type of the specimen with PCR analysis. Although, we were able to show the presence of antibodies by using ELISA test.

CHAPTER 5

CONCLUSION

The 152 clinical specimens (140 serum and blood, 10 cerebrospinal fluid, 1 synovial fluid, and 1 skin biopsy specimens) from 140 suspected LB patients were subjected to ELISA and PCR analysis in this study.

From 140 patients, 45 were found positive (32,1%), whereas ELISA results exhibited uncertainty 25 of 140 (17,9%). IgM-IgG antibodies against *B. burgdorferi* reactions showed that serospecifities were 40,3% for Ankara region, 30,8% for Çorum region, 21,4% for Kastamonu region and 25 % for Yozgat region. Our results support that there is a risk of acquiring LB in different regions of Turkey. Although considerable positive detections were recorded using serologic tests, none of the specimens were positive in PCR analysis.

Characteristic clinical feature is important for diagnosis of LB besides laboratory results. Further studies on molecular methods should be attempted for detection of *B. burgdorferi* sensu lato in patients with a high clinical probability of LB in the early phases. Our results especially indicate that clinical specimens should be taken before antibiotic therapy if the PCR method were to be used for diagnosis.

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